UNIVERSITY OF CALIFORNIA,
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Waterpipe Smoke Exposure and Arterial Plaque Formation:

Effects and Mechanisms Related to Sex, Autonomic Nervous System Changes and Endothelial Permeability in a Mouse Model of Atherosclerosis

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Environmental Health Sciences

by

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2021

DEDICATION

To

my wife, Monika, my parents, and my siblings

in recognition of their support, guidance, and love.

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LIST OF ABBREVIATIONS

AA	Ascending aortic arch
ANS	Autonomic nervous system
BALF	Bronchoalveolar lavage fluid
BaP	Benzo(a)pyrene
AV	Atrioventricular node
BCA	Brachiocephalic artery
CAPs	Concentrated ambient particles
CD36	Cluster of differentiation 36
CNS	Central nervous system
CO	Carbon monoxide
CRP	C-reactive protein
CVD	Cardiovascular disease
DA	Descending aortic arch
DNPH	2,4-dinitrophenylhydrazine
dWPS	Denuded waterpipe smoke
EBD	Evan's blue dye
ECG	Electrocardiogram
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FFT	Fast Fourier Transform
GnRH	Gonadotropic-releasing hormone
HDL	High-density lipoprotein
HF	High frequency heart rate variability
HPG	Hypothalamic-pituitary-gonadal axis
HPHCs	Harmful and potentially harmful constituents
HPLC	High-performance liquid chromatography
HR	Heart rate
HRV	Heart rate variability
IM	Intima-media
kDa	Kilodalton
LCA	Left carotid artery
LDL	Low-density lipoprotein
LF	Low frequency heart rate variability
LF/HF	Low frequency to high frequency ratio
LH	Luteinizing hormone
LOX-1	Lectin-like oxLDL receptor-1
LSA	Left subclavian artery

MCP-1	Monocyte chemoattractant protein 1
MMP-9	Matrix metalloproteinase 9
NN	Normal to normal beat interval
NTS	Nucleus of the solitary tract
OCT	Optimal cutting temperature compound
oxLDL	Oxidized low-density lipoprotein
PAH	Polycyclic aromatic hydrocarbon
PM	Particulate matter
PPM	Parts per million
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RMSSD	Root mean squared of successive differences
SA	Sinoatrial node
SDNN	Standard deviation of normal RR intervals
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SMC	Smooth muscle cell
SOD	Superoxide dismutase
SVOC	Semi-volatile organic compound
TIMP-1	Tissue inhibitor of metalloproteinase 1
TRPA1	Transient receptor potential ankyrin 1
VCAM-1	Vascular cellular adhesion molecule 1
VLDL	Very low-density lipoprotein
VOC	Volatile organic compound
WPS	Waterpipe smoke

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ABSTRACT OF THE DISSERTATION

Waterpipe Smoke Exposure and Arterial Plaque Formation:

Effects and Mechanisms Related to Sex, Autonomic Nervous System Changes and Endothelial Permeability in a Mouse Model of Atherosclerosis

by

Rebecca Marie Johnson Arechavala

Doctor of Philosophy in Environmental Health Sciences

University of California, Irvine, 2021

Professor Robert F. Phalen, Chair

Chronic inhalation exposure to particulate matter (PM) has been well documented to increase the risk of developing and dying from cardiovascular disease (CVD). This risk, however, has been studied mostly in ambient PM and cigarette tobacco. Waterpipes have been used for centuries to smoke tobacco and have recently increased in popularity worldwide, but its cardiovascular toxicity has not been widely studied. Therefore, I aimed to begin to fill this gap by studying the effects of long-term waterpipe smoke (WPS) inhalation on disease progression in an atherosclerosis prone mouse model (ApoE-/- mice).

Methods: Male and female ApoE-/- mice underwent nose-only exposure to WPS for 2 hours per day, 4 days per week for either 8 or 20 weeks. In the 8-week exposure, an additional group exposed to charcoal denuded WPS (dWPS) was included to study the importance of volatile and PM components on cardiovascular toxicity. Atherosclerosis progression was measured by histological assessment of intima-media thickness in the aortic arch, brachiocephalic, left carotid and left subclavian arteries. Inflammation and endothelial

permeability were assessed in plasma, as well as tissue expression of biomarkers and extravasation of Evan's blue dye (EBD), which was injected just prior to euthanasia. Heart rate variability (HRV) was assessed daily throughout the study as a proxy for autonomic nervous system perturbations associated with CVD.

Results: Intima-media thickness was altered mainly in the aortic arch as increased thickness in male ApoE-/- mice but decreased in the female mice after 5 months of WPS exposure. EBD extravasation was increased after only 2 months of WPS exposure in male mice. Inflammation marker, C-reactive protein, was decreased in female mice after 2 months of WPS inhalation. No significant changes were found in other markers of permeability or inflammation. HRV indicated sex-dependent effects, where WPS induced dampening of vagally mediated responses in females. In males, WPS increased vagal stimulation of the heart in a transient manner, seen only on the days of exposure, and not on the non-exposure days of each week.

Conclusions: In summary, despite differences from the initial hypotheses, this dissertation presents results that WPS exposure is able to induce cardiovascular toxicity as assessed by heart rate variability, and that WPS can influence the progression of atherosclerosis differently between male and female mice.

1. INTRODUCTION

1.1 Waterpipe use

Waterpipes, also known as hookahs or narghiles, have been used for centuries throughout the world, with origins likely in Asia and northern Africa (C. Cobb et al., 2010). For over 400 years, waterpipes have been used for tobacco smoking (Knishkowy & Amitai, 2005). Waterpipe use has grown in popularity in the United States with up to 48% of young adults reporting waterpipe use at some point in their life (Soule et al., 2015). Despite overall waterpipe use being one of the least common tobacco products in the United States, this still corresponds to many people who are at risk from potential health effects of waterpipe smoke. In 2019, approximately 2.5 million Americans were regular users of either regular pipe or waterpipe tobacco (Cornelius et al., 2020). In 2020, 2.1% of middle and high school students in the United States, which is an estimated 580,000 students, reported having smoked waterpipe tobacco in the past 30 days (Gentzke et al., 2020). A waterpipe consists of a bowl in which the tobacco, which is moist, often sweetened and flavored, is heated, a partially filled water base, and a stem which connects the bowl to the base. The smoke from the heated tobacco is drawn down the stem and bubbled through the water before being inhaled by the user puffing on the waterpipe hose.

Attractions of waterpipe smoke (WPS), in addition to the variety of available flavors and the social aspects of smoking in a group, include the cooling and humidifying effect of the bubbling process on the smoke, which makes the smoke less harsh, and the misconception that filtration effects provided by the water will remove harmful components of the smoke (Jawad et al., 2013). The perception that waterpipe smoke is less harmful and addictive than smoke from cigarettes probably contributes to the steadily increasing trends in waterpipe use across all age groups

(Bhatnagar et al., 2019; Heinz et al., 2013). In one study, Perraud et al. found that bubbling the smoke through water in a waterpipe does not reduce the number of particles in the mainstream smoke, indicating that the water alone is not a sufficient filter, in contrast to some widely held public beliefs (Perraud et al., 2019).

1.2 WPS composition

WPS is a complex aerosol composed of particulate matter, gaseous compounds and semi-volatile compounds. Studies of the chemical composition of WPS have identified hundreds of compounds, including known toxicants such as tar, polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds, carbonyl compounds and heavy metals (al Rashidi et al., 2008; Perraud et al., 2019; Primack et al., 2016; Sepetdjian et al., 2010; Shihadeh et al., 2012, 2015; Shihadeh & Saleh, 2005). These compounds have been found at similar, and often greater, concentrations than seen in cigarette smoke (Shihadeh et al., 2015). Carbonyls are often found in WPS as they are thermal degradation products of the glycerol which is added to the waterpipe tobacco as a humectant. Specifically, formaldehyde, acetaldehyde, acetone, acrolein, propionaldehyde and butyraldehyde were found in ranges from 1-25 µg/L of smoke, with their concentrations varying slightly depending on the puffing topography utilized (Eddingsaas et al., 2019). Most of the compounds reported to be found in WPS samples are on the Food and Drug Administration (FDA) list of harmful and potentially harmful constituents (HPHCs) in tobacco products and tobacco smoke, classified as carcinogens, respiratory, cardiovascular, reproductive, or developmental toxicants, or as addictive compounds ("FDA Harmful and Potentially Harmful Constituents in Notice of Tobacco Products and Tobacco Smoke; Established List," 2012). The presence of carcinogenic biomarkers, particularly PAHs and VOCs unique to combusted tobacco products, have been found in urine of regular waterpipe users (Etemadi et al., 2019).

WPS differs from other forms of tobacco smoking in that the tobacco is not itself directly ignited, and there is a lit charcoal block placed atop the tobacco. This charcoal is a large contributor of chemical components of the WPS aerosol. Charcoal burning produces carbon monoxide (CO) and PAHs, in concentrations which vary depending on the brand or source of the charcoal (Monzer et al., 2008; Sepetdjian et al., 2010). Heavy metals, such as lead, zinc and chromium, are found in quick-lighting charcoals but not usually in a natural charcoal (Elsayed et al., 2016). However, as the waterpipe is smoked, the concentration of PAHs in WPS substantially increases, with levels of benzo[a]pyrene (BaP) inhaled after one waterpipe smoking session was reported to be equal to that of smoking 38 cigarettes (Sepetdjian et al., 2008). Overall, the large volume of smoke and toxic compounds inhaled from a waterpipe, represents a potential health risk. Although the lungs and respiratory tract are the first tissues to come into contact with inhaled toxicants, decades of research have indicated that there are extrapulmonary responses and that the cardiovascular system is a critical target organ.

WPS is a complex aerosol and, the toxic potential is not only due to the chemical constituents, but the size of the PM in WPS is an important factor in determining the overall toxic potential. The waterpipe used in this study produces mainstream smoke ultrafine particles with a mode diameter around 80 nm at the start of a smoking session, and after 10 minutes the mode was stable at ~200 nm. Of particular note is that one puff from the waterpipe contained approximately 24 times more ultrafine particles than are in a single puff from a conventional cigarette, with an average of 7.6×10^{10} ultrafine particles per puff of WPS (Perraud et al., 2019). The pathologic potential of ultrafine PM is augmented by the large relative specific surface area per unit of mass on which toxic chemical compounds can adsorb, as well as the ability of ultrafine PM to carry toxic agents to the deep lung (Gaté et al., 2017; Hess & Tseng, 2007). The

ultrafine PM in air pollution and tobacco smoke can act as an efficient delivery mechanism for inhaled compounds to the gas-exchange region of the lung, and subsequently to the blood stream.

There is evidence from Oberdörster et al. of extrapulmonary translocation of ultrafine carbon particles to the liver and other organs occurs, indicating that these particles can enter the blood stream and/or lymphatic system and circulate through the body (Oberdörster et al., 2002). Inhaled ultrafine titanium dioxide particles were found to translocate after inhalation exposure into the blood and olfactory bulbs of the brain (Pujalté et al., 2017).

The exact mechanisms by which PM inhalation alters systemic responses, whether as a direct reaction of extrapulmonary tissues with PM or for the cascade of inflammatory mediators initiated in the lung, are not fully understood. Several toxicants identified in cigarette smoke and ambient PM have been shown to exacerbate atherosclerosis and cause other adverse cardiovascular effects (Araujo, 2011; Araujo et al., 2008; Araujo & Nel, 2009; L. C. Chen et al., 2010; Simkhovich et al., 2009; Xie et al., 2015). In summary, WPS aerosol contains toxic chemical constituents which are associated with ultrafine particulate matter and there is evidence WPS can influence extrapulmonary health effects. This study was designed to investigate the potential risks of cardiovascular effects of WPS.

1.3 Cardiovascular disease background

Cardiovascular disease (CVD) is a broad term referring to afflictions of the heart and vessels of the circulatory system. In particular, it is often associated with atherosclerosis, an inflammatory disease of the arteries which involves buildup of lipid deposits, inflammation and even necrosis of the tissue in the arterial walls. Atherosclerosis is initiated by excessive oxidative stress which leads to downstream inflammation and permeability of the vascular endothelium. Free radicals associated with oxidative stress can also interfere with cardiac physiology and can contribute to

CVD. Recently, additional mechanisms and methods have developed that can link autonomic nervous system control of the heart to CVD (Thayer et al., 2010). Heart rate variability (HRV) assessment is a non-invasive method to assess changes in the interval between successive heartbeats. HRV indexes changes to the autonomic nervous system (ANS) which innervates the cardiac muscle and influences the cardiac rhythm. In this way, it is useful in toxicology as a measure of cardiac and autonomic changes. This study tested hypotheses related to effects of WPS exposure on development of atherosclerosis and HRV changes.

1.3.1 Mechanisms of atherosclerosis

Atherosclerosis is an inflammatory disease initiated by the characteristic deposition and accumulation of lipids in the walls of arteries (Ross, 1999). Low-density lipoprotein (LDL) is a particular culprit. LDL is a lipid transport particle in the blood consisting of a phospholipid exterior organized by an apolipoprotein, with a core containing esterified cholesterol, phospholipids, fatty acids, and triglycerides (Pirahanchi et al., 2021). This dense LDL particle delivers lipids to tissues throughout the body. However, when LDL reacts with reactive oxygen and nitrogen species (ROS and RNS, respectively), this produces oxidized LDL (oxLDL). ROS can arise as products of normal cellular aerobic metabolism but are also generated by inflammatory processes. ROS are highly reactive and therefore self-perpetuating, by oxidizing other compounds which can, in turn, generate new ROS or RNS. In functioning systems, ROS are quenched by indigenous antioxidant defensive redox systems in the body, such as glutathione cycling. However, when exogenous inducers of ROS are introduced, such as from tobacco-derived and ambient PMs, these redox systems can be overwhelmed, inhibited, or altered, reducing the body's ability to combat the perpetuation of ROS and subsequent oxidative stress can damage tissues and organs (Kelly & Fussell, 2017; Messner & Bernhard, 2014). The initial

step in lipid accumulation is an increase in permeability of the endothelial cell barrier of the arterial wall. Endothelial permeability allows for both passive and active transport of LDL into the underlying arterial tissue layers and this accumulation contributes to atherosclerosis (Mundi et al., 2018).

Regarding atherosclerosis, when oxLDL is produced and deposited within arterial tissue, the affected endothelial cells respond to this injury by release of cytokines and chemoattractant molecules. These inflammatory signaling cascades recruit monocyte-derived macrophages and lymphocytes to the area. OxLDL also induces expression of various molecules, such as vascular-cell adhesion molecule 1 (VCAM-1) which is involved in the adhesion of recruited macrophages and T cells to the endothelium. These immune cells are internalized into the intimal layer of the vascular wall, signaled by the underlying stimulated smooth muscle cells (SMCs) releasing monocyte chemotactic protein-1 (MCP-1) (Leiva et al., 2015). The stimulated leukocytes, endothelial cells and SMCs release specific cytokines such as IL-6 and TNF-α which signal further cellular recruitment and migration (Fatkhullina et al., 2016). Macrophages differentiate to express scavenger receptors such as cluster of differentiation 36 (CD36) and lectin-like oxLDL receptor-1 (LOX-1) which have a high affinity for oxLDL (Leiva et al., 2015; Pirillo et al., 2013).

The activated macrophages phagocytize the oxLDL in attempt to remove it, but oxLDL is not effectively eliminated from the cell. This process produces foam cells, which are macrophages with high amounts of lipid accumulation. In this activated state, macrophages further promote inflammation through secretion of cytokines and enzymes such as myeloperoxidase. Myeloperoxidase generates localized ROS and RNS which oxidize more nearby LDL (Carr et al., 2000), promoting the cycle of oxidation and uptake of oxLDL by macrophages and

furthering the development of a lipid core in intermediate atherosclerotic lesions. Heavy lipid accumulation overwhelms the capacity of foam cells which induces cell death via apoptosis or necrosis. As the macrophages are trapped in the intimal tissue, the cellular debris is not removed from the lesion (Leiva et al., 2015). Therefore, atherosclerosis progression subverts the protective role of macrophages to phagocytize and remove the offending oxLDL particles, coopting this as macrophages cannot digest or clear the lipid deposits and only further the inflammatory progression of disease.

Below the intima layer of an artery is the media. Media of arteries are characterized heavily by the smooth muscle cells (SMCs) which confer the ability for an artery to relax and contract in response to systemic stimuli. SMCs however also have a role in atherosclerotic lesion progression. In response to oxLDL accumulation and foam cell infiltration SMCs proliferate and migrate into, and thicken, the intima (Shen et al., 2001). However, the structure of the intima-media area is maintained by an extensive extracellular matrix (ECM), consisting of various types of collagens and elastin (Newby, 2006). The ECM must be degraded to facilitate migration of SMCs through the tissue. Several proteinases are involved in this process, and matrix metalloproteinase-9 (MMP-9) expression and activity is vital, as both Galis et al. and Johnson et al. showed that MMP-9 deficiency led to less migration of SMCs in lesions and therefore less thickening of carotid intima-media in a mouse model of atherosclerosis (Galis et al., 2002; Johnson et al., 2005). MMP-9 is secreted by SMCs, macrophages, and foam cell macrophages in particular (Newby et al., 2009). MMP-9 degrades several types of collagen and elastin, and its expression from macrophages is upregulated by many cytokines (Shimizu et al., 2004; Yabluchanskiy et al., 2013). This progression of tissue remodeling produces a fibrotic cap containing the accumulated foam cells, SMCs, collagen, and cellular debris which can all

become a necrotic core (Ross, 1999; Yabluchanskiy et al., 2013). Overall, the stimulation of SMC migration and ECM degradation and reorganization by MMPs leads to formation of an advanced and complex atherosclerotic lesion.

1.3.2 PM induced toxicity

Ambient particulate matter inhalation has been extensively researched with respect to exacerbation of atherosclerosis and cardiovascular outcomes (Araujo et al., 2008; Simkhovich et al., 2009; Xie et al., 2015). Chen et al. concluded that PM from either ambient air pollution or side-stream cigarette smoke, even at low concentrations, elucidate similar effects in the enhancement of atherosclerotic lesion area in mice (L. C. Chen et al., 2010). Decades of research have shown that cigarette smoking induces inflammation and oxidative stress resulting in lipid peroxidation and tissue damage (Conklin et al., 2017, 2019; Mullick et al., 2002; Office of the Surgeon, 2004; Srivastava et al., 2011). Studies show that similar inhalation exposures of ambient PM and cigarette smoke can lead to an exacerbation of disease, but the degree to which WPS will elicit adverse effects on users' cardiovascular systems needs to be determined.

1.3.3 WPS poses a possible risk to exacerbating CVD

WPS increases inflammation markers in the lungs (Javed et al., 2017; Khan et al., 2018), and induces systemic changes to superoxide dismutase (SOD) and glutathione reductase (Ali et al., 2017; Nemmar et al., 2017). This suggests that WPS induces inflammation, impairs antioxidant responses leading to oxidative stress and has been shown to induce systemic effects leading to increased levels of oxidized lipids in the blood, which is consistent with the premise that WPS may cause or exacerbate CVD (Nemmar et al., 2017). Other sources of PM are capable of inducing similar inflammation and oxidative pathways, in addition to changes of HRV, suggesting that WPS is a likely factor in increased risk of developing and exacerbating CVD.

While several review articles have posited the cardiovascular risks which may be associated with WPS (Bhatnagar et al., 2019; Rezk-Hanna & Benowitz, 2019), the literature is lacking in studies assessing the outcomes of WPS exposure in animal models.

Although WPS contains many familiar components which are present in cardiotoxic PM such as that from ambient pollution and cigarette smoke, it is still a different and unique aerosol mixture and differences in exposure habits, with people are exposed to waterpipe smoke less frequently, but receive higher doses than in cigarette users. This makes it difficult to directly predict cardiotoxicity outcomes. One reason that the underlying mechanisms by which WPS interacts with the cardiovascular system are not well documented is that many of the published WPS toxicology studies have been short-term exposures or cross-sectional studies (Bhatnagar et al., 2019; C. O. Cobb et al., 2012; Khan et al., 2018; Nelson et al., 2016). Other target organ systems have also been studied, such as the WPS induction of male reproductive toxicity (Ali et al., 2015, 2017) and pulmonary toxicity (Khan et al., 2018), which do suggest that WPS does pose a risk to health. To begin to fill this gap in knowledge of WPS toxicity, cardiovascular effects of WPS were examined in an atherosclerosis-prone, hyperlipidemic mouse model exposed for 8 and 20 weeks to document whether long-term WPS exposure can exacerbate progression of CVD as indicated by altered development of atherosclerosis and impairment of the autonomic balance of sympathetic and parasympathetic nervous systems which control heart rate (HR) and HRV.

2. Exposure system and study design

2.1 Waterpipe exposure system development

The waterpipe used during this study was a commercially available waterpipe (Anahi Smoke, model “Fantasy”) made of all glass components to limit any contamination of the smoke from the waterpipe materials. Three, 1-inch cubes of a commercially available, coconut shell natural charcoal (Black Diamond) were heated for 10 minutes on an electric heater and placed atop the bowl filled with 10 grams of apple-flavored shisha tobacco (Al Fakher Tobacco Trading, Ajman, UAE) from one of the most commonly used brands (Maziak et al., 2019) and covered in perforated aluminum foil (Starbuzz Tobacco Inc, Starbuzz Premium foil). Although the charcoals do not directly touch the tobacco under the perforated foil, the tobacco reaches a temperature of between 265°C to 318°C (Perraud et al., 2019). The base of the WP was filled with 800 mL of de-ionized water, replaced daily, and the stem was inserted a depth of 39 mm below the water surface. The lab did not have any previously developed or commercial system for waterpipe smoke administration. Therefore, a waterpipe smoking system was created, modified from published diagrams (Khabour et al., 2012; Khan et al., 2018) to simulate the appropriate puffing, dilution and delivery of WPS for the needs of the present study.

The smoking system was actuated by an automated solenoid control and valve assembly that alternately drew smoke from the waterpipe and diluted it with air purified through activated charcoal and a HEPA filter (Figure 1). WPS was generated with a puffing frequency of 4 seconds every 30 seconds through the waterpipe at a volumetric flow of 10 liters per minute. This puffing topography was previously chosen based on a modified Beirut protocol (Shihadeh, 2003) and is reiterated within the ranges measured from recent studies in American populations (Brinkman et al., 2020; Eddingsaas et al., 2019; Maziak et al., 2019). The WPS was pulled

through the waterpipe by a membrane pump (Gast Manufacturing, Inc., Benton Harbor, MI) into a holding volume and then 'pushed' into the mixing chamber and mixed with purified dilution air which provided WPS at a positive pressure to the nose-only exposure chambers (In-Tox Products, LLC, Clinton, MS). To obtain the desired dilution factor, direct input of purified air would result in an excess of air volume and pressure in the system. To avoid this, a portion of the initial whole smoke was exhausted out, and this smaller volume was then further diluted for a final flow rate and pressure that would be appropriate for the mouse exposure. In addition, the lab developed and built mouse-specific restraint tubes modified to fit with the existing nose-only exposure chambers. Nose-only exposure was used to mimic the normal route of exposure with smoking, and minimizes contamination of the animals' skin and fur, which could lead to non-target routes of exposure such as dermal interaction or ingestion of deposited WPS aerosol (Phalen et al., 1984).

Undiluted WPS from this system contains 2.1 (± 0.6) × 10^3 ppm of CO (Perraud et al., 2019). While epidemiologically, CO is an important part of understanding WPS toxicity (Shihadeh & Saleh, 2005), however excess CO could confound and overwhelm chronic effects of inhalation of whole smoke aerosol, including PM and all other VOCs. To minimize the potentially confounding risk of CO-related cardiotoxicity (Lee et al., 2015; Rezk-Hanna et al., 2019), the smoke was diluted approximately 20 times with purified air or enough to maintain a carbon monoxide (CO) content of the smoke below 100 ppm.

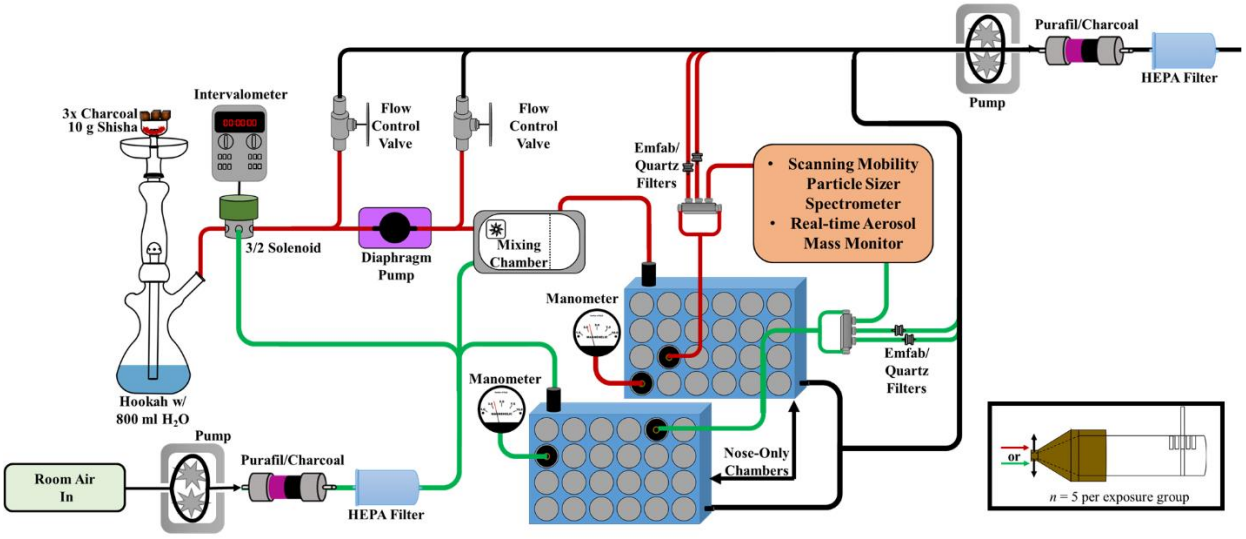

Figure 1. Nose-only waterpipe smoke exposure system. *Inlay image of mouse nose-cone.*

2.2 Study design

The Apolipoprotein E knockout (ApoE$^{-/-}$; B6.129P2-Apoetm1Unc/J, Jackson Laboratories, Bar Harbor, ME) mouse model was utilized for this study as it is a well-established model of CVD and spontaneously develops plaques that are similar to those seen in human atherosclerosis (Meir & Leitersdorf, 2004; Nakashima et al., 1994; Rosenfeld et al., 2000). Dyslipidemia, as is seen with high concentrations of low density lipoproteins (LDL) in ApoE-/- mice, increases the susceptibility of these mice to vascular endothelial dysfunction and subsequent predisposition for atherosclerotic plaque development (Garg et al., 2015; Wengrofsky et al., 2019). VCAM-1, in particular, is upregulated in vascular endothelium at sites prone to develop lesions, where it is not found in wild-type mice (Nakashima et al., 1998). These are some of the underlying mechanisms defining the innate incidence of atherosclerotic lesions in this mouse model.

Both male and female mice were included with each exposure group to allow for assessment of sex-specific differences in WPS responses. Animals were housed in an AAALAC (formerly Association for Assessment and Accreditation of Laboratory Animal Care International;

AAALAC International) accredited vivarium at the UC Irvine Air Pollution Health Effects Laboratory (APHEL) and received water and food ad libitum when not being exposed. A subset of mice (n=3-5 per group; Table 4), were implanted with radiotelemetry devices to monitor electrocardiograms (ECG). Allowing time for recovery from surgery, acclimation to the exposure system, and assessment of baseline ECG collection, exposures began when animals were 12-weeks of age. An additional 6 mice per group were not implanted, for a total of 11 animals per sex for each exposure group whose tissues were used for further biochemical assessments (Tables 2 and 4). The exposure paradigm was first performed, in 2018, for 20-weeks (2 hours per day, 4 days per week) to assess long-term effects of WPS, with purified air as a control. The following year, 2019, the exposure was repeated in an 8-week paradigm which included a third exposure group which was exposed to denuded WPS. Denuded WPS is WPS which went through activated charcoal cartridges in an annular denuder column (Dekati Ltd., Tampere, Finland) run at room temperature to adsorb and remove volatile compounds from the whole WPS aerosol, allowing animals to be exposed to only the particulate fraction of WPS. Studying the responses of both denuded WPS (dWPS) and whole WPS allowed for more specific assessment of the importance of gaseous compounds, versus particulate matter, of the aerosol in subsequent changes in health outcomes. To assess endothelial permeability, a solution of 1% Evan's blue dye (EBD; Sigma-Aldrich, St. Louis, MO) in sterile saline was injected into the tail vein of each mouse just prior to euthanasia. EBD binds to albumin and was allowed to circulate for 30 minutes prior to euthanasia and cardiac perfusion with ice-cold sterile saline. Areas of endothelial permeability would show extravasation of the EBD, which would be retained in the tissue after perfusion. Animals were euthanized approximately 24 hours following their final exposure.

2.3 WPS Exposure characteristics

2.3.1 WPS Constituent analysis

The WPS was continually monitored for particle mass concentration (DustTrak aerosol monitor, TSI, Shoreview, MN), particle size distribution (scanning mobility particle sizer, TSI, Shoreview, MN), and CO concentration (48i-TLE, ThermoFischer, Waltham, MA) during the exposure. Aerosol sampling was performed using a modified nose-cone for the sampling inlet to mimic the location of a mouse's nose and most accurately assess the inhaled WPS.

There were variations in the week-to-week particulate matter (PM) concentrations (Figure 2A) due to intermittent mechanical issues that were identified and repaired during the 2018 study. The particle mass concentration of the diluted mainstream WPS over the entire study averaged 46.46 ± 24.82 mg/m^3 (mean ± SD) and the CO concentration averaged 79 ± 31 ppm (Table 1). In the 2019 study, PM mass concentration was, on average, 16 mg/m^3 lower in the WPS group, and 24 mg/m^3 lower in the dWPS group, compared to WPS concentrations previous year. There were no differences in CO concentrations between exposure groups, or between years. As the exposure system contains dilution of the WPS which was adjusted to maintain a CO concentration below 100 ppm, differences between exposures on the PM concentration is due to dilution factor differences. The loss of particle mass when using the denuder in the 2019 study is as expected from previous studies (Keebaugh et al., 2015).

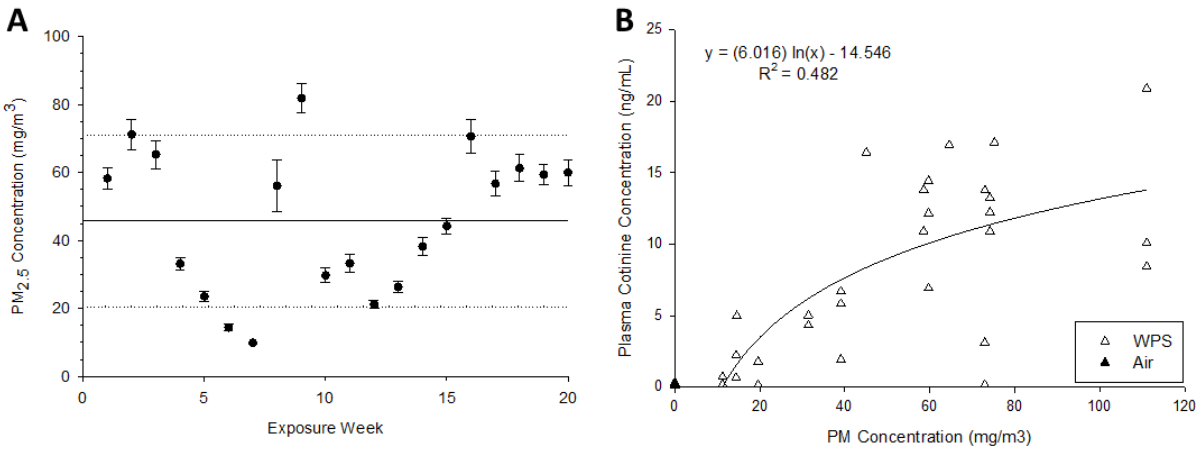

Figure 2. Mass concentration (mg/m3) of diluted WPS *(mean ± SD) measured in the mouse breathing zone during the 20 weeks of exposure (A) as measured from the DustTrak aerosol monitor, averaged weekly with the solid and dashed lines marking the study average and stand.*

	2018 – 20-week study			2019 – 8-week study		
	Particle Number (ptc/cm^3)	Particle Mass (mg/m^3)	CO (ppm)	Particle Number (ptc/cm^3)	Particle Mass (mg/m^3)	CO (ppm)
Air	2.71 ± 1.68	-	-	0.76 ± 2.18	-	-
WPS	-	46.46 ± 24.82	79 ± 31	-	30.53 ± 18.76[b]	83 ± 33
dWPS	-	-	-	-	22.40 ± 17.91[a,b]	85 ± 54

Table 1. Particle concentrations averaged over the exposure period for each study. *Particle number data collected for purified air group only. Particle mass and CO data collected for WPS and dWPS exposures (mean ± SD). [a]$p<0.05$ between WPS and dWPS within the 2019 exposure period. [b]$p<0.05$ compared to WPS during 2018 study.*

2.3.2 Plasma Cotinine Measurements

0.2 mL of blood was collected by retroorbital bleeding within 30 minutes of exposure for each mouse under isoflurane anesthesia. Samples were taken weekly from a rotating subset of animals throughout the month so that each animal was sampled only once per month to follow recommended standards for safe blood drawing. The collected plasma from the 2018 study was

analyzed to quantify the content of the nicotine metabolite, cotinine, using a direct enzyme-linked immunosorbent assay (ELISA) (Calbiotech, El Cajon, CA).

Plasma cotinine levels were compared at baseline and at the end of the 20-week exposure (Table 2). Baseline concentrations of cotinine were not significantly different between air and WPS groups. Following WPS exposures, cotinine levels rose significantly to 10.6 ± 1.9 ng/mL, consistent with exposure to smoke containing nicotine. Despite the day-to-day variations in WPS PM concentration (Figure 2B), there was generally good correlation of plasma cotinine to WPS exposure concentration.

In humans, after smoking cigarettes, it is expected that plasma cotinine levels will rise above 100 ng/ml (Benowitz, 1996). A significant difference between humans and mice however is the rate of nicotine metabolism and the subsequent half-life of nicotine and cotinine. In humans, the half-life of nicotine is 2-2.5 hours, compared to only 9 minutes in C57Bl/6 mice. Cotinine, the main metabolite of nicotine, has a half-life of 17 hours in humans, but only 37 minutes in C57Bl/6 mice (Benowitz, 1996; Siu & Tyndale, 2007). Therefore, despite taking care to collect plasma samples from the mice within one hour after the exposure ended, it is likely that 1-2 half-lives of cotinine had passed, decreasing the measured amount. The higher elimination rate in mice also means that at the same exposure levels, humans would reach higher cotinine levels than would mice. In addition, the WPS was diluted 20 times on average, which will also lower the dose of nicotine inhaled by the mice in the exposure system, further differentiating their plasma cotinine levels from those expected in humans directly after smoking. Given the metabolism rates in mice and the dilution factor of the WPS, these concentrations are comparable to those found in mice after undiluted WPS exposure (Khan et al., 2018).

	Baseline Mean ± SEM (ng/mL)	**Final** Mean ± SEM (ng/mL)
Purified Air	0.20 ± 0.02	0.20 ± 0.03
WPS	0.25 ± 0.07	10.6 ± 1.9
p-value	0.262	<0.001

Table 2. Plasma cotinine concentrations. *Group averages for the baseline and values after 5 months of purified air or WPS exposure. Measurements from 2018 exposure study.*

2.3.3 WPS chemical constituent analyses

Perraud et al. found that the aerosol from the waterpipe and tobacco used in the present study contains large amounts of glycerol and monosaccharide in the vapor and particle phases. Nicotine content was mainly in the particle phase and was also not altered by the process of bubbling the smoke through the water. The WPS is a very diverse and complex mixture. Many compounds of concern to health were found in both WPS and in smoke from a 3R4F reference cigarette such as formaldehyde, methanol, formic acid, acrolein, benzene and toluene. However, due to the differences in combustion mechanisms in cigarettes and waterpipes, where lower temperatures are reached, the concentration of combustion byproducts were lower in WPS. Waterpipe tobacco is unique with a heavy presence of glycerol which lead to large amounts of its decomposition products, including acrolein, acetaldehyde and benzene found in the aerosol (Perraud et al., 2019). The glycerol humectant was also found to have a role in the generation of particulate matter, which is supported by literature (Bernd et al., 2019).

Currently, FDA regulations of waterpipe tobacco pertain only to notifying users of nicotine content (21 CFR Part 1143, 2021), and do not specify uniformity or labeling of chemical composition (Haddad et al., 2016). However several studies have published the presence of chemicals with known toxicities in WPS. As there is no uniformity on the preparation method or

additives in waterpipe tobacco. Carbonyl compounds were assessed from the WPS after collecting filtered mainstream WPS using impingers containing a solution of 2,4-dinitrophenylhydrazine (DNPH). The DNPH solution was analyzed using high-performance liquid chromatography (HPLC) and concentrations of analytes were calculated from calibration curves against a standard carbonyl solution. Carbonyl analyses were also performed on a commercially available cigarette for comparison (Table 3).

		***Waterpipe Tobacco**	****Cigarette**
Formaldehyde	(μg/g)	73.3 ± 5.7	22.2 ± 1.7
Acetaldehyde	(μg/g)	115.7 ± 7.2	631.5 ± 16.2
Acrolein	(μg/g)	16.3 ± 2.3	66.3 ± 2.5
Acetone	(μg/g)	10.8 ± 2.2	248.7 ± 6.4
Propionaldehyde	(μg/g)	13.2 ± 0.7	57.6 ± 2.0
Crotonaldehyde	(μg/g)	14.8 ± 1.4	30.6 ± 1.2
2-Butanone/ Butyraldehyde	(μg/g)	7.1 ± 0.5	53.2 ± 2.1
Valeraldehyde	(μg/g)	n.d	n.d.
p-Tolualdehyde	(μg/g)	60.2 ± 5.6	n.d.
Hexaldehyde	(μg/g)	n.d	n.d.

Table 3. Carbonyl concentration in whole tobacco smoke. *Sampling was performed on the Al Fakher waterpipe tobacco used in the present study and assayed using HPLC methods. * μg/g Tobacco (60 puffs, 4 sec puff duration, puff frequency: 30 sec). ** μg/cigarette (9 puffs, 2 sec puff duration, puff frequency: 1 min), single Camel Filter cigarette with a weight of ~1g/cigarette. Unpublished work, reproduced with permission from Norbert Staimer.*

Formaldehyde, acetaldehyde, acrolein, acetone, propionaldehyde, crotonaldehyde, butyraldehyde and *p*-tolualdehyde was found in the WPS. For comparison purposes, concentrations are given as mass of the compound per gram of tobacco smoked, not as aerosol concentrations. Given that, the concentration of most of the compounds was lower in the WPS than in the cigarette smoke, except for formaldehyde, which was 3.3 times greater in WPS and p-Tolualdehyde, which was

not found in combustion cigarettes. p-Tolualdehyde (4-methylbenzaldehyde) is toxic and irritating to the mucus membranes of nasal, oral and upper respiratory tract tissues. Approximately 2 grams of tobacco are consumed every 30 minutes of a waterpipe smoking session using the 4 second puff duration and 30 second frequency, as used in the present study, for a total of 8 grams of tobacco consumed over the 2-hour WP exposure duration.

3. Intima-medial thickness is affected by WPS exposure in ApoE-/- mice

3.1 Introduction

ApoE-/- mice are a well-studied model of atherosclerosis since the first publications on the subject 30 years ago (Getz & Reardon, 2016). The significant marker of this mouse model is the elevation of plasma cholesterol levels, from around 80 mg/dl in wild-type mice up to 400 mg/dl in ApoE-/- mice (Maeda, 2011). High circulating cholesterol predisposes the mice to develop atherosclerotic lesions, which does not occur in wild-type mice. Cholesterol levels, and lesion progression, can be exaggerated by administering a western-type diet in the ApoE-/- mouse model. The initial stage of lesion formation is adhesion of monocytes to the vascular endothelium, which occurs between 8- to 10-weeks of age. Development of foam cell aggregation and fatty streak lesions in ApoE-/- mice fed a normal chow diet begins around 10 weeks of age and can be found at this stage for several months. Lesions can begin to progress into intermediate lesions at 15 weeks, and fully developed fibrous plaques are found at 20 weeks (Nakashima et al., 1994; Reddick et al., 1994). As the animals age, all lesion types can be found simultaneously, and are most commonly found at areas of bifurcation in the aorta and carotid arteries. The aortic arch and brachiocephalic artery are regions with consistent rates of disease progression, and therefore present good models of monitoring atherosclerotic lesion progression (Seo et al., 1997). In the present study, inhalation exposure to WPS began at 12-weeks of age and histological changes assessed in the arteries at 32 weeks. The WPS exposure overlaps with timing of foam cell lesion initiation and covers the progression of lesions into intermediate and fibrous plaques.

3.2 Methods

3.2.1 Tissue processing

Prior to necropsy, mice were euthanized by overdose with pentobarbital sodium and perfused with ice-cold, sterile saline via cardiac puncture. The aortic arch was then removed *en bloc* with a portion of the brachiocephalic, left common carotid, and left subclavian arteries attached. The tissue was cleared of any remaining blood and embedded in optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetek, USA), and quickly frozen over a bath of dry ice and 2-methylbutane. Frozen samples were stored at -80C for future sectioning and analysis. Tissue was sectioned using a cryostat (Avantik, Pine Brook, NJ) at 10 µm thickness, placed immediately onto glass slides and stored at -80C until fixed and stained. Each slide contained three sections, and every 5^{th} slide was stained with Masson's Trichrome (ab150686, Masson's Trichrome stain, Abcam, Waltham, MA) following the manufacturers protocol. Briefly, slides were fixed in Bouin's fluid, and incubated with Weigert's iron hematoxylin to stain nuclei, Biebrich Scarlet to stain muscle, and Aniline Blue to stain collagen. Assessing progressive slides allowed visualization of arterial health and plaque development throughout the entire arterial block.

3.2.2 Image processing and artery section analysis

Images were taken at 20X using brightfield microscopy on a Keyence BZ-X800 microscope (Osaka, Japan), and stitched when necessary, using the microscope-associated software. Image files were exported and analyzed on ImageJ (Abràmoff et al., 2004), using the "polygon selection" tool to measure the areas of the lumen and entire artery (inside the adventitia), and the "segmented line" tool to measure the circumference of the artery section in a blinded manner. Intima-medial (IM) thickness and IM area, indicators of arterial health, were calculated from these measurements. IM area was calculated as the artery area, less the lumen area. IM thickness

was calculated as IM area divided by the circumference of the artery. Measurements were performed on an average of 5 and 10 sections, in small arteries and aortas respectively, per animal (Table 4). The aortas assessed were from the mice with radiotelemetry implants. Aortae from five animals were assessed per group apart from WPS exposed females which only had aorta samples from 3 animals. Representative images of a healthy and atherosclerotic plaque are below (Figure 3). An incidence score was calculated for each animal as the sum of artery regions with visual presence of an atherosclerotic plaque. The score ranges from zero to five, as five artery regions were assessed.

		2018 study			
		Air F	WPS F	Air M	WPS M
n animals		5	3	5	5
n of sections analyzed per group for each artery region	BCA	24	25	25	23
	LCA	27	25	26	31
	LSA	14	14	20	36
	AA	40	47	60	48
	DA	56	45	64	64

Table 4. Number of animals in lesion analysis. *Number of animals and sections analyzed for each exposure group for the assessment of morphology in arteries by Masson's Trichrome stain. BCA, brachiocephalic artery; LCA, left carotid artery; LSA, left subclavian artery; AA, ascending aortic arch; DA, descending aortic arch.*

3.2.3 Statistics

All data were expressed as means ± standard error of the mean (SEM), unless otherwise stated. Statistical analyses were performed using GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California USA. Significance was assessed at $p<0.05$ using unpaired, parametric t-tests to compare to the purified air control group for each measure.

3.3 Results

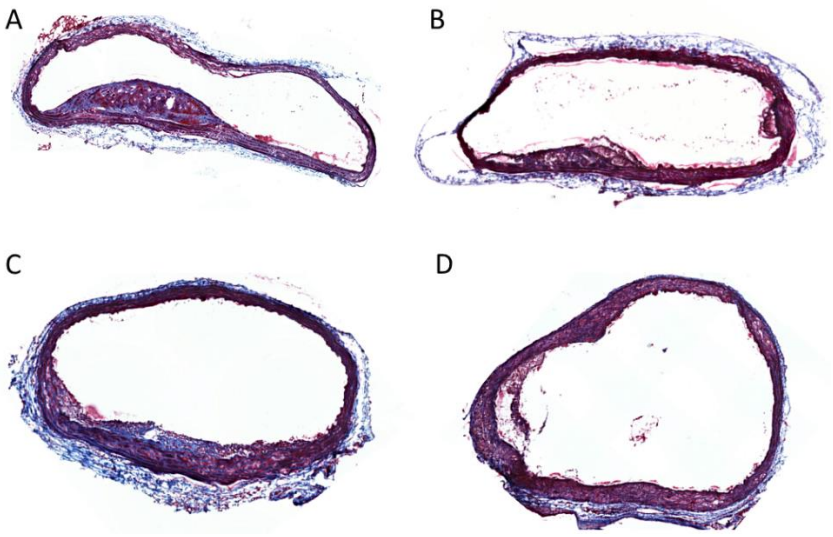

Figure 3. Representative images of aortic arch cross-sections. *(A) air-exposed female, (B) air-exposed male, (C) WPS-exposed female, (D) WPS-exposed male.*

3.3.1 Intima-media measurements

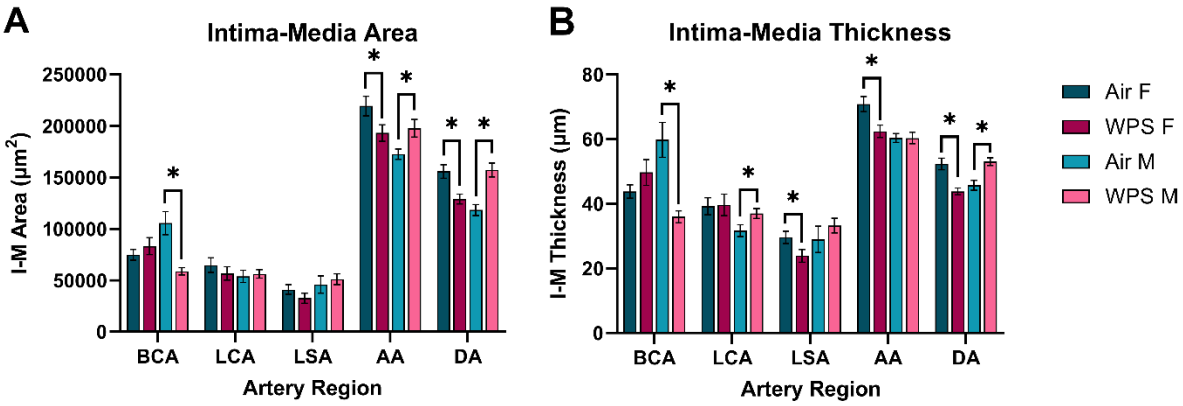

Figure 4. Intima-medial (IM) area and thickness *from aortae and aortic branches of ApoE-/- mice exposed to 5-months of either purified air or whole WPS. BCA, brachiocephalic artery; LCA, left carotid artery; LSA, left subclavian artery; AA, ascending aortic arch; DA, descending aortic arch. *p<0.05*

The area and thickness of the IM regions were proportional to each other, with consistent trends of effect (Figure 4). Comparisons are made between WPS and Air animals within each sex.

Female ApoE-/- mice exposed to WPS show thinner IM of the aortic arch, both descending (DA) and ascending (AA) portions, as well as the left subclavian artery (LSA). There was no change in the left carotid artery (LCA), and a slight trend of thicker IM in brachiocephalic arteries (BCA) of WPS-exposed females. In contrast, the ascending and descending portions of the aortae of male mice exposed to WPS show greater area, and thicker IM in DA and LCA. The IM of BCA in WPS-exposed males are significantly smaller in area and thickness.

	Comparison Group	Brachio-cephalic Artery	Left Carotid Artery	Left Subclavian Artery	Ascending Aorta	Descending Aorta
Intima-media area (μm^2)	Female	0.25	-0.23	-0.19	-0.32	-0.65**
	Male	-1.24**	0.07	0.23	0.39*	0.99**
	Air	-0.9††	0.31	-0.05	0.69††	0.72††
	WPS	0.68†	0.01	-0.42	-0.07	-0.93††
Intima-media thickness (μm)	Female	0.37	0.03	-0.79*	-0.5**	-0.93**
	Male	-1.01*	0.52*	0.27	0	0.69**
	Air	-0.68†	0.64†	0.04	0.67††	0.56†
	WPS	0.88††	0.2	-0.76†	0.15	-1.2††

Table 5. Cohen's d effect size on intima-media area and thickness. *Effect size between WPS and Air inhalation, and between Female and Male within exposure groups, on intima-media area and thickness. *p<0.05, **p<0.005 comparing Air and WPS within each respective sex. †p<0.05, ††p<0.005, comparing Female to Male within each respective exposure group.*

The effect sizes of the exposure driven differences in IM area and thickness were most apparent in the BCA and DA regions, as shown in Table 5. Regionally, WPS affects the small (BCA, LCA, and LSA) arteries in a contrasting manner of the larger aortic vessels studied here (AA and DA). WPS-exposed males in particular show a very large decrease in BCA IM area, and large decrease in BCA IM thickness, with a contrasting large and medium increase in DA IM area and thickness, respectively. In female mice, the effect of WPS was seen the most in the LSA and in the aorta, both AA and DA, with medium to large effects.

Irrespective of the effect of inhalation exposure group, there are distinctive sex-differences in the area and thickness of the IM across different regions (Table 5). In the control groups, female mice had significantly smaller IM in the BCA, and significantly larger IM in the AA and DA. In WPS groups, female mice developed significantly thicker and larger IM in the BCA. IM thickness was considerably thinner in the DA of females in the WPS groups.

	BCA	LCA	LSA	AA	DA	Incidence Score (Mean ± SEM)	p-value
Air F	2/5	0/5	0/5	4/5	3/5	1.8 ± 0.37	
Air M	3/5	2/5	1/5	4/5	2/5	2.4 ± 0.68	
WPS F	3/3	2/3	0/3	3/3	1/3	3 ± 0.0	0.0529
WPS M	1/5	3/5	2/5	3/5	2/5	2.2 ± 0.37	0.8027

Table 6. Plaque incidence rates in each artery. *BCA, brachiocephalic artery; LCA, left carotid artery; LSA, left subclavian artery; AA, ascending aortic arch; DA, descending aortic arch. p-value calculated in comparison to Air group of the same sex.*

The influence of WPS on the incidence rate of atherosclerotic plaques was also examined (Table 6). The incidence score serves as a proxy of plaque burden of the number of artery regions containing lesions in each animal. The incidence score neared statistical significance (p=0.0529) for an increase in plaque burden in females exposed to WPS. There was no difference induced by WPS in males, nor was there a significant difference due to sex differences alone within each exposure group.

3.4 Discussion

This study assessed the effect of WPS inhalation on the progression of atherosclerosis in mice. Overall, ApoE-/- mice show sex- and location-dependent changes in atherosclerotic plaque deposition after chronic WPS inhalation. At 32 weeks old, atherosclerotic lesions were evident in all groups regardless of exposure atmosphere. Five arteries near the heart were assessed to find

the best location for identifying WPS exposure-induced effects on IM area and thickness. IM thickness is a well-studied endpoint for atherosclerosis and as an indicator of future development of negative health outcomes (Chambless et al., 1997). Of the five arterial regions assessed, an opposing direction of change after WPS exposure was found between males and females, and in small versus large arteries. Where it was shown that male IM area or thickness decreased in response to WPS, female mice showed a thickening of the IM. The regions with the greatest significant, and largest effect size of sex-and exposure- dependent changes in IM area and thickness were the brachiocephalic artery and the descending aorta. This suggests that in future studies, these would be the most appropriate arterial regions to focus on understanding the mechanisms behind these changes. In addition, the study should be replicated in the future with larger group sample sizes to assess these measures.

Comparing in tandem the IM thickness and plaque incidence rate results allows a better understanding of the effect of WPS on atherosclerosis progression. Although WPS females had thinner IM, they also had a greater plaque burden overall in comparison to the controls. This indicates that in females, WPS induced more atherosclerotic plaques throughout the aortic arch and artery branches, but that they were, on average, smaller than those seen in controls. WPS exposure in males induced a thickening of IM, but did not alter the overall plaque burden. This suggests that the average thickening of IM from WPS is due to greater progression and worsening of atherosclerotic lesions, but not a change in the initiation of new plaques in males. Overall, five months of WPS inhalation results in divergent responses in male and female ApoE-/- mice.

The patterns of atherosclerotic lesion development are inherently different between sexes within each inhalation exposure group, and the pattern shows sex-driven opposing responses to WPS.

Literature supports that control female mice at this age may be expected to have greater plaque burden than male ApoE-/- mice (Man et al., 2020). However, in the presence of WPS, female ApoE-/- mice in this study seem to be protected from WPS-induced increases in exacerbation of plaque development, with an indication of smaller IM in the DA and LSA. This protection may be due to differences in estrogen related antioxidant responses in females and males (Borrás et al., 2010). This study highlights the importance of including multiple sexes in toxicology studies as statistically separate groups, where opposing effects could be masked if assessed only by exposure group, as would be the case in the present study.

Sex differences have been previously found in the normal progression of atherosclerotic lesions in ApoE-/- mice since at least 1999 (Caligiuri et al., 1999), however very limited research has been done to understand the mechanisms behind this. Over the period from 2006 to 2016 a literature search study found that less than 25% of published animal studies included both male and female animals, and only 44.8% of those studies statistically assessed sex as a biological variable in atherosclerosis (Man et al., 2020). While sex-differences in lesion size is seen in these animal studies, it is variable and dependent on diet, timing and the method used to assess atherosclerosis (Caligiuri et al., 1999; Man et al., 2020).

Much of the existing literature on the toxicological effects of particulate matter exposures and atherosclerosis have been performed exclusively in one sex. The results of WPS-induced exacerbation in males found the present study is supported by results found from exposure to ambient PM and cigarette smoke. Tani et al. found that 8 weeks of cigarette smoke exposure increased IM thickness in male ApoE-/- mice (Tani et al., 2004). Previous exposures by our laboratory in ApoE-/- mice have shown that 8-weeks of exposure to concentrated ambient PM

increases plaque size and lipid deposition in males, consistent with the present WPS exposure (Keebaugh et al., 2015).

The results of lessening IM thickness in WPS-exposed females are not widely supported by studies assessing the effect of ambient PM or cigarette smoke on atherosclerosis. Boue et al. found that cigarette smoke exposure for six months increased plaque area of the aortic arch by 41%, and this was accompanied by greater, and more diverse, lipid accumulation in the arterial wall (Boué et al., 2012). Although the exposure concentration was very high in comparison to the WPS, at an average of 600 mg/m^3, as they did not dilute the smoke prior to animal exposure. Possible differences in cigarette smoke exposure outcomes could be due to the different chemicals, PM concentrations or also the higher CO levels in undiluted smoke. In a study of concentrated ambient PM exposure on ApoE-/- female mice, PM at an average concentration of 123 $\mu g/m^3$, the percentage of endothelial cells of the aortic arch which were positively stained for lipids, indicating increased lipid deposition (Luderer et al., 2021).

Outside of the context of toxicological impact on atherosclerotic development, there is not a consistent consensus on the sex-differences in ApoE-/- lesions. Both Caligiuri et al. and Liu et al. published in depth articles on assessments of atherosclerosis development in ApoE-/- mice, fed a normal chow diet, in both males and females, however they found opposing results in their research done 17 years apart. Caligiuri et al. found that at 16 weeks old, females had larger and more progressed plaques than seen in males, who only showed fatty streak development. As the animals aged though, plaques progressed in males and there was no longer a sex-difference found at 48 weeks (Caligiuri et al., 1999). Liu et al. concluded that for the first 2 months, there is no difference between male and female ApoE-/- aortic lesion area. At ages 3 months or older however, females consistently showed significantly less lesion area in the aorta through to the

end of the study at 8 months of age (Liu et al., 2016). These widely opposing conclusions indicate that there may be differences in the phenotype of the ApoE-/- mouse model which are changing over time. Though there is not a consensus on the expected direction of sex-differences in atherosclerosis, even in control animals, it is widely apparent that progression of disease in male and female animals, particularly in the context of toxicological studies, needs to be better studied.

The average IM thickness of all groups in this study are greater than that expected in wild-type mice, where the average IM thickness of the carotid artery in a C57BL/6 mouse is 20 µm, which is approximately half the thickness found in all groups (Berg et al., 2006). This suggests that although the effect of WPS was not uniform between groups, all animals in this study had IM thickness indicative of increased lesion formation over their wildtype counterparts. Overall, 5 months of repeated exposure to WPS is able to affect the normal progression of atherosclerosis in ApoE-/- mouse model, in a sex-dependent manner.

4. Heart rate variability in ApoE-/- mice

4.1 Introduction

Heart rate variability (HRV) analysis describes the changes in the inter-beat interval of successive heartbeats. Modern interest and analysis of HRV began in the 1960s and 1970s, and in 1996 the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology came together to organize standards in HRV assessment in terms of nomenclature, methods, physiological interpretation, and clinical application of these HRV measurements (Billman, 2011; Malik et al., 1996). Overall, HRV is useful as a non-invasive tool to investigate autonomic nervous system (ANS) responses. The ANS consists of two branches: sympathetic and parasympathetic. In broad terms, the sympathetic branch increases heart rate, and the parasympathetic branch sends signals which decrease heart rate. However, ANS influence is not simply a switch from one branch to the other. The opposing systems are both acting on the heart simultaneously and it is their net effect that is seen when measuring heart rate alone. Mathematically separating the minute changes in inter-beat intervals enables us to parse out the inputs from the parasympathetic nervous system in particular.

The ANS branches are discerned by pathways of innervation involved. Signals from the ANS are sent from the medulla oblongata in the brainstem. Sympathetic preganglionic neurons connect the medulla to the ganglia of the spinal cord. From there, the postganglionic neurons innervate the heart at the sinoatrial (SA) and atrioventricular nodes (AV), as well as diffusely throughout the ventricular muscles. Norepinephrine is the neurotransmitter that conveys the sympathetic signal. In contrast, the parasympathetic signal exits the medulla via the vagus nerve, or cranial nerve X, on both the left and right sides of the medulla. The signal is more direct, with the postganglionic vagal nerves located in the cardiac tissue. The vagus nerve innervates the SA and

AV nodes, utilizing acetylcholine as the neurotransmitter (Hainsworth, 2004). With respect to the cardiac cycle, the SA and AV nodes are the vital cellular areas which initiate regulation of normal heart rhythms (Mangoni & Nargeot, 2008). Therefore, for HRV assessments, only cardiac cycles which are not arrhythmic and initiate in these vital nodes of the heart are evaluated.

The measures used for assessment of HRV are designated as either time- or frequency-domain measures. In the time-domain, standard deviation of normal inter-beat intervals (SDNN) and the root mean squared of successive differences (RMSSD) are both simple mathematical calculations based on the time between two successive, normal R-R peaks on an ECG. SDNN is interpreted as total variability of the HR. RMSSD is interpreted as the influence of the parasympathetic nervous system on the heart. To assess frequency domain measures, the R-R interval cycles undergo mathematical transformation using Fast Fourier Transform (FFT) and the resultant frequency spectra is divided into regions of high and low frequencies (HF and LF, respectively). HF HRV is very highly correlated with RMSSD and is also interpreted as another marker of vagal input to the heart (Malik et al., 1996). LF HRV is more difficult to interpret and is likely the result of both sympathetic and parasympathetic nervous system outflows (Billman, 2011; Shaffer et al., 2014).

The main driver allowing differentiation of sympathetic and parasympathetic inputs via HRV is the variation in the timing of the cardiac response to nervous system outflow. Due to the pathway of the neurons in the sympathetic nervous system as well as transduction by norepinephrine, there is a delay of approximately 5 seconds until acceleration of heart rate begins. It takes another 20-30 seconds of continuous stimulus to fully reach a stabilized increased heart rate (Hainsworth, 2004). The vagal pathway is able to affect the cardiac cycle almost instantaneously

and continues to affect 1-2 heartbeats after the onset of the stimulus (Hainsworth, 2004; Levy et al., 1970). This is due to the speed with which acetylcholine is both released into the synaptic cleft, and subsequently either undergoes reuptake or degradation. With respect to the speed with which each branch can influence the cardiac cycle, a sympathetic stimulus is slow, at a low frequency, and the vagal stimulus is fast, at a beat-to-beat frequency.

Two of the HRV measures are interpreted as indexes of the parasympathetic nervous system input to the heart: RMSSD and HF HRV. As both are measures of quick changes to the inter-beat interval, they indicate vagal changes (Malik et al., 1996). This has been assessed in humans by chemically inducing a complete vagal block which led to an overall reduction in HRV, with a complete elimination of HF and a reduction in LF HRV (Akselrod et al., 1981; Minarini, 2020; Pomeranz et al., 1985). This supports the conclusions that HF is the result of vagus nerve influence and LF is the result of a combination of sympathetic and parasympathetic inputs to the heart.

Translation of this system to rodents has been studied and reviewed for the last several decades (Rowan 3rd et al., 2007; Thireau et al., 2008). Pham et al. assessed the effect of chemical blockade of each branch of the ANS independently. They found that blocking the sympathetic nervous system by atenolol decreased SDNN, but not RMSSD. In contrast, parasympathetic blockade by methylatropine resulted in significant decrease in RMSSD, but not SDNN (Pham et al., 2009). Just et al. characterized that the bands of the frequency spectra representing LF and HF HRV in mice are approximately 10 times higher than those obtained in humans. Furthermore they verified using chemical autonomic blockades that the HF range corresponded to vagal cardiac inputs (Just et al., 2000). This directly mimics results found in humans, canine, and other mammals, supporting the validity of the interpretation of HRV in rodent models (Akselrod et al.,

1981; Pomeranz et al., 1985; Randall et al., 1991; Rosenberg et al., 2020). In summary, RMSSD, which is directly proportional to HF HRV, is a direct proxy of vagus nerve inputs to the heart. SDNN is a marker of total HRV and is more influenced by the sympathetic nervous system in mice.

4.2 Methods

4.2.1 Animals

Twelve-week-old male and female hyperlipidemic ApoE-/- mice were exposed to waterpipe mainstream smoke (WPS) or purified air (n=3-5 per group; Table 7) by inhalation. Exposures were nose-only, 2 hours per day, 4 days per week for 20 weeks or 8 weeks during the 2018 study and 2019 studies respectively.

Study Year	Air F	dWPS F	WPS F	Air M	dWPS M	WPS M
2018	4	-	3	4	-	5
2019	5	5	5	4	4	3

Table 7. Number of animals assessed for HRV measures *which completed the full exposure period. Variations in n due to either death of the animal or insufficient battery life in the implanted radiotelemetry monitor to allow for data analysis for the entire exposure period.*

4.2.2 Radiotelemetry implantation and data acquisition

Animals were implanted with radiotelemetry electrocardiogram (ECG) monitors (ETA-F20, Data Sciences Inc., St. Paul, MN) to determine heart rate and HRV. Aseptic implantation surgery was performed under 1.5% isoflurane anesthesia; the body of the ECG unit was implanted in the intraperitoneal cavity and sutured in place against the abdominal muscle. The ECG leads were placed in a subcutaneous lead II configuration and sutured in. Immediately post-surgery, analgesics and antibiotics were administered, and the mice were monitored and allowed to recover for 10-14 days. At this time, there is negligible influence of surgical implantation on HRV (Thireau et al., 2008). Each animal was singly housed, and ECG data were recorded using

the easyMATRIX3 (EMKA Technologies, Falls Church, VA) acquisition system and analyzed using the ecgAUTO post-processing software (EMKA Technologies, Falls Church, VA) for heart rate and heart rate variability changes. Mice were acclimated to the exposure system and restraints over one week while being exposed to purified air followed by another week of purified air exposure during which ECG recordings began to be collected.

4.2.3 Heart Rate Variability Analysis

HRV was analyzed on ECG data recorded during the evening hours of 19:00 to 23:00. This overnight period was chosen as the active period for mice, which are nocturnal, in addition, beginning recordings at 19:00 avoids possible circadian rhythm influences which may impact HRV at 18:00 when the room lights are turned off.(Rowan 3rd et al., 2007) Analysis was performed both on exposure days (Monday through Thursday), and on non-exposure days (Friday and Sunday) when animals remained in their housing the entire day. To optimize the battery life of the radiotelemetry implants, ECG recordings were limited to 6 days a week throughout the exposures. ECG data were assessed following a modified protocol from that described by Thireau et al. to determine the most appropriate duration and sampling time for frequency domain analyses. It was determined that for the present data 3-minute epochs every 30 minutes was most appropriate, consistent with published findings (Thireau et al., 2008). Data were analyzed for heart rate (HR) and normal beat-to-normal beat (N-N)-interval data which was then used to assess the magnitude of variance explained in the time-domain of the heart's rhythm as measured by SDNN (standard deviation of N-N intervals) and RMSSD (root mean squared of successive differences of N-N intervals). To assess frequency domain measures of HRV, periodicity of oscillations in heart rate were analyzed using FFT that separated the single ECG curve into its components made up of sinusoidal waves of specific frequencies (Rowan 3rd et

al., 2007). Frequency domain spectra were analyzed with resampling every 50ms and linear interpolation, in a 50% overlapping with a Hamming window for segment lengths with 512 points (Fenske et al., 2016; Thireau et al., 2008). The power of the high-frequency (HF, 1.5-5.0 Hz) band has been used to represent cardiac vagal control which drives the parasympathetic nervous system (Fenske et al., 2016; Just et al., 2000; Liao et al., 1996). The physiological interpretation of heart period oscillations at low frequency (LF, 0.15-1.5 Hz) are less well understood, and therefore are not reported (Malik et al., 1996). Epochs were excluded from analysis if there were noise or artifacts, in the ECG data acquisition, or an insufficient number of normal successive beats.

HRV data acquired for one week prior to the start of WPS exposures were used to establish individual baselines for each animal. HRV outcomes during the exposure period were reported as changes in terms of percent changes from baseline which enabled us to normalize for variability between individual mice. The percent changes from baseline for each respective animal were averaged by exposure group and sex to test the hypotheses that WPS-exposed mice would differ from air-exposed mice with respect to HRV and that both males and females would be similarly affected.

4.2.4 Statistics

All data were expressed as means ± standard error of the mean (SEM). Heart rate and HRV data were expressed as percent changes from baseline for each parameter. Statistical analyses were performed using SPSS® (IBM, Armonk, NY, USA) and GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California USA. Equality of variance was assessed using Levene's test. Significance was assessed at $p<0.05$ using the students t-test as compared to the purified air control group each exposure week.

4.3 Results

4.3.1 HR

Heart rate (HR; Figures 5-6) and heart rate variability (HRV; Figures 7-XX) were determined from the ECG recordings. Over the 20-week purified air exposures, male and female control mice exhibited a trend towards reduced HR, consistent with the expected progression of CVD in ApoE-/- mice (Figure 5) irrespective of exposure day. On exposure days, WPS-exposed mice of both sexes showed statistically significant slowing of the HR, with an abrupt drop of 12-18% during weeks four through nine. To date, in spite of extensive investigation, the cause of the drop is unexplained. From week 10 through 20 of exposure, HR in WPS-exposed males was reduced compared to air controls, however there was no statistically significant HR difference between air-exposed females and WPS-exposed females. On non-exposure days, there was no significant difference between air controls and WPS-exposed males (Figure 5C), WPS-exposed females (Figure 5D) exhibited significantly increased HR compared to air non-exposure days during weeks 12 through 20. To summarize, by the end of the 20-week exposure period, compared to air-exposed, WPS-exposed males showed a significant decrease in HR on exposure days, but were not significantly different on non-exposure days. Females showed no exposure-related HR differences on exposure days, but on non-exposure days did exhibit increased HR after WPS, as compared to air.

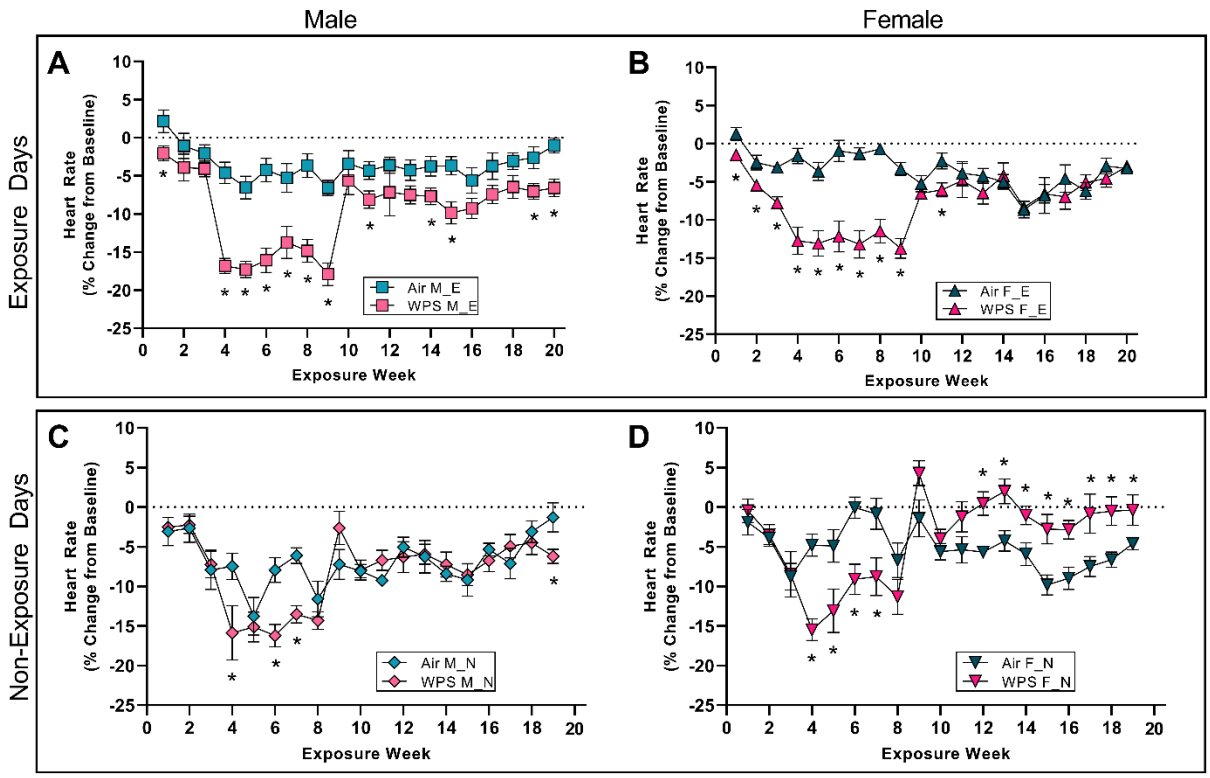

Figure 5. Heart rate – 2018 study. *HR is represented as percent change from baseline for air and WPS exposed ApoE-/- mice on the evenings of exposure days: male (A) and female (B), and non-exposure days: male (C), and female (D) during 20 consecutive weeks of exposure. *P<0.05 compared to purified air.*

In the 2019 study, in Figure 6, male animals, showed a lower percent change in both WPS and dWPS on exposure days. This continued on non-exposure days, but not consistently for dWPS males as it is for WPS males. In females, there is no significant difference between HR in the different exposure groups, except for in week 4, dWPS had a lower percent change in HR. On non-exposure days, there is a trend of less HR change in dWPS females, that becomes significant during weeks 4 and 5, but returns to the same value as air animals.

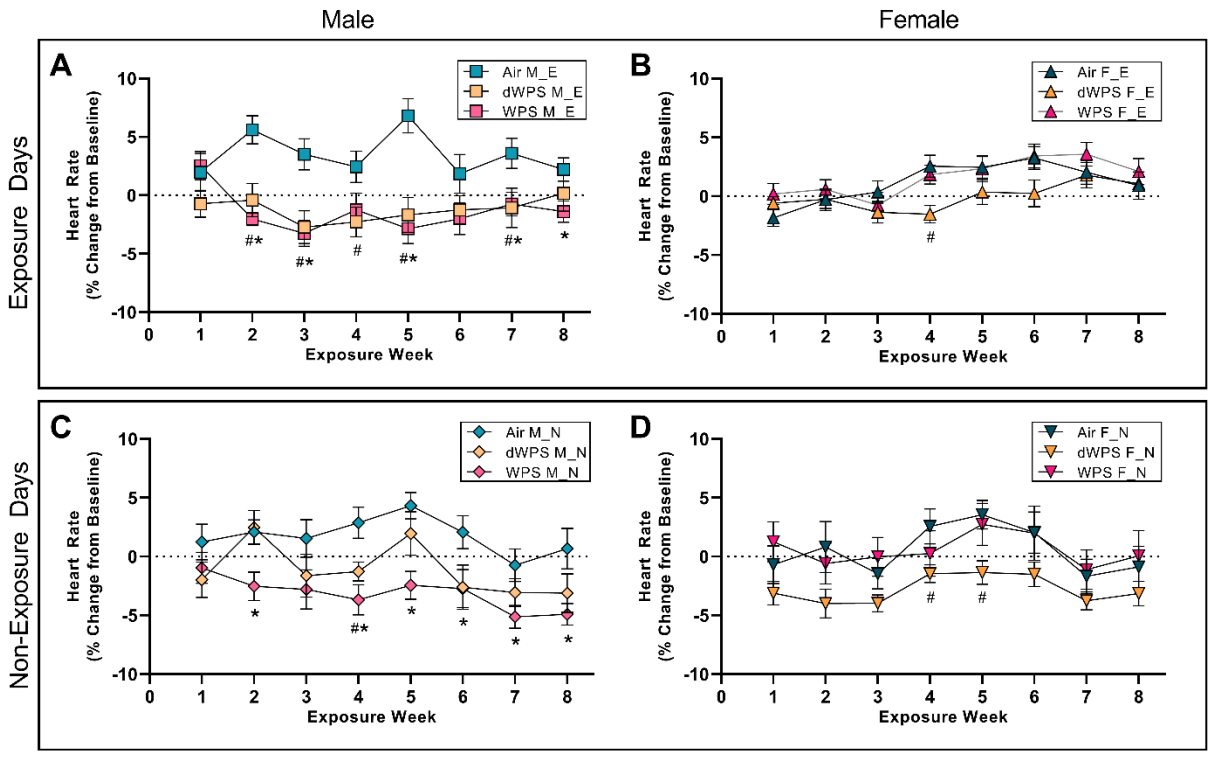

Figure 6. Heart rate - 2019 study. *HR is represented as percent change from baseline for air,* dWPS and *WPS exposed ApoE-/- mice on the evenings of exposure days: male (A) and female (B), and non-exposure days: male (C), and female (D) during* 8 *consecutive weeks of exposure. *P<0.05 compared to purified air.*

4.3.2 Time-domain HRV

4.3.2.1 SDNN

Total HRV, measured in the time domain, was characterized by the standard deviation of N-N beat intervals (SDNN). As shown in Figure 7A, throughout the exposure days, WPS increased SDNN in male mice. However, on the non-exposure days (Figure 7C) there was no statistically significant difference between SDNN in males. Figure 7B shows that other than week 1, SDNN for WPS-exposed females were not significantly different from air-exposed females on exposure days. However, on non-exposure days (Figure 7D), WPS significantly decreased SDNN in females relative to air controls from weeks 10 to 20 of exposure. To summarize, the HRV

decrease in males on exposure days was eliminated during the non-exposure days, but females exhibited HRV decreases only during the non-exposure days.

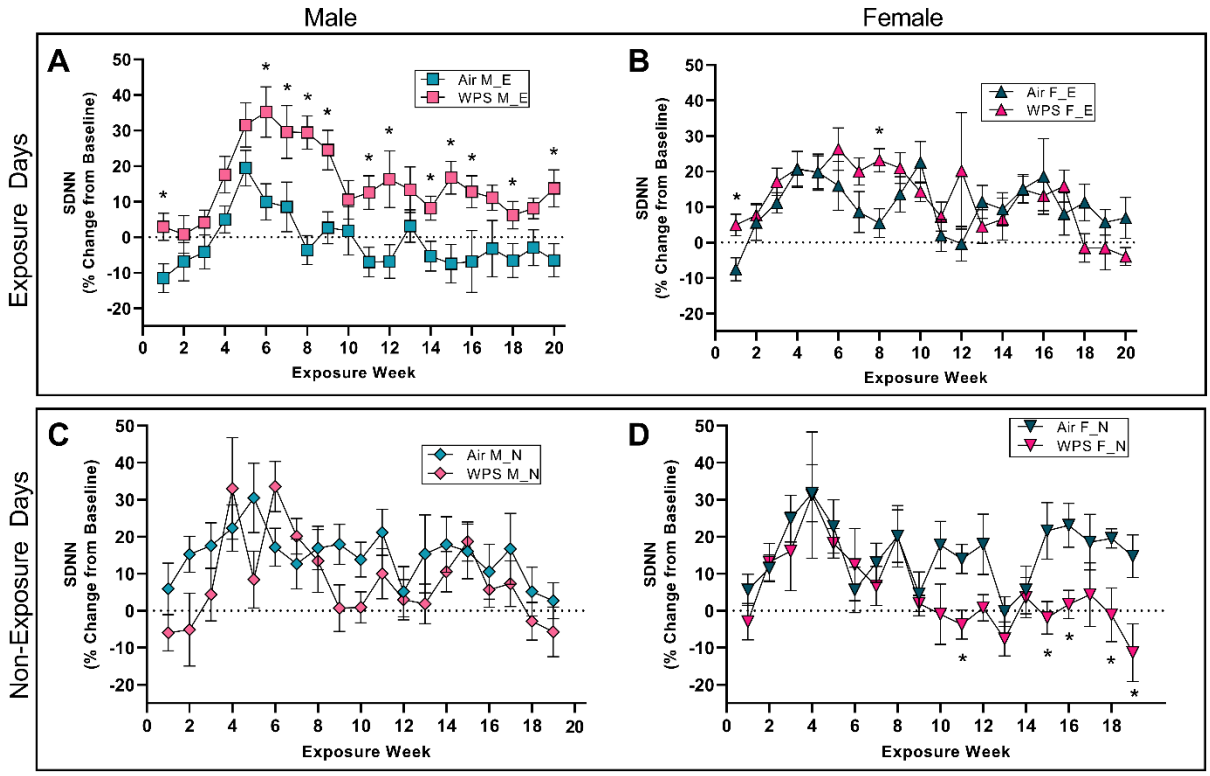

Figure 7. Standard deviation of normal R-R intervals (SDNN) - 2018 study. *SDNN, the time domain measure of total HRV, represented as percent change from baseline for air and WPS exposed ApoE-/- mice on the evenings of exposure days: male (A) and female (B), and non-exposure days: male (C), and female (D) during 20 consecutive weeks of exposure. *P<0.05 compared to purified air.*

Measurements of SDNN during the 2019 study are shown in figure 8. In males on exposure days SDNN is higher in WPS exposed animals during weeks 3 and 5 but returns to the air levels for the remainder of the study. In week 1 only, there is a decrease in SDNN percent change of dWPS males (Figure 8A). On non-exposure days, there is no difference between any of the male exposure groups (Figure 8C). Throughout the study period, the SDNN of WPS exposed females was significantly lower than that of air, while no difference in SDNN was induced by dWPS

(Figure 8B). On non-exposure days of week 1, WPS animals had a lower change of SDNN, while dWPS animals had a greater SDNN percent change than air. For the remaining non-exposure days, SDNN was not different between groups until the final week where dWPS animals again had a greater SDNN percent change from the other groups (Figure 8D).

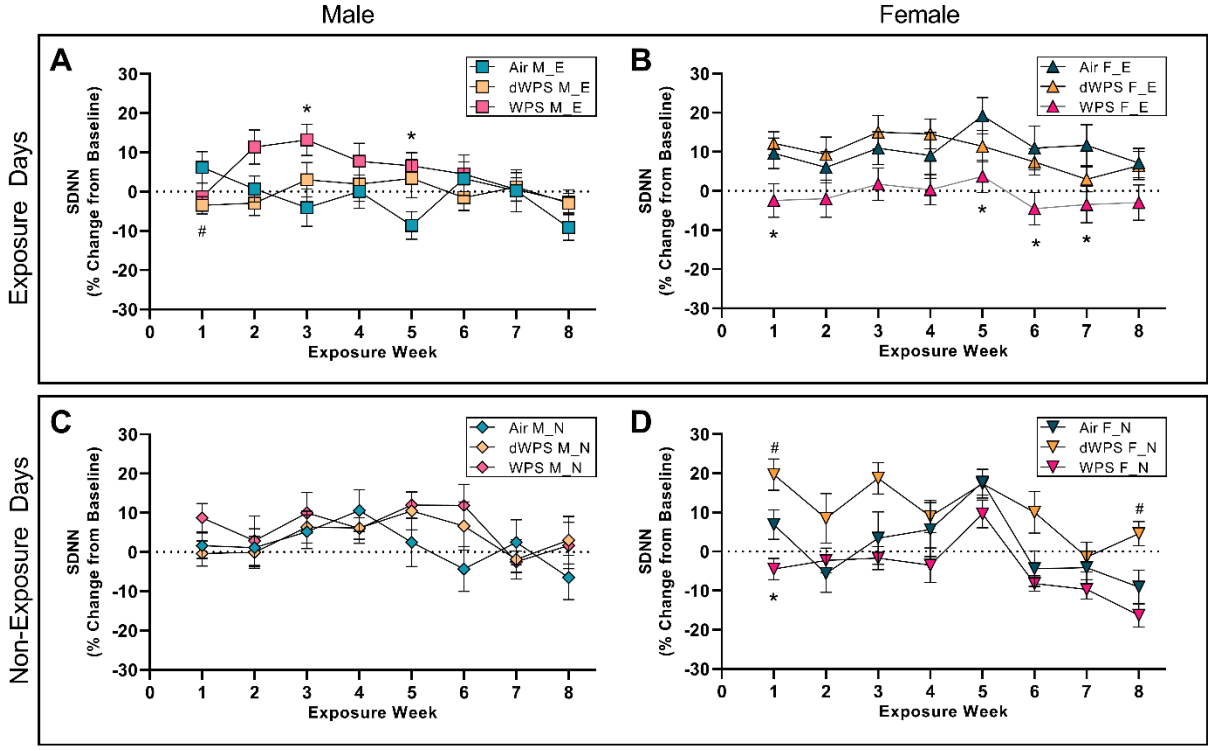

Figure 8. Standard deviation of normal R-R intervals (SDNN) - 2019 study. *SDNN, the time domain measure of total HRV, represented as percent change from baseline for air,* dWPS and *WPS exposed ApoE-/- mice on the evenings of exposure days: male (A) and female (B), and non-exposure days: male (C), and female (D) during* 8 *consecutive weeks of exposure. *P<0.05 compared to purified air.*

4.3.2.2 RMSSD

RMSSD, another time-domain measure of HRV which represents parasympathetic nervous system input to the heart showed statistically significant increases on exposure days in male mice exposed to WPS after week 6 and continued for the remainder of the 2018 study (Figure 9A). Figure 9C shows no differences in RMSSD in males on non-exposure days. RMSSD in females

had an initial increase in WPS exposure during week 1 on exposure days (Figure 9B). During the exposure days of the final weeks of the study, there was a statistically significant decrease in RMSSD of WPS exposed females as compared to air controls. On non-exposure days, WPS-exposed females were similar to air controls until week 11, when RMSSD decreased for the second half of the study (Figure 9D).

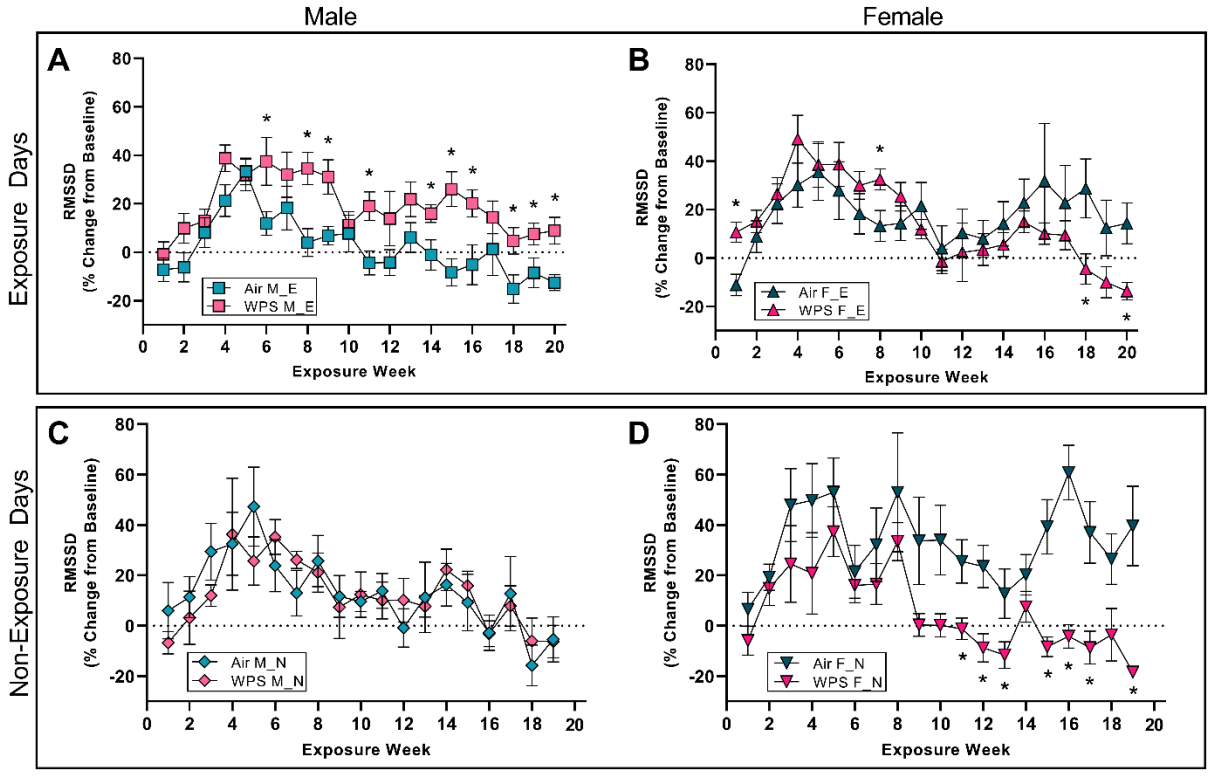

Figure 9. Root mean squared of successive differences (RMSSD) - 2018 study. *RMSSD, a time domain measure of parasympathetic-dominant HRV, represented as percent change from baseline for air and WPS exposed ApoE-/- mice on the evenings of exposure days: male (A) and female (B), and non-exposure days: male (C), and female (D) during 20 consecutive weeks of exposure. *P<0.05 compared to purified air.*

In males during the 8-week study, RMSSD change from baseline was significantly lower in both WPS and dWPS during week 1. Week 3 indicated an increase in RMSSD of WPS exposed males, followed by a return to air values for the remainder of the study. During week 4, dWPS

exposed males had a negative percent change in RMSSD that was significantly lower than that of air controls (Figure 10A). On NE throughout the study, there was a trend of both WPS and dWPS RMSSD change being lower than that of air, however significance was only reached in WPS males (Figure 10C). Female WPS exposed animals had significantly less RMSSD change than air animals on exposure days (Figure 10B), and on NE, the change was no longer apparent. During weeks 1 and 2, dWPS females had greater positive percent change of RMSSD than controls (Figure 10D). This effect did not persist.

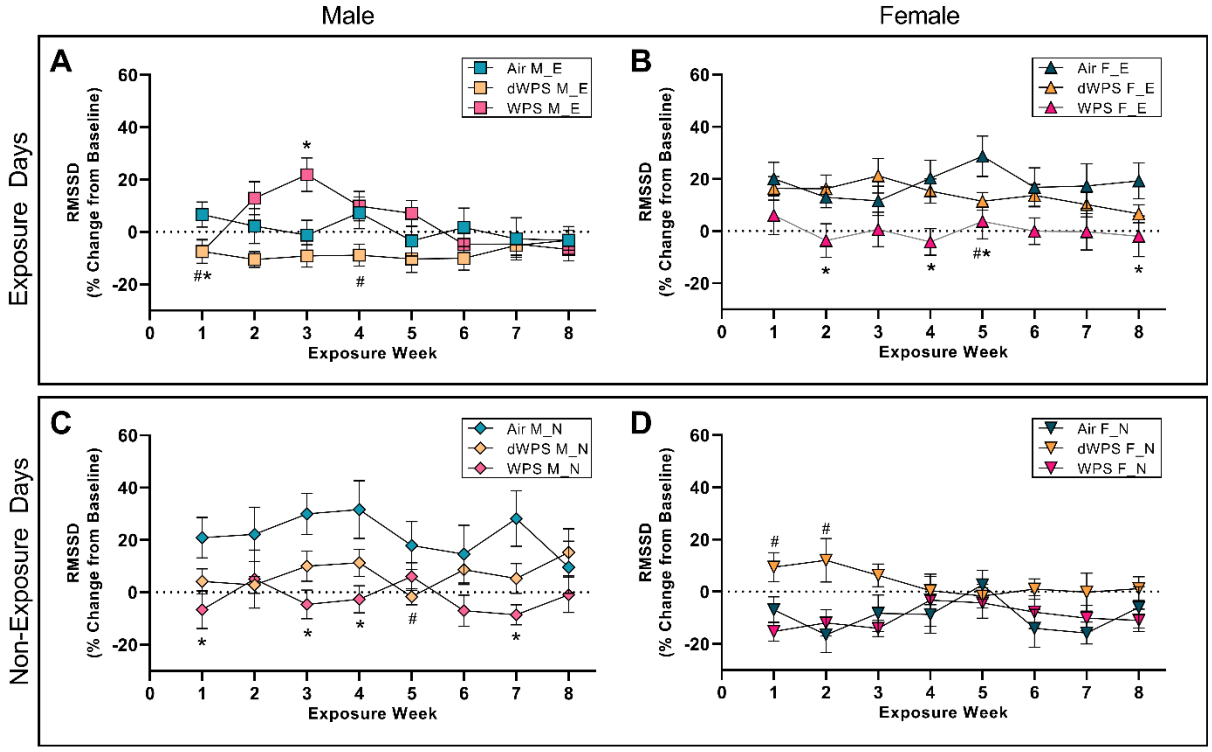

Figure 10. Root mean squared of successive differences (RMSSD) - 2019 study. *RMSSD, a time domain measure of parasympathetic-dominant HRV, represented as percent change from baseline for air,* dWPS and *WPS exposed ApoE-/- mice on the evenings of exposure days: male (A) and female (B), and non-exposure days: male (C), and female (D) during* 8 *consecutive weeks of exposure. *P<0.05 compared to purified air.*

4.3.3 Frequency-domain HRV

4.3.3.1 HF

HRV can also be analyzed through the frequency domain of the ECG signal. In the 2018 study, the trends for HF HRV held in a similar pattern to other HRV measures. In males, after week 8 WPS they began to show statistically significant increases in HF HRV on exposure days (Figure 11A). Figure 11C shows that there was no statistically significant difference in HF HRV of males on non-exposure days. Figure 11B shows that WPS-exposed females had an initial increase in HF HRV during week 1 and week 8 exposure days, which flipped during the final weeks as HF decreased in WPS-exposed females as compared to air. In contrast, on non-exposure days (Figure 11D) HF HRV was decreased from the controls after WPS exposure in females.

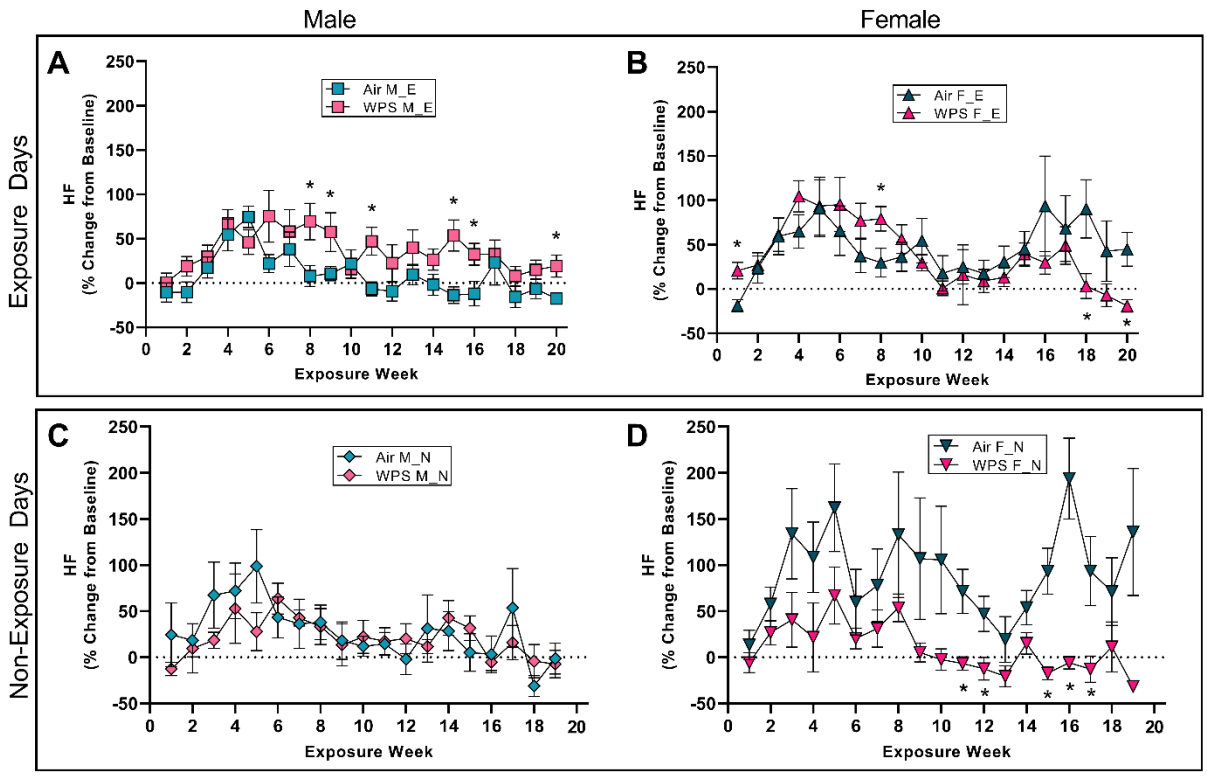

Figure 11. High-frequency (HF) HRV - 2018 study. *HF HRV, a frequency domain measure of parasympathetic-dominant HRV, represented as percent change from baseline for air and WPS exposed ApoE-/- mice on the evenings of exposure days: male (A) and female (B), and non-exposure days: male (C), and female (D) during 20 consecutive weeks of exposure. *P<0.05 compared to purified air.*

High frequency variability changes during the 2019 study were similar to those seen in RMSSD. Male animals showed little effect of WPS on HF changes during exposure days (Figure 12A), but on NE, HF was significantly lower in WPS throughout the study, and periodically dWPS was also significantly lower than the change seen in air control males (Figure 12C). Female mice had significantly less HF change after WPS exposure on exposure days during weeks 2, 4 and 5. dWPS also affected HF change on week 5 (Figure 12B). On NE however, only during week 1 was HF change greater in dWPS females. For the remainder of the study, there was no difference between groups (Figure 12D).

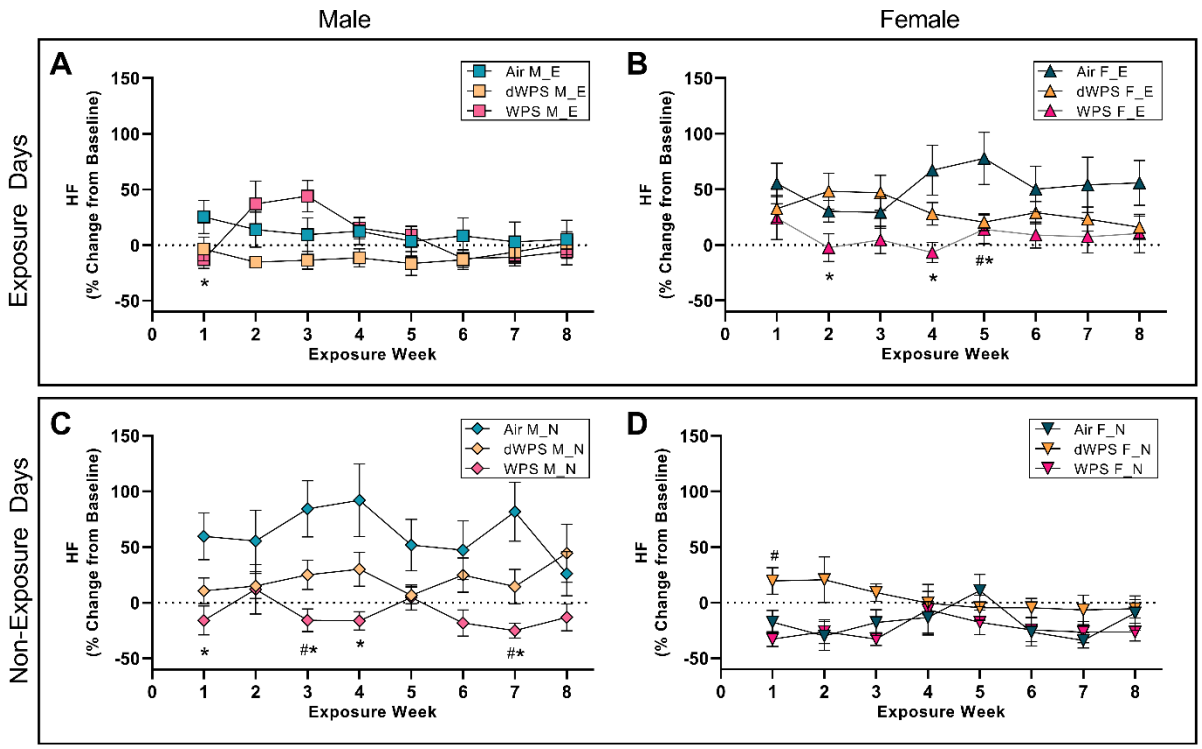

Figure 12. High-frequency (HF) HRV - 2019 study. *HF HRV, a frequency domain measure of parasympathetic-dominant HRV, represented as percent change from baseline for air,* dWPS *and WPS exposed ApoE-/- mice on the evenings of exposure days: male (A) and female (B), and non-exposure days: male (C), and female (D) during* 8 *consecutive weeks of exposure. *P<0.05 compared to purified air.*

4.3.3.2 LF

Low frequency variability in 2018 was affected by WPS in males on exposure days, particularly increased during the last 9 weeks of the exposure, but there was no difference in LF on non-exposure days (Figure 13A and 13C). In females, LF change was lower after WPS exposure from week 5 and onward throughout the study (Figure 13B). The trend of lower LF in the WPS females persisted through to non-exposure days, but was only significant periodically on weeks 5, 11, 12, 16 and 19 (Figure 13D).

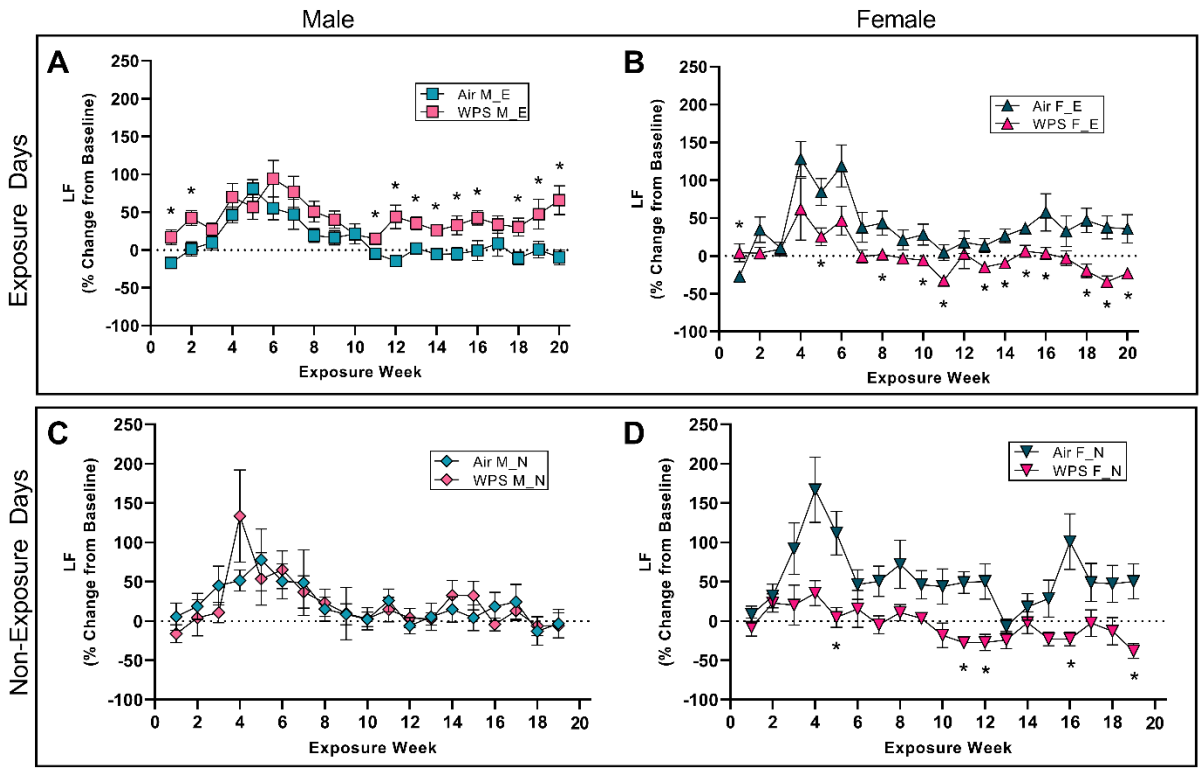

Figure 13. Low-frequency (LF) HRV - 2018 study. *LF HRV, represented as percent change from baseline for air and WPS exposed ApoE-/- mice on the evenings of exposure days: male (A) and female (B), and non-exposure days: male (C), and female (D) during* 20 *consecutive weeks of exposure. *P<0.05 compared to purified air.*

Low frequency variability was minimally affected by WPS or dWPS inhalation in male mice on exposure days during the 2019 study (Figure 14A). On non-exposure days, WPS trended to induce lower LF change than air, reaching significance during weeks 3 and 7 (Figure 14C). Female animals exposed to both WPS and dWPS had significantly less percent change of LF on weeks 4 and 5 on exposure days, and on the non-exposure days of week 5 as well (Figure 14B and 14D).

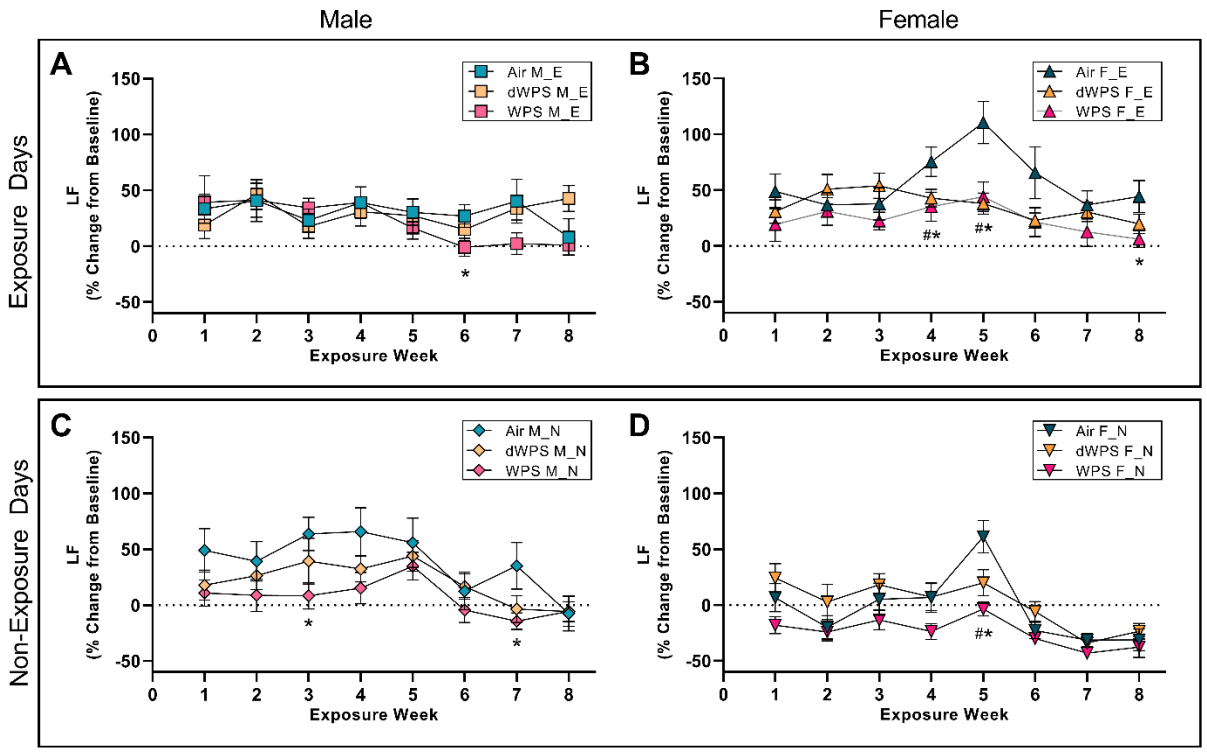

Figure 14. Low-frequency (LF) HRV - 2019 study. *LF HRV, represented as percent change from baseline for air,* dWPS *and WPS exposed ApoE-/- mice on the evenings of exposure days: male (A) and female (B), and non-exposure days: male (C), and female (D) during* 8 *consecutive weeks of exposure. *P<0.05 compared to purified air.*

4.3.3.3 LF/HF

In the 2018 study, despite significant alterations of LF and HF variabilities individually in males, there was minimal difference of LF/HF ratio in WPS exposed males (Figure 15A and 15C). Weeks 1 and 3 at the beginning of the study, and weeks 14 and 20 in the latter half of the study all showed that LF/HF was higher after WPS inhalation. In females, LF/HF ratio was lower after WPS exposure from weeks 4 through 8, after which it was no longer different from the change seen in air females (Figure 15B). On non-exposure days towards the end of the study, LF/HF ratio had a greater change in WPS females, despite no change on exposure days (Figure 15D).

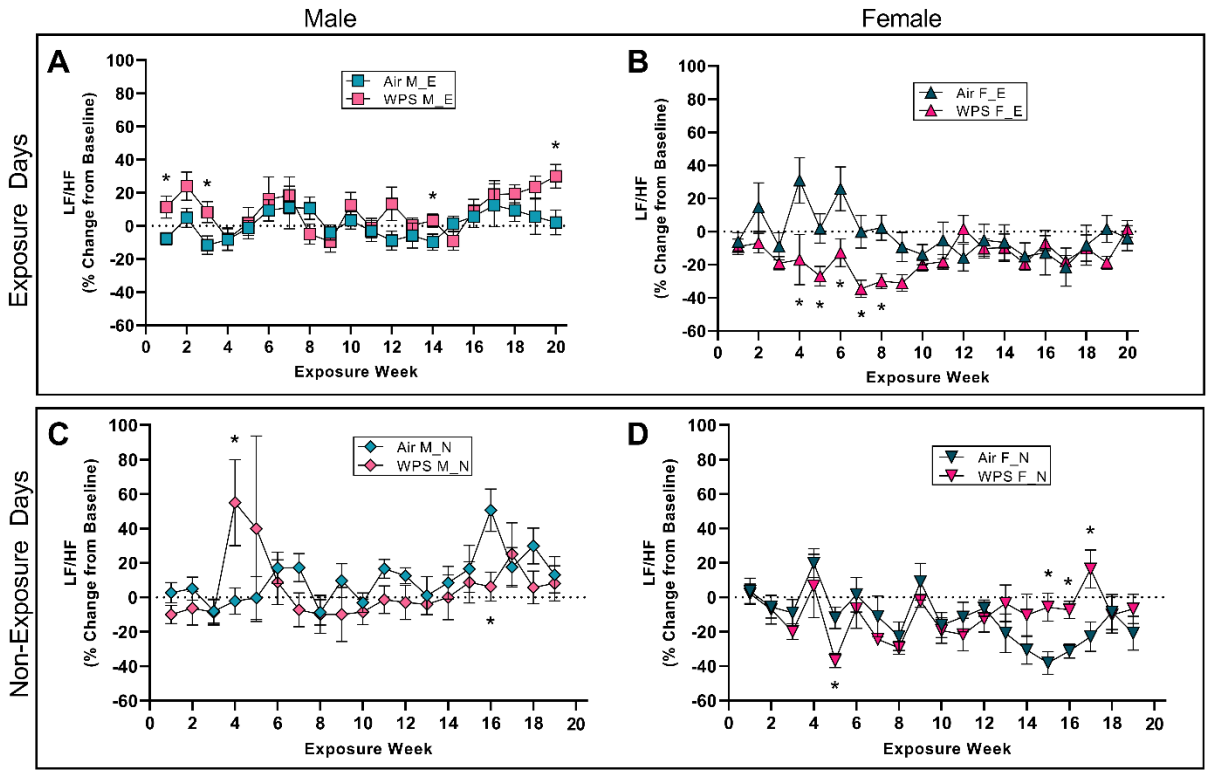

Figure 15. Low- to high-frequency ratio (LF/HF) - 2018 study. *LF/HF represented as percent change from baseline for air and WPS exposed ApoE-/- mice on the evenings of exposure days: male (A) and female (B), and non-exposure days: male (C), and female (D) during* 20 *consecutive weeks of exposure. *P<0.05 compared to purified air.*

Given the respective changes in LF and HF variability in the 2019 study, LF/HF ratio was minimally affected in either sex on exposure days (Figure 16). Males also had no difference in LF/HF on non-exposure days as a result of WPS inhalation. On non-exposure days, the female WPS group had lower LF/HF percent change from week 4 onwards, and a trend in the same direction for the dWPS group, which reached significance as well during week 7.

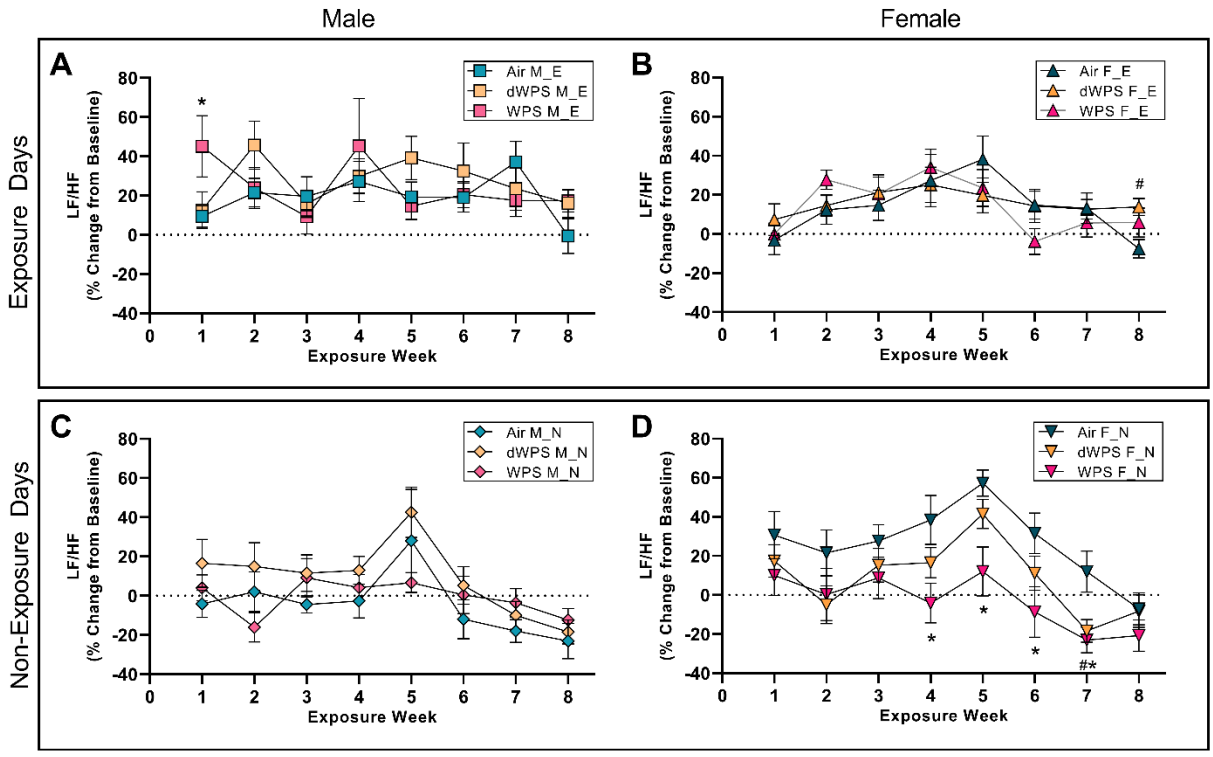

Figure 16. Low- to high-frequency ratio (LF/HF) - 2019 study. LF/HF *represented as percent change from baseline for air,* dWPS *and WPS exposed ApoE-/- mice on the evenings of exposure days: male (A) and female (B), and non-exposure days: male (C), and female (D) during* 8 *consecutive weeks of exposure. *P<0.05 compared to purified air.*

4.4 Discussion

HRV refers to the variation in the beat-to-beat interval of normal sinus rhythm. Assessment of HRV, specifically RMSSD and HF, provides a means to quantitate changes to the parasympathetic, or vagal, branch of the autonomic nervous system (Rowan 3rd et al., 2007), which plays a role in vascular function. Perturbation of the autonomic balance can influence the progression of oxidative stress, atherosclerosis, and CVD (Rhoden et al., 2005). Ambient PM exposures elicit changes to HRV (Liao et al., 1999; Pope et al., 1999). Induction of oxidative stress responses and HRV changes in mice (Rhoden et al., 2005) after exposure to various PM sources indicate the importance of these endpoints in determining the health effects of WPS inhalation.

This study adds to the current body of published literature as it reports on the effects of chronic WPS inhalation exposure on HR and HRV as well as the WPS-induced cardiovascular responses in hyperlipidemic male and female mice. The chronic 20-week exposure experiment better replicates the effects of repeated exposure of humans to WPS than short-term studies. The data show that acute and chronic effects of WPS inhalation on HRV can differ, even in the same animals, and that results from short-term studies may not accurately represent effects of chronic exposures. WPS inhalation in female mice shows an underlying trend towards decreased HRV over time, which is more apparent after chronic exposure. Male animals respond differently to WPS, with increases in HRV measures that become more evident with longer exposure. Denuding the WPS, which removes some of the volatile component of the aerosol, does induce a different autonomic response than whole WPS, particularly in females. This indicates that the autonomic responses to whole WPS are the result of both volatile components and the particulate matter. Monitoring the chemical composition of both the particle and gas phases in future studies will be vital to better understanding WPS-induced toxicities.

4.4.1 Comparison of results between studies

There is some inconsistency with the direction of WPS induced HRV changes between the 8- and 20-week studies, particularly in male animals. The 2019 study replicated the decreases in HRV measures seen in female animals after WPS inhalation, however, this was only apparent on exposure days, as the non-exposure day differences found in the 2018 study only became evident after the 8-week time point, so this is consistent. In males, greater changes in HRV measures were only seen on exposure days during the 2018 study. However, in the 2019 study, the significant changes were lower percent changes in HRV, and the timing of these changes was different, with the main effects occurring on the non-exposure days. The biggest limitations in

comparing the two exposure studies are the differences in the length of exposure and in the concentration of PM. PM concentrations (Table 1) were lower overall in 2019, however they were still in the range of two orders of magnitude higher in comparison to exposures to concentrated ambient PM which also show autonomic disruption by PM.

4.4.2 HRV interpretation

Associations of decreased HRV and late-stage stenosis of the arteries and heart have been previously reported in humans (Hazari et al., 2014; Kurhanewicz et al., 2017). Increases in HRV, as observed in males exposed to WPS, are consistent with previously reported inhalation exposures to respiratory irritants such as acrolein (Hazari et al., 2014; Kurhanewicz et al., 2017) and ultrafine PM,(Rhoden et al., 2005; Zareba et al., 2009) which are components WPS (al Rashidi et al., 2008; Hammal et al., 2015; Monn et al., 2007; Perraud et al., 2019; Schubert et al., 2012). Changes to HRV, in either direction, from expected levels can indicate an imbalance of the autonomic nervous system as a result of pathology or exposure (Ghelfi, 2011). Elevated HRV has been related to an increase of risk for negative cardiac outcomes and associated mortality in elderly people as well (Stein et al., 2005). There is no consensus on whether increased or decreased HRV is a measure of better or worsened health; an imbalance of cardiac autonomic activity in either direction could be a response signifying worsened disease or an effect of environmental exposure.

The drastic drop in HR in weeks 4-9 of the 2018 study is suspected to be due to the sudden decrease in WPS concentrations. If the mice had been acclimated to a level of nicotine in the first 3 weeks, one could speculate that the drastically lower nicotine level could have ‘shocked’ the system. However, the stability of the decreased HR during those weeks is difficult to reconcile with the fluctuations in PM concentration (Figure 5). This inconsistency with PM

concentration is particularly evident with the drop in PM concentration during weeks four through seven followed by a spike over the following two weeks, and the return to similar values and after this PM spike (Figure 2). However, the HR decrease in both males and females was stable during weeks four through nine, indicating that WPS can affect HR, but the elucidation of the dose-response relationship may be complicated by cumulative effects, aging effects and the normal progression of CVD in this animal model. The role of daily variation in PM on changes to HR is supported by the 2019 study, where the particle concentration was much more stable, and the subsequent changes in HR were smoother (Figure 6).

SDNN represents the overall variability of the normal RR-intervals during the sample. The power of the HF band and RMSSD are indications of parasympathetic nervous system influence on the heart. LF HRV is more difficult to interpret as it is influenced by both sympathetic and parasympathetic nervous system stimulations, and therefore it is useful in physiological interpretations only in the context of other vagal-specific changes (Malik et al., 1996; Minarini, 2020; Rowan 3rd et al., 2007; Shaffer & Ginsberg, 2017).

WPS affects the HRV measures, SDNN, RMSSD, HF and LF, which can be concluded as alterations to the parasympathetic nervous system balance. In male animals vagally-mediated increases are occurring mainly on the days of WPS exposure in both studies, and the effect is greatest after 2 months of exposure. WPS also altered parasympathetic nervous system markers in female mice, however in the opposite direction, as a decrease in vagal stimulation. For females, the drop in RMSSD and HF indicate parasympathetic nervous system depression on non-exposure days, but not on days of WPS inhalation indicates a latency in exposure response. There is recovery of the vagal changes seen immediately after inhalation of WPS. Depression of parasympathetic nervous system does not pervade into exposure days until four months of

exposure. After four months, the nervous system changes become persistent and WPS inhalation does not recover or alter back to normal levels. Interestingly, LF HRV is decreased in females on exposure days beginning at 5 weeks of WPS exposure, possibly indicating that changes to the sympathetic nervous system are driving these early changes. Remarkably, the WPS-induced vagal imbalance varies between sexes despite both sexes having a similar decrease in HR during the study, highlighting the importance of assessing underlying HRV changes.

Very few rodent studies have been published on HRV response after tobacco smoke inhalation. There is also minimal consensus on the expected direction of change of HRV after ambient PM exposure in mice independent of sex (Hadei & Naddafi, 2020). The present results are supported by a study on secondhand smoke exposure in male C57BL/6 mice, where only after exposure to a high dose of secondhand smoke was it seen that SDNN and RMSSD were significantly reduced. Their exposure parameters were very similar to this WPS exposure with a total PM concentration of 30 mg/m^3 and CO concentration at an average of 107 ppm, and HRV measures were recorded overnight after each exposure, as done in the protocols described in the present study (C.-Y. Chen et al., 2008). The decrease in SDNN and RMSSD is consistent with the changes seen in females in this study, not with the males, despite the study by Chen et al. being performed in males. Although their smoke was created from cigarettes not a waterpipe, and the exposure only lasted four days, this is supportive of a similar exposure dose inducing changes to SDNN and RMSSD in mice, as seen in the present study.

The discrepancies between different studies on the direction of HRV change after inhalation exposures or due to the chemical composition of the exposures themselves which are highly variable between studies. Some studies have reported HRV changes after exposure to individual components of tobacco smoke or air pollution aerosols (Farraj et al., 2012; Hazari et al., 2014;

Kurhanewicz et al., 2014, 2017). Keebaugh et al. reported that exposure to concentrated ambient PM significantly decreased the percent change from baseline in SDNN, HF and LF, compared to the increases in those measures seen in the control ApoE-/- mice. Those HRV responses were likely dependent however on the presence of semi-volatile organic compounds (SVOCs) on the PM as the effect disappeared when the ambient PM was thermo-denuded prior to exposure (Keebaugh et al., 2015). This was similarly seen in the present study, although the dWPS was not heated to fully desorb SVOCs from the PM, the treatment was aimed to remove the already volatile components of the WPS. SDNN and RMSSD changes seen in the whole WPS groups, were dampened in the dWPS exposed mice for both males and females. This supports the idea that the chemistry of the entire aerosol, as well as the PM content is important for driving cardiovascular responses.

4.4.3 Time-dependent effects

Male and female ApoE-/- HRV results both indicate that time is an important variable in measuring the effect of WPS on the autonomic nervous system. There are changes over the duration of the study with greater effects after a cumulation of weeks of WPS inhalation. In addition, on a weekly basis there are significant differences in the response between the exposure and non-exposure days which are also driven by the sex of the animal. To the best of my knowledge, no other study has reported effects with the combined effect of sex and time latency, as many HRV toxicology studies assess immediate changes which occur during or immediately after an exposure.

It is important to note that HRV is assessed approximately 6-10 hour after the WPS exposure while the animals are resting. This allows us to measure the persistent effects of WPS inhalation, and not just the immediate response to the stress of the exposure or to nicotine alone. At this

time, the half-life of nicotine and its metabolite cotinine would have passed. In mice, the half-life is 9 minutes for nicotine and 35-50 minutes for cotinine (Siu & Tyndale, 2007). Therefore, the HRV changes seen here are not the result of immediate and direct chemical responses of nicotine, which will have already been metabolized, on the autonomic nervous system but are latent responses to the exposure.

Particle clearance rates from the lung offer a possible explanation for this phenomenon of delayed response. Volatile components are quickly absorbed into the tissues of the respiratory tract and begin their cascades of response and metabolism, or they are exhaled in successive breaths. As the size distribution of the ultrafine PM in the WPS has a mode diameter of 200nm with a range from 4-600nm (Perraud et al., 2019), when inhaled, the PM deposits in all regions of the mouse's lung, reaching the alveolar region (Kolanjiyil et al., 2019; Kreyling et al., 2012). Many factors in addition to the size of the PM can affect the deposition and regional dosimetry in the mouse, such as exposure methodology, inhalability, respiratory parameters and differences in anatomy between strains of mice (Asgharian et al., 2014; Bauer et al., 2020; Phalen et al., 1984). Once deposited, there are mechanisms in place to clear the insoluble PM, which are region specific.

Mucociliary clearance is the dominant mechanism of healthy, larger airways of the tracheobronchial region, with physical beating of cilia driving the deposited PM upwards to eventually be swallowed (Asgharian et al., 2014; Bustamante-Marin & Ostrowski, 2017; Phalen et al., 2010). The bulk of this process takes place during the first 24-48 hours after inhalation, however there is some slow clearance PM from mucociliary clearance as well (Phalen et al., 2010; Smith et al., 2008). Cigarette smoke exposure is able to disrupt this process (Cohen et al., 2009; Navarrette et al., 2012). In the deep, alveolar region of the lung, alveolar macrophages

phagocytize PM and remove it from the lung either through the mucociliary clearance pathway or through transportation to the lymphatic system. The smallest nanoparticles may also be cleared from the airways by directly translocating through the interstitial space, particularly in rodents (Kreyling et al., 2012; Oberdörster et al., 2002).

Despite these several active mechanisms of particle clearance from the lungs, there is a large amount of retention, between 20-80%, of slowly dissolving ultrafine and fine particles which remain for weeks to months after inhalation (Kreyling et al., 2012; Möller et al., 2012). These diverse and complex mechanisms of particle clearance are occurring with the PM in the lungs of the WPS-exposed mice. The latency of hours to days to weeks of removal of the PM provides plausible explanation for the delayed and differing responses between exposure and non-exposure days as the exposure to PM continues long after they are removed from the exposure system. In addition, the accumulation of PM in the lungs, as they are repeatedly inhaling large doses of WPS PM before the previous dose can be cleared, a buildup of PM can occur in the lungs, further altering clearance mechanisms and perpetuating the exposure.

Overall, this experiment provided new evidence to the field of WPS toxicology that WPS can induce sex- and time-dependent changes to the parasympathetic nervous system control of the heart, as measured by heart rate variability.

5. Molecular mechanisms underlying the cardiovascular outcomes from WPS exposure

5.1 Introduction

Atherosclerosis development and progression has many complex and integrated underlying mechanisms. To further understand the changes which were seen in IM thickness after WPS exposure, the levels of protein expression of disease biomarkers were investigated in plasma and tissue. CRP and MCP-1 are involved in the perpetuation of the inflammatory cascade of cellular migration and activation that is occurring at sites of atherosclerotic lesion formation. VCAM-1 is additionally important in the adhesion of monocytes and lymphocytes to the vascular endothelium. As biomarkers of tissue remodeling and endothelial permeability, the expression levels of the collagenase MMP-9 and its inhibitor, TIMP-1 are important to assess. These biomarkers, assessed in both circulation and at the tissue level allow insight into possible mechanisms of atherosclerotic lesion progression that may be altered by WPS inhalation.

5.2 Methods

5.2.1 Plasma Biomarker Measurements

Plasma levels of biomarkers were measured using commercially available ELISA kits for c-reactive protein (CRP, cat. # MCRP00, R&D Systems, Minneapolis, MN) and MCP-1 (cat. # MJE00B, R&D Systems, Minneapolis, MN) (Table 8). Manufacturer's protocols were followed.

5.2.2 Tissue Homogenization

Artery tissue samples were stored at -80°C embedded in Tissue-Tek (cat. # 4583, Sakura Finetek, Torrance, CA) OCT compound. Samples were carefully thawed, removed, and cleared of any remaining OCT compound attached to the tissue in 1X-PBS. To achieve sufficient protein

concentration for Western Blot analyses, samples were pooled in batches of two for each homogenate with a final n=3-5 samples assessed per exposure group (Table 6). Fewer samples were homogenized from the 2018 study year, as a subset of samples underwent histological assessment as described in chapter 3. Tissue samples were placed in tubes containing 1.4mm ceramic beads (cat. # 15-340-153, FisherScientific, Pittsburg, PA) with 75 µL of Tissue Protein Extraction Reagent (cat. # 78510, Thermo Scientific, Rockford, IL) as well as 1% protease inhibitor cocktail (cat. # 784540, Thermo Scientific, Rockford, IL). Homogenization was performed by aggressively shaking in a FastPrep-24 (MP Biomedicals, Irvine, CA) at 4 m/s for 20 seconds, repeated 4 times with a 5-minute rest on ice between each run.

	Study Year	Air F	dWPS F	WPS F	Air M	dWPS M	WPS M
Artery Western Blot Analyses (n samples)*	2018	3	-	3	3	-	3
	2019	5	5	3	5	5	3
Plasma Biomarkers (ELISA; n animals)	2019	7	6	7	7	5	7

Table 8. Number of samples and animals assessed for biomarker measurements. **Note that the n for samples assessed by Western Blot are samples containing pooled sets of tissue from 2 animals within the same group to provide sufficient protein content for analysis.*

The homogenate was carefully removed from the bead tube and centrifuged at 10,000g for 5 minutes. The supernatant was then transferred to a fresh tube, followed by protein concentration assessment in duplicate by the Pierce™ BCA Protein Assay Kit (cat. # 23225, Thermo Scientific, Rockford, IL) following the manufacturer's protocol for a microplate procedure to minimize use of sample. Protein concentration was normalized for all samples to 800 µg/µL allowing for 30 µg of protein from each sample to be loaded in each well. Samples were mixed with 6X sodium dodecyl sulfate (SDS) Protein Loading Buffer (cat. # LB0100, Morganville Scientific, Morganville, NJ) and denatured at 95°C for 10 minutes before being quenched on ice briefly and loaded into the wells for electrophoretic separation.

5.2.3 Western Blot Procedure

Electrophoretic separation of proteins was performed using 4-15% gradient polyacrylamide gels (4–15% Mini-PROTEAN® TGX™ Precast Protein Gels, cat. # 4561084, Bio-Rad, Hercules, CA). Gel electrophoresis was run at 60V for 15 minutes to stack proteins in the samples, followed by 120V for 45 minutes to fully separate proteins. Dry transfer of proteins from the gel to a polyvinylidene difluoride (PVDF) membrane was done using the Trans-blot Turbo system (Bio-Rad, Hercules, CA) at 25V and 1A for 30 minutes. The membranes were then blocked with TBS blocking buffer (LI-COR Biosciences, Lincoln, NE) in a dark box for 1 hour at room-temperature.

As sample volume was limited by the small size of mouse aorta tissue, membranes were cut and analyzed in a multiplex manner. Membranes were cut horizontally at a protein size of approximately 45 kDa to allow for larger and smaller proteins to be assessed separately. Each half of the membrane was incubated with one primary antibody at a time. Primary antibodies were chosen to have different host species from the other antibodies to be used on the same portion of the membrane (Table 9). This, in concert with use of both the 700 and 800 nm channels on the LI-COR Odyssey CLx imaging system, allowed for all protein targets for any given sample to be assessed from a single gel, which minimized variation between analyses.

Briefly, membrane sections were hybridized with primary antibodies, diluted in TBS blocking buffer containing 0.3% Tween-20, for 18 hours at 4°C with gentle rocking. After washing with 0.1% TBS-Tween, membranes were hybridized with secondary antibodies at a 1:20,000 dilution in a solution of 0.2% TBS-Tween, 0.01% SDS and 5% non-fat milk. Membranes were incubated with secondary antibody at room temperature for 1 hour before being washed and imaged at the appropriate wavelength using the LI-COR Odyssey CLx imaging system. Following imaging,

membranes were immediately probed for the next target protein and the process repeated until all proteins had been measured.

Target Protein	Host species	Vendor	Catalog #	Size (kDa)	Concentration
VE-cadherin	Mouse	Santa Cruz	Sc-9989	130	1:1,000
MMP-9	Rabbit	Abcam	ab38898	105	1:500
VCAM-1	Goat	R&D Systems	AF643	100	1:800
GAPDH	Rabbit	Proteintech	10494-1-AP	37	1:5,000
TIMP1	Goat	R&D Systems	AF980	27	1:200
CRP	Mouse	Abcam	ab50861	25	1:2,000

Table 9. Primary antibody vendor and concentration information for Western Blot analyses.

5.2.4 Statistics

All data were expressed as means ± standard error of the mean (SEM), unless otherwise stated. Statistical analyses were performed using GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California USA. Outliers were identified using the ROUT method, with a false discovery rate of 0.5%. Significance was assessed at $p<0.05$ using unpaired, parametric t-tests to compare to the purified air control group of the same sex for each measure. Western blot data were normalized within each membrane, to a standard sample loaded equally for each gel, and compared to the control group of the same sex. As samples are normalized to the fold-change of 1 for controls, it is not possible to compare values between males and females quantitatively.

5.3 Results

5.3.1 ELISA

Plasma levels of C-reactive protein were not significantly affected by WPS exposure condition or by sex of the animals (Figure 17A). Similarly, MCP-1 also was not significantly altered by

exposure or sex (Figure 17B). MCP-1 did show a trend of higher concentrations in the control females compared to control males. However this did not reach statistical significance.

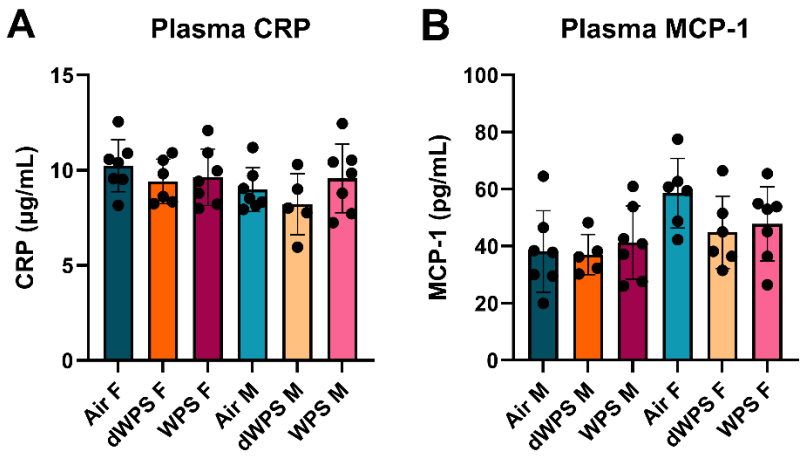

Figure 17. Plasma concentrations of CRP and MCP-1 *after 8-weeks of inhalation exposure.*

5.3.2 Protein expression after 5-month WPS exposure

Overall, WPS inhalation did not induce any statistically significant changes to protein concentrations of CRP, VCAM-1, MMP-9, or TIMP-1 in artery tissue (Figure 18). However, due to the small sample size and limitations associated with pooling tissue, discussion of the trends in changes may still be informative to future research questions. WPS did not alter CRP in males or females (Figure 18A). VCAM-1 increased slightly in females, while decreasing in males (Figure 18B). MMP-9 was increased almost 3-fold after WPS exposure in females, and unchanged in males (Figure 18D). TIMP-1, the inhibitor of MMP-9, showed a slight increase in females and a slight decrease in males (Figure 18E). When assessing the ratio of MMP-9/TIMP-1, this translates to a higher overall MMP-9/TIMP-1 ratio in all WPS exposed groups (Figure 18F). The uptake of Evan's blue dye (EBD), as measured via imaging on the Western membrane, was

approximately 1.75-fold greater in WPS exposed females, but this did not reach significance. No change in EBD uptake was found in WPS males (Figure 18C).

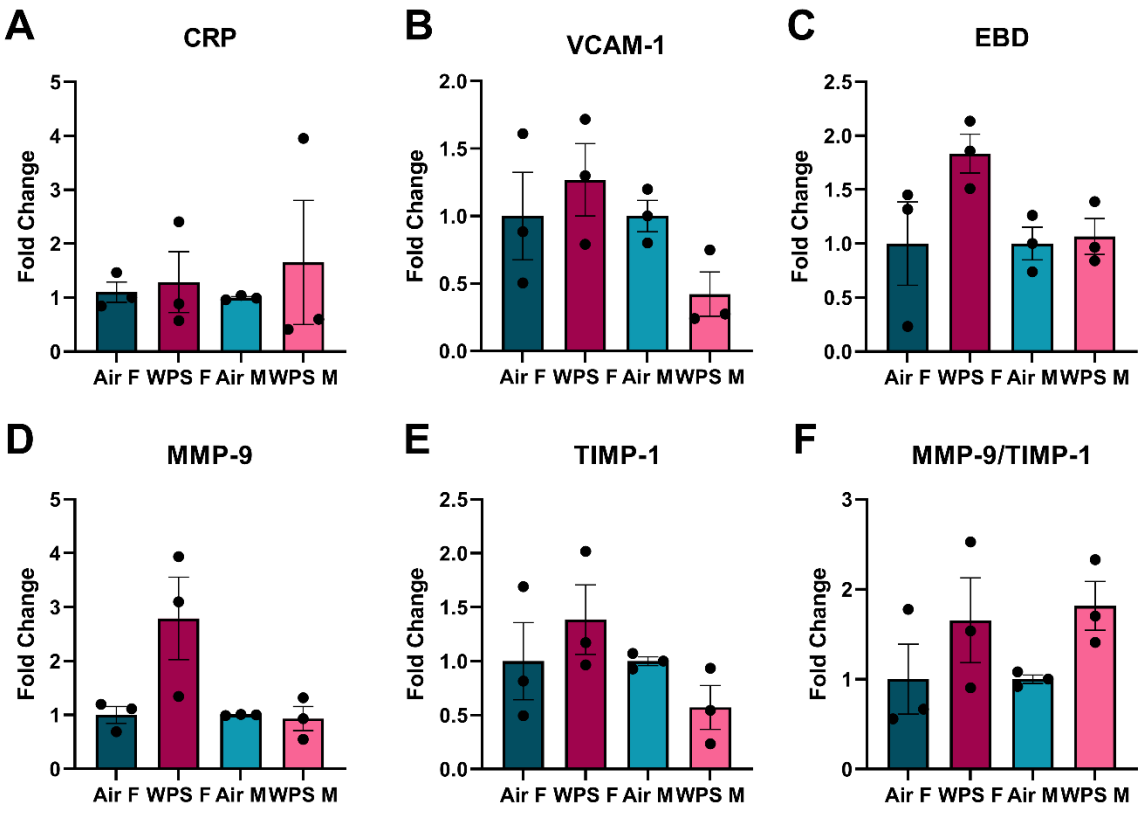

Figure 18. Protein expression levels in artery homogenate – 2018 study.

5.3.3 Protein expression after 8-weeks of dWPS or WPS exposure

In artery tissue homogenate from the 8-week exposure, protein expression varied (Figure 19). The acute phase protein CRP was significantly decreased by 67% in WPS females. A non-significant decrease of 33% was found in females exposed to denuded WPS. dWPS and WPS showed similar non-significant decreases in males (Figure 19A). VCAM-1 had a non-significant trend of a decrease incrementally with dWPS and WPS in females. There was no difference in VCAM-1 in males (Figure 19B). No statistically significant changes were evident in MMP-9 or

TIMP-1 expression, however, there were some interesting trends (Figure 19D-F). In female mice, MMP-9 and TIMP-1 showed a decrease after dWPS exposure, which was further exacerbated in the WPS group. Male mice did not show any substantial trends in exposure related changes of MMP-9 or TIMP-1 expression. As MMP-9 and TIMP-1 showed parallel changes within each exposure group, the ratio of the two proteins did not significantly change. Evan's blue dye uptake into the artery was significantly increased in WPS males, with an opposing trend towards less EBD uptake in WPS females.

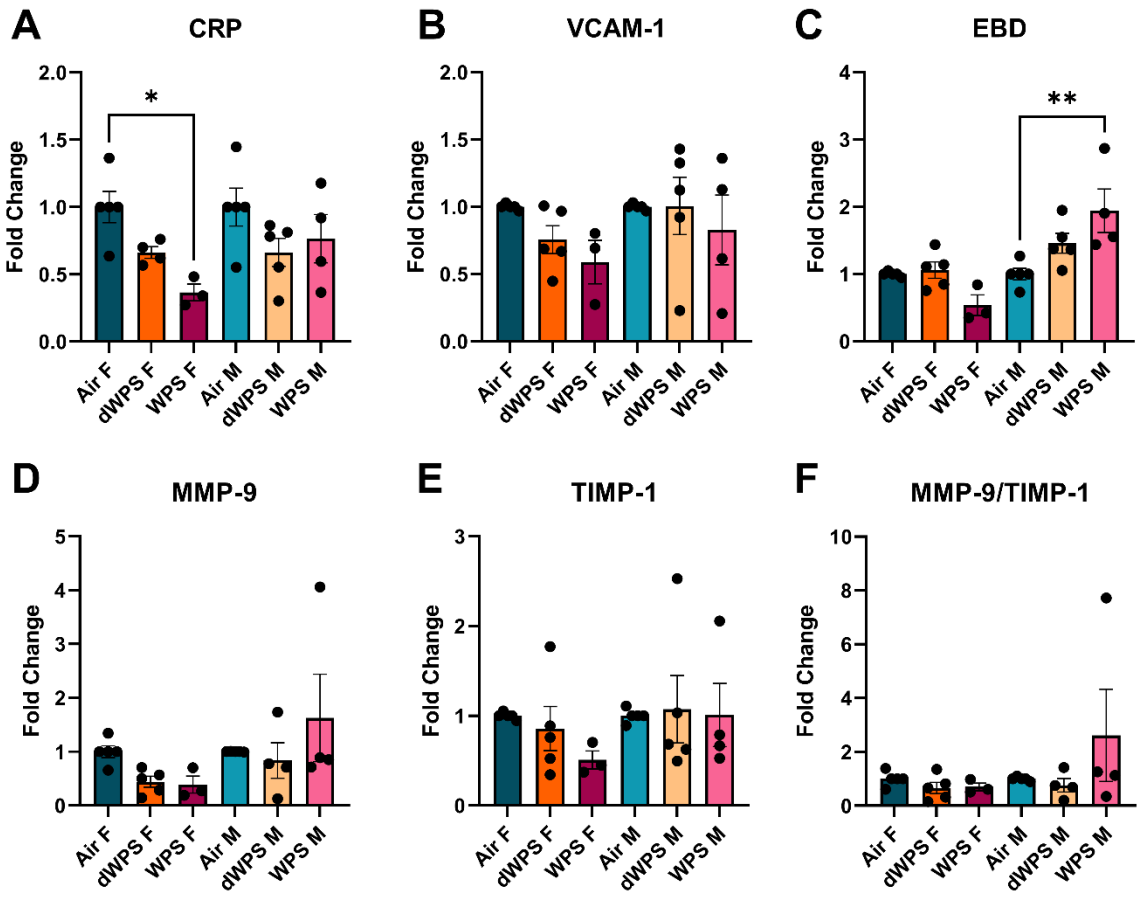

Figure 19. Protein expression levels in artery homogenate – 2019 study. **p<0.05 comparisons to air control group within each sex.*

In addition to protein expression in the arteries, western blot analysis was performed to assess protein expression levels of VCAM-1 and CRP in the heart and lung tissue of mice exposed during the 8-week study in 2019 (Table 10). In support of the decrease in arterial CRP in female mice exposed to WPS, there was a significant decrease in cardiac CRP in dWPS females, and a trend in the same direction in WPS females. Similar, non-significant, decreases were seen in cardiac VCAM-1 in females. Males exposed to WPS had a trend suggesting an increase in CRP and VCAM-1 in cardiac tissue. In the lung, no statistically significant changes were found in expression of CRP or VCAM-1, although a non-significant 24% increase in VCAM-1 was indicated after WPS exposure in females.

Table 10. Protein expression in heart and lung tissue - 2019 study.

		Male			**Female**		
		Air	dWPS	WPS	Air	dWPS	WPS
Heart	VCAM-1	1.00 ± 0.06	0.93 ± 0.21	1.32 ± 0.38	1.00 ± 0.16	0.76 ± 0.10	0.76 ± 0.14
	CRP	1.00 ± 0.06	1.10 ± 0.11	1.28 ± 0.25	1.00 ± 0.10	0.72 ± 0.09*	0.84 ± 0.10
Lung	VCAM-1	1.00 ± 0.13	0.95 ± 0.16	1.17 ± 0.22	1.00 ± 0.11	1.09 ± 0.19	1.24 ± 0.24
	CRP	1.00 ± 0.03	1.05 ± 0.10	1.02 ± 0.08	1.00 ± 0.05	1.05 ± 0.11	1.05 ± 0.09

5.4 Discussion

5.4.1 WPS influence on biomarkers of cardiovascular disease

Although a full panel of circulating biomarkers was not assessed, CRP, as an acute phase protein, has a longstanding association with atherosclerosis and inflammation (Badimon et al., 2018). Circulating levels of CRP are very sensitive to the presence of an inflammatory stimulus. Concentrations can dramatically rise hundreds- to thousands-fold in the span of hours and then fall equally as fast (Badimon et al., 2018; Baltz et al., 1985; Vigushin et al., 1993). Despite the hypothesis that WPS, as a tobacco source of PM, would induce inflammation, there was no change in circulating CRP levels after 8-weeks of WPS inhalation. Nemmar et al. also found that plasma CRP was not altered by a short, 5-day exposure to WPS (Nemmar et al., 2015). Tissue

expression of CRP was decreased, and consistent, in both artery and hearts of WPS-exposed females. This is supported by a study in humans, which found a slight, but non-significant increase in plasma CRP in regular waterpipe users (Khan et al., 2019). Although, the study did not report or control for the time since the participants last smoked, which would greatly affect the expected CRP levels. The lack of change in plasma CRP in the present animal study may be due to differing roles and rates of metabolism of CRP in mice as compared to humans, where its importance in atherosclerosis mechanisms was initially developed.

Teupser et al. assessed the effect of knocking out CRP in ApoE-/- mice on lesion development. Their results indicated that knocking out CRP substantially increased lesion size in males by 71%, while not altering lesions in female mice. In ApoE-/- mice, they found no evidence of age-related, and therefore lesion progression related, increased concentrations of CRP in either males or females (Teupser et al., 2011). In addition, the metabolism rate of CRP is very different between species, with a half-life of 19 hours in humans, but only 4 hours in C57BL/6 mice (Baltz et al., 1985; Vigushin et al., 1993). This indicates that although CRP is an indicator of systemic inflammation, it is produced in the liver, where it may be an indicator of pro-atherogenic influences but CRP may not be atherogenic in and of itself in ApoE-/- mice as it is hypothesized to have in humans. Therefore CRP is not a reliable marker of progression, or resolution of disease in this study.

It is important to note that all mice in this study, including controls, are hyperlipidemic mice so perhaps it is just that WPS is not further altering inflammation at this stage to a level any different than expected at the given stage of disease progression. In addition, blood collection for these analyses was performed approximately 24 hours after their final exposure. As the half-life for some cytokines is within this period (Vigushin et al., 1993). at this point WPS induced

changes may no longer be measureable. The levels of CRP and MCP-1 at this time point may be more indicative of only atherosclerosis induced changes to cytokines, which may be more localized to the tissues themselves, rather than systemic, and be lower in concentration.

Tani et al. showed increased intima-media thickening after cigarette smoke exposure in ApoE-/- mice, but an abnormal inflammatory response to oxLDL and other circulating cytokines, while there were no changes to serum antioxidant potential (Tani et al., 2004). This supports the conclusion that tobacco smoke does not always produce the expected biochemical changes, even when tissue level damage and lesions are affected, as seen in the WPS exposed ApoE-/- mice, and that the underlying mechanisms are likely more complex.

Rababa'h et al. found that acute and chronic exposures to WPS produce different biomarker fingerprints. Initially, after 2 weeks of exposure, MMP-9, MMP-3, TIMP-1 and myeloperoxidase were upregulated in murine cardiac tissue. However, after 8 weeks of WPS exposure, these markers indicative of cardiac distress were no longer elevated above those found in control animals. The only biomarker measured which increased at both 2- and 8-week exposure time-points was endothelin-1, a vasoconstrictor with evidence of involvement at several stages of atherosclerosis (Rababa'h et al., 2019; Sutton et al., 2019). While the authors describe their results in the context of atherosclerosis, they only examined tissue from hearts, and did not assess protein expression levels in aortic or any artery tissue, nor was the study performed in animals prone to atherosclerosis, making it difficult to compare to the present study. In humans who have atherosclerotic plaques and chest pain, the levels of serum MMP-9 have been associated with sex, suggesting that MMP-9 may have differing expression mechanisms in atherosclerosis, which supports the present results (Gu et al., 2017). The resolution of MMP

upregulation and oxidative stress changes after 8-weeks of WPS exposure is consistent with the results seen in the present study.

In a short, 10 day, WPS exposure study, Khan et al. found a significant increase in lymphocyte and neutrophils in bronchoalveolar lavage fluid (BALF), in addition to increases in pro-inflammatory cytokines in BALF. These results were affected by the strain and sex of the mice being studied. Particularly, lipid peroxidation of lung tissue, a marker of oxidative stress, was increased in male mice, but decreased in female mice, further suggesting that there are sex-dependent mechanisms underlying WPS immune response (Khan et al., 2018). Longer exposures to WPS have been done to investigate chronic immune responses. Reyes-Caballero et al. recently published that in C57BL/6 mice, 6 months of repeated WPS exposure suppressed pulmonary immune responses. Unexpectedly, significantly fewer lymphocytes and neutrophils were found in BALF. Levels of chemokines involved in the recruitment and activation of immune cells in the lung were also significantly reduced (Reyes-Caballero et al., 2019). Together, these studies suggest that mice have a dynamic response to WPS, and that the length of exposure is important in assessment of damage likely with an initial increase in inflammation signaling, followed by a resolution, and further suppression of immune response. Further studies should be done to better understand the implications of this possible WPS-induced immune suppression on the body's ability to fight infections.

VCAM-1, an important molecule in mediating the adhesion of circulating lymphocytes and monocytes to the vascular endothelium at sites of injury, was slightly increased in females after 5 months of WPS exposure, but decreased in males at that time point, and decreased in females at the earlier time point of 8-weeks of exposure. Altered trends in CRP and VCAM-1 in the arterial tissue during the 8- and 20-week exposures could indicate that WPS is interfering with normal

inflammatory and cellular recruitment pathways in the vascular system, as suggested by the assessment of WPS-induced immune suppression in the lungs.

In the current study, with longer exposure period, the trends in biomarkers shift; females exposed to WPS showed trends towards greater inflammation and cell recruitment. Although the results were not statistically significant, possibly due to the small number of samples that were available for homogenization, the direction of changes can help to inform future research. At 32-weeks old, the animals in the 5-month exposure group are expected to have lesions in the intermediate and fibrous plaque development stages (Nakashima et al., 1994). This is validated by the histology results from Chapter 3 on the same groups of animals. Histological analyses showed visible deposition of collagen throughout the atherosclerotic lesion areas. Being in this fibrous lesion stage likely explains that there is greater relative MMP-9 activity in the older animals, as collagenase activity is involved in fibrotic tissue remodeling.

In summary, WPS did not elicit overt or consistent increase in biomarkers of inflammation in systemic circulation, nor in the tissue of the aortic arch, heart or lungs. Female ApoE-/- mice exposed to WPS overall had the most sensitive response, with suppression of CRP expression in cardiac and aortic tissues after 8-weeks of WPS inhalation and an increase in MMP-9 expression in aortic arch after chronic WPS inhalation. These results indicate that WPS can influence inflammation and tissue remodeling in a dynamic and sex-differentiated manner.

5.4.2 Limitations

There are several limitations that should be considered with the results of biomarker expression. These results should be replicated and supported by further studies containing greater animal numbers and statistical power to verify the conclusions. Furthermore, collection of the entire abdominal aorta would provide greater tissue mass to assay in each animal, removing the need to

pool samples and further increasing the sample size of Western blot analyses. As atherosclerosis develops in ApoE-/- mice along the descending abdominal aorta, this region would also be useful in further understanding of molecular changes induced by WPS. Performing the necropsy of animals immediately after the conclusion of their final exposure may also give greater insight into the WPS-induced changes in systemic inflammatory markers.

6. Summary Discussion

6.1 Summary of Key Findings

The experiments and studies described within this dissertation expand on and add to the current scientific knowledge of the cardiovascular toxicity of WPS inhalation exposure. It was found that despite WPS being bubbled through water prior to inhalation, the tobacco smoke alters the autonomic nervous system and development of atherosclerosis in hyperlipidemic mice. Long-term inhalation of WPS induced an overall drop in vagal influence on HRV in female ApoE-/- mice. The vagal branch of the ANS was also affected in male mice, with WPS inducing greater vagal inputs to the heart immediately following WPS exposure. These sex-dependent changes to the vagal mediation of the heart are affected moderately by the removal of SVOCs from WPS, particularly in female ApoE-/- mice. WPS induced greater endothelial permeability of the aortic arch in male ApoE-/- mice after 8-weeks of smoke exposure. Atherosclerosis, measured after 5 months of repeated WPS exposure, was overall increased in all animals. Greater atherosclerotic plaque incidence, with less severity of IM thickness, was found in female mice. In male mice however, arterial walls were thickened after WPS inhalation, with no change in the incidence of plaque formation, indicating an overall increased progression of atherosclerosis. In all, the studies described herein support that WPS can induce cardiovascular and autonomic nervous

system toxicities in a hyperlipidemic mouse model, and should be considered for further study of the sex-dependent mechanisms underlying WPS toxicity.

This study addressed effects of exposure of an animal model of cardiovascular disease, hyperlipidemic ApoE-/- mice, to either 8 or 20 weeks of WPS and 8 weeks of denuded WPS (Figure 20). To the best of my knowledge, there have been no published studies uniquely assessing the combined effects of WPS and hyperlipidemia on heart disease. Methods and use of a newly developed inhalation exposure system designed specifically for waterpipe studies were presented. In addition, this dissertation provides uniquely developed methods for assessment of heart rate variability in chronic mouse studies, which prioritize reproducibility and accuracy in studies such as this which produce large amounts of ECG data. The proposed hypotheses at the onset of these experiments were that WPS would increase inflammation and endothelial permeability leading to exacerbation of atherosclerotic lesion progression. It was hypothesized that these systemic perturbations would also lead to changes in heart rate variability measures indicative of a loss of parasympathetic stimulation of the heart as an indication of further WPS-induced exacerbation of cardiovascular disease.

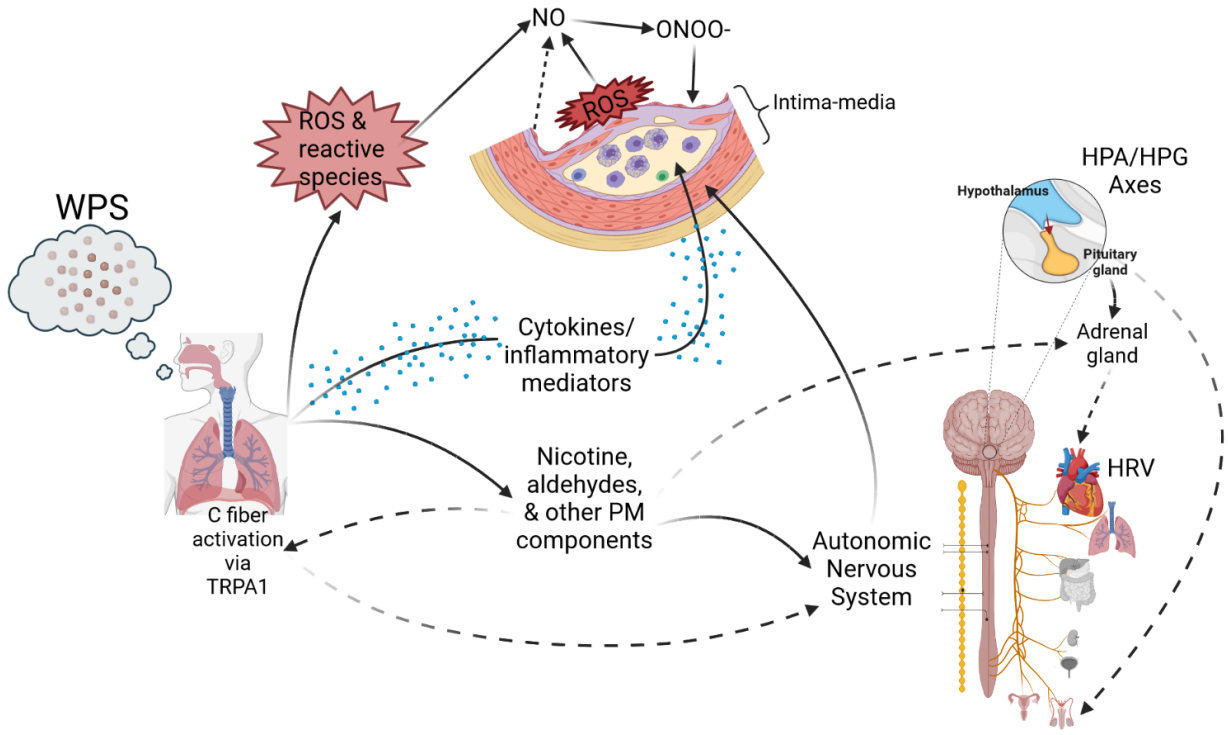

Figure 20. Diagram of key findings and proposed mechanisms of WPS induced cardiovascular toxicity in ApoE-/- mice. *WPS is inhaled and contains ROS and many chemical and PM components. WPS inhalation induction of ROS and inflammatory mediators lead to progression of atherosclerosis development. WPS induces greater vagal influences on the heart in male ApoE-/- mice, while depressing vagal input to the heart in female ApoE-/- mice. Proposed mechanisms, indicated by dashed lines, involve C fiber activation in the respiratory tract, hypothalamic-pituitary axes activations, and toxicity to the reproductive systems.*

As the study progressed it became apparent that the combined effect of WPS and cardiovascular disease is complex. Although no initial hypotheses were made regarding differences between males and females, adequate numbers of both were included. The results highlighted that sex of the animal, as well as time, both time since last exposure and cumulative time of WPS inhalation, are important cofactors in determining the effect of WPS inhalation. It was hypothesized that animals which showed an increase in IM thickness of the arteries and aortic arch, would show complementary decreases in vagally mediated HRV measures. However, the result was the opposite, and dependent on sex. Male mice developed thicker IM as well as

increases in HRV measures in response to WPS. Female mice alternatively had thinner IM in the WPS-exposed group than the controls, while progressively showing decreases in HRV measures.

Inflammation and endothelial permeability were also not as impacted as had been hypothesized. There was only a trend of increased CRP mediated inflammation after 5 months of WPS exposure. However, after 8 weeks of exposure to whole WPS, male mice did show significant evidence of increased endothelial permeability as measured by extravasation of Evan's blue dye. The lack of plaque development in female mice compared to the control animals could be driven by the types of altered responses seen in the biomarkers collected after the 8-week exposure study. Evidence of less inflammation and vascular adhesion markers would lead to less overall infiltration of inflammatory cells into the arterial wall, which is the result found during histological assessment of the arteries after 5-months of WPS inhalation. In summary, despite differences from the initial hypotheses, this dissertation presents results that WPS exposure is able to induce cardiovascular toxicity as assessed by heart rate variability, and that WPS can influence the progression of atherosclerosis in a sex-dependent manner.

6.2 Possible mechanisms underlying WPS induced cardiotoxicity

6.2.1 Systemic effects of WPS

In the last decade, as scientists have become more aware of the widespread use of waterpipe by people worldwide, the studies investigating the toxicity of WPS have also increased. Pulmonary toxicity has been determined in that WPS exposure increases proliferation of epithelial cells in the alveoli of the lung, increasing cellular turnover (Shihadeh et al., 2014). In animal studies, WPS exposure has shown capable of inducing oxidative stress as seen in doubling lipid

peroxidation levels in the lungs and liver, in addition to collapsing activity of superoxide dismutase, glutathione peroxidase and catalase, all which are mechanisms in the body's antioxidant pathways (Charab et al., 2016; Khan et al., 2018). Studies have also shown decreases to antioxidant pathway mechanisms in other tissues such as the heart (Nemmar et al., 2013, 2015), and the testes (Ali et al., 2017). In addition, several different groups have reported that WPS accelerates coagulation and risk of thrombosis mice (Alarabi et al., 2020; Nemmar et al., 2015). Although these endpoints were not assessed in the present study, the results of these studies suggest that the increases in IM thickness seen in the male mice exposed to WPS may be driven by similar increases in oxidative stress via reduction of antioxidant capacity and changes to blood chemistry.

Increases in oxidative stress could also influence the changes seen in HRV measures. Rhoden et al. performed an experiment in which pre-treatment of rats with an antioxidant, n-acetylcysteine, was able to prevent HRV changes induced by ambient PM exposure. In addition, they assessed the pathway connecting oxidative stress and HRV in reverse, by showing that pre-treatment with chemical blockers of either branch of the ANS before PM exposure significantly reduces the expected cardiac oxidative stress response (Rhoden et al., 2005). This study highlights that the ANS is linked to systemic changes in oxidative stress. With studies showing WPS reduces antioxidant capacity in mice, this indicates that increases in oxidative stress are a probable mechanism by which WPS is inducing autonomic nervous system changes. Similar mechanisms have been proposed by Mladěnka et al. to explain the actions of cigarette smoke, and nicotine, to induce endothelial damage in the vascular system, disruption of the autonomic nervous system, activation of the adrenal gland, and disruption of normal cardiac rhythms (Mladěnka et al., 2018).

6.2.2 Stimulation of TRPA1 chemoreceptor by the WPS aerosol

The vagus nerve is not only important in the efferent pathways responsible for producing parasympathetic nervous system stimulation, but it is also involved in afferent pathways transmitting sensory signals from the body to the brain. One relevant portion of this vagal afferent system are the C fibers located in the bronchopulmonary regions of the lung (Undem & Sun, 2019). These C fibers express receptors which sense particular chemicals as well as pain within the respiratory tract. One function of these nerves is to induce cough responses to expel a noxious inhaled compound, or excess mucous which may have been produced from infection or trauma. Specifically of interest however is the transient receptor potential ankyrin 1 (TRPA1) ion channel, which is expressed on these bronchopulmonary C fibers (Nassenstein et al., 2008). TRPA1 acts as a chemoreceptor particularly sensitive to aldehyde compounds such as those found in the WPS aerosol like formaldehyde and acrolein.

TRPA1 activation has recently been highlighted as one of the foremost mechanisms by which acrolein induces systemic and autonomic nervous system responses. Acrolein, and other aldehydes, act as electrophiles and subsequently activate TRPA1 (Achanta & Jordt, 2017). TRPA1 is an ion channel which opens, allowing an influx of calcium into the neuron and inducing an action potential to send a signal to the nucleus of the solitary tract (NTS) in the brainstem. The NTS acts as a hub for acquiring the afferent signals from the body and passing them on to the central nervous system, such as the hypothalamus, as well as to the efferent vagal pathways (Boscan et al., 2002). The hypothalamus is involved in the central autonomic network which connects the pre-frontal cortex to the peripheral autonomic nervous system responses, such as those measured in HRV (Thayer et al., 2009). In this way, acrolein, and other inhaled aldehydes, are capable of triggering the ANS via the TRPA1 receptor in the respiratory tract.

The ability for acrolein to induce ANS changes has been repeatedly found in animal exposures. Hazari et al. has shown that inhalation of acrolein induces autonomic imbalance, arrhythmia, and a dampening of the vagal baroreflex signals. Through administration of an agonist of TRPA1, they verified that these ANS effects were mediated by the TRPA1 receptor (Hazari et al., 2011, 2014). Furthermore, when exposed to very high levels, 100-275 ppm, of acrolein, 75% of the C57BL/6 male mice died within 24 hours, compared to only 10% loss in the female mice. This indicates that female mice have some protection against acrolein induced toxicity. However, when TRPA1 is knocked out the female mice are no longer resistant to acrolein toxicity (Conklin et al., 2017). This suggests that there is a sex-dependent response to acrolein exposure which is mediated by TRPA1.

Female mice, although resistant to death induced by high concentrations of acrolein, still produce acrolein mediated stimulation of vagal control of the heart. Kurhanewicz et al. exposed radiotelemetry implanted female mice to low, 3ppm, levels of acrolein. This exposure induced increases in SDNN, RMSSD and HF HRV measures, which were not evident at all in the TRPA1 knockout animals (Kurhanewicz et al., 2017). This study highlights that inhalation exposure to acrolein, which is known to induce HRV changes, is reliant upon signaling via the TRPA1 pathway.

As acrolein, as well as several other aldehydes which also activate TRPA1, were found in the WPS (Table 3) it is plausible that these compounds, and the subsequent neurological pathway, are responsible for triggering the ANS responses seen. The differences between HRV responses in dWPS and WPS exposed animals may also be explained by this mechanism. Whole WPS contains both the gas and particle phase components, whereas dWPS has had much of the gas phase removed. Perraud et al. found that aldehyde content was split between both phases, with

acetaldehyde, acrolein and propionaldehyde dominating in the gas phase, and formaldehyde remaining associated with the PM as it is a highly soluble chemical (Perraud et al., 2019). As dWPS only contains a portion of the aldehyde compounds, it is expected that the response would be diminished, as seen in the HRV responses from the 2019 study (Figures 8, 10, 12, 14, 16).

In addition to mediating HRV responses, chronic exposure to acrolein has also been shown to increase lesion size in atherosclerosis of male ApoE-/- mice, by increasing plasma cholesterol and activation of vascular endothelium (Srivastava et al., 2011). Although this study used oral ingestion of acrolein, rather than inhalation, and therefore the mechanism may be different than the triggering of bronchoalveolar chemoreceptors. However, although the direct mechanism may not be via TRPA1, as previously discussed, particle clearance pathways in the respiratory tract utilize macrophages and mucociliary clearance to bring deposited PM, which in the WPS contains formaldehyde, upwards in the lungs to be eventually swallowed. Through this mechanism, ingestion becomes a secondary route of exposure, through which the WPS PM can further induce toxic effects.

Overall, although TRPA1 involvement was not specifically tested in this WPS exposure study, the presence of several compounds which are well known to trigger TRPA1 signaling are present in the WPS. Therefore, TRPA1 activation of the NTS and hypothalamus is a plausible pathway instilling changes to the ANS after WPS inhalation.

6.3 Sex-differences in WPS cardiotoxicity

Overall exposure to WPS in ApoE-/- male and female mice induced inconsistent results between outcomes in accordance with the initial hypotheses. WPS was hypothesized to increase inflammation and oxidative stress leading to a worsening of atherosclerotic lesions measured as greater IM thickness and area (Araujo & Nel, 2009). As a result of greater atherosclerosis, it was

expected that HRV would decrease by means of altered baroreflex sensitivity and by altered CNS regulation of the ANS branches from the systemic inflammation and oxidative stress (Nasr et al., 2005). An overall decrease in HRV in female animals occurred after extensive exposure to WPS, however the IM thickness was not worsened, and in fact had a better overall atherosclerosis outcome. The male animals, who showed greater IM thickness of the aortic arch had greater HRV on the exposure days, with no changes in HRV on non-exposure days. This indicates that the changes to HRV by WPS in males was transient and not likely to be due to the chronic development of worsening lesions. Overall, the directions of change in both atherosclerotic endpoints and HRV were opposite between males and females. Baroreceptor activity is possibly not altered in either male or female mice, as the expected connection between lesion development and vagal changes to HRV are inconsistent and therefore WPS may be acting on each of these outcomes in an independent manner (Nasr et al., 2005). Correlation assessments linking HRV and IM thickness found no significant relationships or trends, further supporting that they are influenced via independent mechanisms.

6.3.1 Sex- differences in HRV

Differences in the resultant response to WPS could be driven by underlying sex-differences in the mechanisms of HRV changes. As sex was not initially planned as a variable to be empirically tested in the present study, estrogen levels and expression of estrogen receptors were not measured. However, connections and pathways can be taken from literature for generation of future exposures to further elucidate the mechanisms behind the sex specific WPS responses. In humans, a meta-analysis was performed that found that at rest, women have less variability in time domain measures, such as SDNN. Women were also found to have less total power in the

frequency domain, but greater HF power (Koenig & Thayer, 2016). This meta-analysis indicates that at rest, the ANS of women is more dominated by a higher vagal stimulation than in men.

Mechanistically, there is evidence that autonomic function is directly regulated by estrogen via the estrogen receptor α and G protein-coupled estrogen receptor (Brailoiu et al., 2013; Dart et al., 2002). Estrogen receptors are present throughout the central nervous system and are highly expressed in regions involved in the top-down control of the autonomic nervous system such as the hypothalamus and amygdala (Simerly et al., 1990). Brailoiu et al. showed that estrogen receptors mediate vagal neuron activity and affects cardiac rhythm. This suggests that the sex dependent HRV responses to WPS are plausibly due to differing levels of estrogen between males and females. It is likely that WPS inhalation could alter estrogen levels in females as well throughout the progression of the exposure period. Luderer et al. found that exposure to concentrated ambient PM greatly reduced follicle counts and subsequently decreased estrogen production from the ovaries in ApoE-/- animals (Luderer et al., 2021). As estrogen is known to function in anti-oxidant pathways (Regitz-Zagrosek & Kararigas, 2017). and Rhoden et al. showed that ROS and oxidative stress may act as a pathway from PM exposure to changes in HRV, estrogen's role as an anti-oxidant may explain the divergence in the WPS response pathway to autonomic outcomes (Du et al., 2006; Rhoden et al., 2005). In addition, if ovarian follicle depletion is occurring in WPS-exposed females, the progressive loss of follicles and estrogen production over the course of the exposure could explain the changes in short-term to long-term WPS toxicity. As the cumulative effects may alter estrogen levels, therefore altering the function of mechanistic pathways.

6.3.2 Sex-differences in inflammation

This study was not initially designed with the aim of assessing sex-differences of response to WPS. Given that, hormones and sex-dependent mechanisms were not explicitly tested. However, a look towards the literature surrounding this topic can help to explain the effects seen in the present study. Although differences in hormones, namely estradiol, are the first conclusion made by many to explain sex differences in cardiovascular disease outcomes, that is likely well oversimplified. Hormones alone cannot explain many of these responses (Bernardi et al., 2020), and where they do, it is difficult to replicate or explain differences across species. In humans, there are differences in innate immune responses of males and females (Klein & Flanagan, 2016). In a study of healthy adults, circulating levels of proatherogenic cytokines, IL-1β, TNF-α, and IL-6, were higher in males than females on average, but not associated with estradiol or testosterone levels (Bernardi et al., 2020). Ter Horst et al. also found that sex hormones do not explain differences in circulating cytokine levels (R. ter Horst et al., 2016). However, neither study controlled for the hormonal changes throughout menstrual cycle phases, where estradiol concentrations can increase 10-fold throughout various phases (Reed & Carr, 2018), nor did they control for use of hormonal contraceptive use, which could greatly alter the results. In a prospective crossover study in women, the use of either of combined oral contraceptive significantly increased circulating levels of CRP, but not IL-6 or TNF-α. In contrast, IL-6 levels decreased slightly with one of the two contraceptives used (Rooijen et al., 2006). This indicates that alteration of estradiol levels can induce changes to cytokine signaling in an unorthodox manner, as IL-6 and TNF-α induce the liver's production of CRP (Badimon et al., 2018).

6.3.3 Reproductive toxicity of WPS

Linking the WPS-induced alterations to HRV and atherosclerotic endpoints is a neurohormonal stress response by inducing changes to sex hormones, cytokines, and the central and autonomic nervous systems. The central structure in these systems is the hypothalamus, which conveys signals to the gonads via the hypothalamic-pituitary-gonadal axis (HPG), as well as to the cardiovascular system as part of the central autonomic network which controls HRV (Thayer et al., 2009). A similar neurohormonal response has also been found to link air pollution derived systemic responses (Kodavanti, 2016).

Although hormone levels were not measured in the present study, research on ambient PM inhalation has found that female Balb/c mice were susceptible to loss of ovarian follicles, which contain the cells responsible for estradiol production, and greater chance of irregular estrous cycling (Mohallem et al., 2005; Veras et al., 2009). Furthermore a recent collaborative study with our research group assessed female reproductive toxicity endpoints in ApoE-/- mice exposed to concentrated ambient PM for three months. PM exposure caused significant depletion of ovarian reserve with over 40% loss of both primordial and primary ovarian follicles, as well as 35% of the mice exhibiting abnormal estrous cycling, which did not occur in exposure controls. PM exposed mice also had less than half of the ovarian estradiol concentration as air-exposed controls (Luderer et al., 2021). The results of Luderer et al. support that excessive damage to the ovary can occur with PM inhalation exposure. It is important to note that the ambient PM mass concentration in the exposure was approximately 350 times less than the WPS exposure presented here. While dose-response of PM induced ovarian toxicity is unknown, similar outcomes may be occurring in WPS exposed female ApoE-/- mice described here.

Reproductive toxicity of inhaled PM is not exclusive to females. Ali et al. studied the reproductive toxicity of WPS on male BALB/c mice and found that WPS exposure for 6 months significantly decreased both testosterone and estrogen levels, while increasing the circulating levels of luteinizing hormone (LH). Histological damage was found in the testes as well as reduced spermatogenesis and abnormal morphology of spermatozoa after WPS exposure (Ali et al., 2017). These results replicated previous studies by their group which demonstrated similar results of male reproductive toxicity and hormonal changes after only one month of WPS exposure (Ali et al., 2015). Although female mice were not included in their WPS exposure studies, it indicates that chronic WPS inhalation can induce toxicity in gonadal tissue and alter hormonal production. LH is a hormone released by the pituitary gland in response to hypothalamic release of gonadotropic-releasing hormone (GnRH). LH is responsible for triggering gonadal hormone production in both testes and ovaries. This endocrine pathway is referred to as the hypothalamic-pituitary-gonadal axis, or HPG axis, and is self-regulating with negative feedback loops by gonadal hormones acting back on the pituitary and hypothalamus (Oyola & Handa, 2017). The drop in testosterone levels after WPS exposure means there is less negative feedback and results in the increase of LH in an attempt to signal the testes to release more hormones.

Leptin is another important signaling molecule which can be altered by WPS. Leptin is a cytokine secreted by white adipose tissue and is known for its endocrine functions throughout the body such as in hunger and satiety. It can also influence the central nervous system (CNS) by acting on neurons in the forebrain upstream up the hypothalamus and inducing the release of GnRH therefore altering the signals of the HPG axis (Quennell et al., 2009; Ratra & Elias, 2014). Leptin also acts on the NTS (Ciriello & Moreau, 2013), a vital region of the brainstem that

integrates afferent and efferent signals from the various bodily systems such as baroreceptors in vasculature, chemoreceptors, vagus nerve, etc, and transmits those signals to the hypothalamus and central nervous system in a feedback cycle (Boscan et al., 2002; Kodavanti, 2016; G. J. ter Horst et al., 1984). Synaptic transmission within the NTS has been shown to be altered, in concert with HRV changes, after exposure to either ambient PM or environmental tobacco smoke, further supporting the role of the NTS region in linking inhalation exposure to changes within the nervous system (Pham et al., 2009; Sekizawa et al., 2008).

Circulating leptin increases the neuronal firing within the NTS which directly projects to the areas of sympathetic nervous system activation in the rostral ventrolateral medulla as well as areas of parasympathetic nervous system activation in the dorsal motor nucleus of the vagus (Ciriello & Moreau, 2013). These stimulations of neuronal firing by leptin have been repeatedly shown to evoke cardiovascular changes mediated by both branches of the ANS (Arnold et al., 2009; Ciriello & Moreau, 2012). Leptin dampens the baroreflex sensitivity as well as depresses SDNN and HF HRV measures, indicating that the cardiovascular outcomes are vagally mediated (Arnold et al., 2009).

WPS has been shown to alter circulating leptin concentrations in several animal exposures. Ali et al. found that WPS exposure for either one or six months resulted in a significant drop in circulating leptin levels in male mice (Ali et al., 2015, 2017). WPS exposures in lactating rats however showed differing results. The dams did not have altered leptin concentrations, but the pups, who were not directly exposed via inhalation, but rather via ingestion of milk from the WPS-exposed mothers, had an almost 4-fold increase in leptin concentrations over controls (Al-Sawalha et al., 2021). These studies differ in that the WPS composition itself may vary between systems, and that the exposures were in different species, for different lengths of time and

outcomes were assessed at different developmental stages. Even though the direction of change was inconsistent, the result that WPS is able to significantly alter leptin concentrations highlights the importance of understanding its effects on the hypothalamus and its downstream changes to sex hormone levels and HRV and the possibility of leptin mediated WPS toxicity.

Leptin also has a role in promotion of atherosclerosis. Chronic levels of increased leptin stimulate macrophages, endothelial cells and smooth muscle cells of atherosclerotic plaques. In animal studies, leptin receptors have been found to be expressed throughout lesions (Raman & Khanal, 2021). In support of the role of leptin in atherosclerosis, when ApoE-/- mice have leptin receptors are knocked out as well, the overall lesion area, as well as the level of progression is depressed (Chiba et al., 2008). However, the WPS exposure done by Ali et. al. indicated that male mice exposed to WPS had a significant drop in leptin levels. As lower leptin levels are associated with less atherosclerotic plaque formation, this does not support the results of the present study where male mice exposed to WPS had thicker arterial walls.

While reproductive organs were not assessed in the present study, there is sufficient evidence from literature that PM inhalation, and WPS inhalation in particular, is likely able to induce toxicity to the gonads of both male and female animals. This reproductive toxicity could result in altered hormone levels, and subsequently lead to the sex-specific changes in WPS toxicity. It is important to note, that there is likely no single mechanism or pathway solely responsible for conferring the toxicity of WPS. This supports that the present study resulted in unexpectedly opposing results of ANS and atherosclerotic outcomes. It is more likely that a combination of many effects and pathways involving several physiological systems are responsible for the toxic effects of WPS, which is a very complex and diverse aerosol.

6.4 Key Limitations

The largest limitation of this study is that although sex-related differences were seen in WPS responses however, as that was not an initial aim of the study, sex hormones and organs were not collected nor assessed in relation to WPS induced toxicity to the reproductive system. Another limitation is that there is no standard waterpipe, tobacco or puffing topography to allow for direct comparison between this study, and those from other researchers. Puffing topography and flow rates alone can alter exposure paradigm, causing changes to the concentration of PM, the size of inhaled PM, as well as the composition of VOCs in the WPS aerosol, so this study is only a limited view on the overall risks associated with WPS (Eddingsaas et al., 2019). Interpretation of results of WPS influence on inflammation is limited by the fact that only two time-points were assessed, and only a small number of cytokines were measured. The sample sizes of the groups analyzed for all measures were small and the study would benefit from being repeated to verify and bolster confidence in the conclusions described herein. Future studies should include assessment of a greater panel of inflammatory cytokines.

6.5 Overall Significance

This study is among the first to examine long-term effects of WPS inhalation by ApoE-/- mice on cardiovascular toxicity. The results of this study have shown WPS to significantly alter autonomic nervous system inputs to the heart, in a sex- and time-dependent manner, and also suggest the importance of sex as a biological variable in the mechanisms underlying cardiovascular responses to WPS. This study also highlights the importance of long-term exposures and monitoring in both sexes to better understand responses to WPS inhalation that can inform regulatory and public health actions in the future.

6.6 Future Directions

This dissertation resulted in a large addition of knowledge on the cardiovascular effects of WPS inhalation. The other side of gaining more insight though is that more questions were also brought up. Future directions of this research should aim to study the mechanisms underlying the sex-dependent differences. A repeat of the present exposure studies with female mice which have undergone ovariectomy would be one route to assess the influence of sex hormones on the relationship of WPS and HRV. Further study of the mechanisms behind male and female cardiovascular and oxidative stress responses to chronic WPS is vital to creating accurate and relevant public health responses to waterpipe smoking. In addition, more in depth staging of the atherosclerotic lesions, as well as utilization of immunohistochemical methods could help to elucidate the differences seen between WPS-induced lesion progression of male and female mice.

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