Effects of *in utero* Diesel Exhaust Exposure on Development of Atherosclerosis in Hyperlipidemic Mice

Rebecca C. Ticknor

A thesis

submitted in partial fulfillment of the

requirements for the degree of

Master of Science

University of Washington

2014

Committee:

Michael Rosenfeld (Chair)

Terry Kavanagh

Michael Chin

Program Authorized to Offer Degree:

Public Health - Environmental and Occupational Health Sciences

©Copyright 2014
Rebecca C. Ticknor

University of Washington

Abstract

Effects of *in utero* Diesel Exhaust Exposure on Development of Atherosclerosis in Hyperlipidemic Mice

Rebecca C. Ticknor

Chair of the Supervisory Committee:
Professor, Michael Rosenfeld
Department of Environmental and Occupational Health Sciences

Coronary artery disease, commonly manifested as atherosclerosis, is a leading cause of death in western countries. There is substantial evidence of the detrimental effect of particulate matter (PM) exposure on cardiovascular morbidity, and diesel exhaust (DE) is a substantial contributor to urban atmospheric PM levels. However, there is little evidence of the impact of *in utero* exposure to PM on long-term health effects. It is hypothesized that *in utero* exposures could permanently affect fetus health through an epigenetic phenomenon termed "fetal programming," in which fetal exposures alter gene expression through adulthood. The current study aims to elucidate the effect of *in utero* DE exposure on atherosclerosis development in adulthood in hyperlipidemic Apo E -/- mice. The study consisted of three treatment groups: a control group in which pregnant mice were exposed to filtered air (FA) during pregnancy and the pups were exposed to FA post-birth (FA/FA); a group in which pregnant mice were exposed to DE during pregnancy and the pups were exposed to FA post-birth (DE/FA); and a group in which pregnant mice were exposed to DE during pregnancy and the pups were exposed to DE

post-birth until weaning, at which point they were switched back to FA (DE/DE). Thoracic aortas were analyzed for RNA sequencing in male mice at 4 weeks of age. Histologic analysis of atherosclerotic plaque development (lesion area) in the aortic sinus was conducted at 16 weeks of age. A significant increase in week 1 body weight was observed in the DE/DE group compared to the DE/FA and FA/FA groups. The DE/DE group was also associated with a significant increase in HDLs when compared to the DE/FA and FA/FA groups. Histological results showed no significant impact of *in utero* DE exposure on atherosclerotic lesion area. RNA sequencing studies indicated downregulation of 137 genes and upregulation of 15 genes due specifically to *in utero* DE exposure when compared to the FA/FA group. Overall results of the impact of *in utero* DE exposure were inconclusive and further studies should be conducted in order to fully elucidate the effect of *in utero* DE exposure on atherosclerotic progression.

Acknowledgements

I would like to acknowledge Dr. Michael Rosenfeld for his advice, mentorship, and financial support throughout the entirety of the project. I would also like to acknowledge Drs. Terry Kavanagh and Michael Chin for their involvement in project direction and overall guidance. The assistance of James Stewart and Timothy McMillen in diesel exhaust exposures and lipid profiling, respectively, is greatly appreciated. Thank you to Jesse Tsai, James MacDonald, Dr. Theo Bammler, and Dr. Fred Farin for their involvement in the RNA sequencing and analysis. I would also like to acknowledge the members of the Rosenfeld lab that assisted in the overall planning and execution of project objectives: Jerry Ricks, Will Driscoll, and Jenna Harrigan.

Table of Contents

List of Figures

List of Appendices

Introduction

Air Pollution and Diesel Exhaust

Recent epidemiological studies have raised concern around the impact of traffic-related air pollution on cardiovascular health. Traffic is near unavoidable in industrialized areas, particularly large cities. A considerable percentage of the population, especially those with low socioeconomic status, is frequently exposed to traffic related air pollution either by direct participation in transportation or living near high-traffic areas. A significant route of personal exposure includes directly spending time in traffic. For example, the average US citizen over 15 years of age will spend approximately 55 minutes each day in motor vehicles. Chronic exposure to traffic-related air pollution can also result from living within 500 meters of a major road or freeway.[1] There are six air pollutants that are designated criteria pollutants by the Environmental Protection Agency (EPA). Emissions of these pollutants are regulated to meet National Ambient Air Quality Standards (NAAQS) as a result of the Clean Air Act. The current regulated concentrations of NAAQS are tabulated in Appendix 1. The six criteria pollutants include ozone, particulate matter, carbon monoxide, nitrogen oxides, sulfur dioxide, and lead. Other common air pollutants include ammonia, nitric acid, methane, formaldehyde, acetaldehyde, non-methane hydrocarbons and other volatile organic compounds, propane, benzene, 1,3-butadiene, sulfate, nitrate, organic carbon, elemental carbon, and polycyclic aromatic hydrocarbons.[2]

Particulate Matter

Air pollution has been linked to early and acute cardiovascular morbidity and mortality, as concluded by previous American Heart Association reviews[2]. Specifically, particulate matter (PM) contributes to reduced life expectancy if exposure remains elevated over prolonged periods

of time[3]. PM can cause acute cardiovascular toxicity, specifically where particles contact the lung, based on a wide variety of characteristics such as chemical composition and size. Specific characteristics that can impact exposure and subsequent toxicity include oxidative stress potential, solubility, charge, surface area, particle count, lung deposition, and stability within the atmosphere and biological environment. PM is often categorized based on the size of the particulates measured by aerodynamic diameter. Particles that can penetrate the lung are generally less than 10 μm (PM_{10}); these include course particles between 2 and 10 μm, fine PM less than 2.5 μm in diameter ($PM_{2.5}$), and ultrafine particles (UFPs) less than 0.1 μm in diameter. PM exposure is of concern to human health due to the size of the particles. When inhaled, $PM_{2.5}$ can penetrate the deep branches of the lung, including the alveolar region, and therefore cannot be efficiently removed by mucociliary clearance mechanisms[4,5].

Substantial sources of atmospheric PM include fuel combustion and high-temperature industrial processes. The source of formation also impacts the composition of the PM. For example, combustion PM is composed of multiple compounds including, but not limited to, organic carbon species, elemental or black carbon, and trace metals (e.g. lead and arsenic). The size of combustion PM is also variable depending on the specific source. Combustion PM ranges between a few nanometers to 1000 nanometers in diameter. The larger PM clusters (200 – 1000 nanometers) are large enough to cause light to scatter. $PM_{2.5}$ is produced in the highest concentration by the combustion of fossil fuels.

The U.S. EPA has set a clean air standard of 12 $\mu g/m^3$ for daily $PM_{2.5}$ exposure based on evidence of increased risk of myocardial infarction, stroke, arrhythmia, and heart failure in susceptible populations after $PM_{2.5}$ exposure spanning hours and days[6]. Other components of air pollution, such as ozone, carbon monoxide, sulfur dioxide, and nitrogen oxides, have also been

associated with increased risks for cardiovascular morbidity and mortality.[2,7,8] Categorizing air pollution based on one component (e.g. PM or ozone) is an inadequate measurement of total pollution because air pollution is a highly complex composition of gases, liquids, and solid particulates that are constantly changing due to climate, location, and atmospheric interactions[2]. However, this study will focus on PM because of its contribution to traffic-related diesel exhaust composition and toxicity[9]. The ninth-ranked cause of disease worldwide and the fourth-ranked cause of disease in East Asia have recently been listed as $PM_{2.5}$, further illustrating the current focus on PM exposures[10].

Diesel and Particulate Matter

Diesel exhaust (DE) is a particularly relevant source of air pollution in highly populated urban areas. Levels of DE exposure vary depending on geographical location with urban areas having higher levels of DE than rural areas. DE is primarily produced by on-road sources like motor vehicles and construction equipment but can also be produced by non-road sources like farm equipment, railway locomotives, and marine uses. Atmospheric DE concentrations will also vary depending on patterns of atmospheric dispersal, which can be impacted by the number and types of engines utilizing diesel fuel. Rates of individual exposure to DE will also vary depending on one's level of activity (i.e. ratio of indoor to outdoor activity).[4] The composition of DE depends on several factors such as engine type, fuel type, and operating conditions, which makes it difficult to discern specific human exposures[11]. In general, there are three basic components of DE: aggregates of elemental carbon particles (otherwise referred to as diesel exhaust particulate, DEP), organic matter and sulfates, and a mixture of gas and vapor containing mainly nitrogen gas, oxygen gas, water, carbon monoxide, carbon dioxide, nitrogen oxides,

sulfur dioxide, other sulfur compounds, and low-molecular-weight hydrocarbon. DE can also contain other hazardous air pollutants including formaldehyde, acetaldehyde, acrolein, benzene, 1,3-butadiene, and polycyclic aromatic hydrocarbons. DEP can be formed directly from incomplete combustion of diesel fuel (primary DEP) or from atmospheric alterations to the gaseous compounds emitted from diesel engines (secondary DEP). DEP can account for up to roughly 90% of total fine PM levels in ambient air in major cities and is considered a particularly hazardous component of DE[12,13]. DEPs consist of 80-95% $PM_{2.5}$ by particle mass with a mean particle aerodynamic diameter of 0.2 μm. UFPs contribute 1-20% of the diesel particle mass with a mean particle aerodynamic diameter of 0.02 μm and account for 50-90% of total particle number of DEP.[4,14] DEP has a high percentage of elemental carbon per unit mass, which can be used to distinguish between noncombustion and diesel-combustion PM in the atmosphere. Elemental carbon can contribute up to 50-75% of DEP mass whereas organic carbon can contribute up to 19-43% of DEP mass.[4] Overall national atmospheric DEP concentrations have been estimated at a median concentration of 1.53 $\mu g/m^3$, average concentration of 2.06 $\mu g/m^3$, and a 95th percentile concentration of 5.34 $\mu g/m^3$, however, ranges and maximum peaks can significantly vary depending on geographical and occupational location.[15]

There are currently thought to be at least three main mechanisms by which DEP could impact cardiovascular health: instigating a proinflammatory response in which inflammatory cytokines enter the circulatory system, dysregulation of the autonomic nervous system, and direct translocation of the particulates into the circulatory system, thus altering vascular function[2]. Because $PM_{2.5}$ or smaller cannot be removed via the mucociliary escalator, these particulates must be removed by macrophage engulfment[5]. The process of DEP-induced macrophage engulfment can stimulate secretion and overexpression of proinflammatory cytokines, activation

of antioxidant response pathways within the airways and lungs, and neutrophilic airway inflammation[16–22]. Local airway and lung inflammation have been linked with systemic vascular inflammation and increases in serum proinflammatory cytokine concentrations[22–25]. It is hypothesized that DEP induces pulmonary inflammation due to its ability to elicit oxidative stress. There is evidence that a small fraction of inhaled DEPs are translocated from the alveolar space into capillaries and pulmonary venules, which allows access to systemic and coronary circulations[26–28]. However, it is more likely that systemic reactions to DEP inhalation stem from released inflammatory factors rather than direct interaction between DEP and vascular endothelium[5]. Effects in the circulatory system can manifest in physiological symptoms such as endothelial dysfunction and vasoconstriction, increased blood pressure, prothrombotic and coagulant changes, systemic inflammatory and oxidative stress response, autonomic imbalance and arrhythmias, and the progression of atherosclerosis[2].

Atherosclerosis Development

Coronary artery disease (CAD) a the leading cause of death worldwide, and atherosclerosis is the primary cause of CAD[29]. Atherosclerosis is a disease in which lesions, commonly referred to as plaques, develop in arterial vessels and narrow the lumen of the artery. It has been shown to primarily develop from chronic inflammation. The anatomy of arteries consists of three layers: the tunica intima, tunica media, and tunic adventitia. The tunica intima is the space around the endothelial layer that forms the barrier to the lumen of the artery. The intima contains connective tissue like proteoglycan and collagen, elastic fiber, smooth muscle cells, and inflammatory factors and cells. Due to its close contact with blood, the intima is the layer in which atherosclerosis develops. The intima is able to adapt to changes in pulse rate, blood pressure, arterial geometry, flow rate, and resistance to flow by altering thickness, generally referred to as compensatory remodelling.[30] Atherosclerosis is shown to induce endothelial dysfunction, thus altering the vessel's ability to undergo compensatory remodeling[31] .

Development

There are roughly three stages of atherosclerotic development: initiation of the fatty streak, transition of the fatty streak to an atheroma, and progression and destabilization of the plaque leading to plaque rupture and occlusive thrombosis. The first stage, initiation of the fatty streak, involves trapping and retention of lipoproteins at lesion-prone sites in the arterial intima.[32] Lesion-prone sites are generally branches where blood flow is slowed and lipoproteins, specifically low-density lipoproteins (LDLs) experience an extended residence time at locations where plaques will subsequently develop, aiding in increased absorption[33]. Under normal conditions, LDLs rarely bind to vessel walls, however, modified lipoproteins (via oxidation,

acetylation, or other mechanisms) can bind and accumulate in vessels. These modifications can occur via lipoxygenases.[34] There is also leukocyte adherence to the endothelial surface and diapedesis, resulting in localization of leukocytes within the intima. Expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and E-selectin aid in the adherence of leukocytes to vessel walls[35,36]. The upregulated expression of these adhesion molecules could be due to oxidative stress initiated by the accumulation of oxidized lipoproteins[37,38]. Initiation of the fatty streak also involves differentiation of monocytes, foam cell formation, and foam cell activation. After initial trapping of LDLs, monocytes enter the artery wall and rapidly differentiate into tissue macrophages. Some of the macrophages transform into foam cells via engulfment of the oxidized LDLs. Macrophages have several receptors that recognize modified lipoproteins, commonly called "scavenger receptors". Expression of these receptors is generally upregulated by the presence of cytokines and oxidized lipids.[32] After foam cells are formed, they can be activated to express other factors that impact the atherogenic process. Apolipoprotein E, lipoprotein lipase, interleukin-1 (IL-1), monocyte chemotactic protein-1 (MCP-1), macrophage-colony stimulating factor (MCSF), tumor necrosis factor (TNF), platelet-derived growth factor (PDGF), tissue factor, and stress proteins are examples of factors that can be expressed by activated foam cells. Some of these factors stimulate macrophage proliferation while others attract other inflammatory cells, thus furthering progression of the inflammatory process.[39,40]

The next stage of atherosclerosis is transition of the plaque. Progression includes cellular migration, proliferation, connective tissue synthesis, and cell necrosis and apoptosis. The main hallmark of this stage is the formation of a fibrous cap and consequential thickening of the arterial wall. It is hypothesized that the fibrous cap is an adaptive response of smooth muscle

cells (SMCs) to altered blood flow and an attempt to reduce the absorption of lipoproteins and inflammatory cells.[41] The migration of SMCs from the media into the intima and proliferation of SMCs that secrete large amounts of connective tissue contribute to the formation of the fibrous cap[42]. Oxidative stress and expression of PDGF, fibroblast growth factor (FGF), and tumor growth factor-beta are all thought to play a role in the stimulation of smooth muscle cell migration, proliferation, and connective tissue synthesis[40]. A second hallmark of the transitional phase of the plaque is the formation of a lipid-rich necrotic core[41]. The necrotic core forms at the core of the plaque, can expand to the margins of the plaque, and is thought to be a result of cell death due to perpetual oxidative stress from oxidized lipids[40]. The necrotic core can also contain a substantial amount of extracellular lipid and calcium deposits[41]. Reduced availability of nitric oxide (NO) is also indicative of the transitional stage and tends to cause dysregulation of normal vascular tone[43]. Due to the influx of cells and connective tissue synthesis that cause the plaque to invade the medial layer of the artery, this stage is also associated with medial thickening[44]. Thickening of the artery can result in decreased lumen diameter and altered blood flow when the vessel's inherent ability to dilate cannot sufficiently compensate[45].

The final stage of plaque development is plaque progression, rupture, and formation of occlusive thrombi. Plaque rupture is a result of plaque destabilization. Plaque destabilization occurs when the necrotic core expands, involving continued foam cell death and extracellular lipid and bone salt deposition. When the necrotic core expands, the fibrous cap tends to thin. In humans, thinning of the underlying media affects the ability of the artery to maintain compensatory remodeling. [32] Smooth muscle cell death and proteolytic degradation of connective tissue is also associated with fibrous cap thinning and could be the result of apoptotic signals due to inflammation and oxidative stress[40,46]. Plaques generally rupture where the cap is

the thinnest: along lateral margins where inflammatory cells are abundant[47,48]. Once the plaque ruptures, the internal debris can be exposed to the blood where clotting factors, including oxidized LDLs, forms a scaffold for thrombus formation[49–51].

Composition

Atherosclerotic plaques can be quite diverse in development and composition depending on the species, individual, and location. However, most plaques have a core cellular composition that appears to be relatively conserved across broad populations. Cells that are generally involved in the atherosclerotic process include leukocytes (specifically monocytes, lymphocytes, and macrophages), foam cells, endothelial cells, and smooth muscle cells. Leukocytes represent the earliest observable event in the initiation of the atherosclerotic plaque. As previously mentioned, adhesion molecules, such as VCAM-1 and E-selectin stimulate clustered leukocyte adhesion to the endothelium of vessels.[52] Monocytes represent the majority of leukocytes that transmigrate to the intima after adhesion molecule expression by activated endothelium[53,54]. It is theorized that monocytes enter the intima in order to scavenge the trapped lipoproteins. After transmigration into the intima, some monocytes differentiate into macrophages, which is facilitated by MCSF. Macrophages, using scavenger receptors, can then internalize the oxidized lipoproteins but cannot digest them, thus forming foam cells. The binding of the modified lipoproteins stimulate an inflammatory response in the foam cells.[52] Under normal conditions, endothelial cells align their long axes in the direction of flow; however, endothelial cells associated with atherosclerotic tissues tend to lose their rigid pattern and become rounded, contain increased levels of stress fibers, and become multinucleated cells[41,55,56]. Endothelial cells also secrete extracellular matrix components like fibronectin, type IV and type V collagen,

laminin, and proteoglycans[57]. Smooth muscle cells are the most abundant cell type in the blood vessel and have a vital role in all stages of atherosclerosis. As mentioned above, SMCs are involved in migration, proliferation, matrix synthesis (i.e. collagen, elastin, and proteoglycan,[30] inflammation, and foam cell formation[58]).

The composition of the extracellular matrix (ECM) also significantly impacts atherosclerotic development. Important components of the ECM are collagens, elastin, fibronectin, and laminin. Collagens are involved in endothelial attachment and therefore are partially responsible for endothelial integrity. Elastin aids in the pliability of vessels and their ability to regain their original shape after contraction or relaxation. Fibronectin plays a role in cell-cell and cell-substrate adhesion, cell motility, and targeted molecule binding whereas laminin is a key component of the basement membrane underlying the endothelium.[41]

Genetics of Atherosclerosis

Because of the complexity of atherosclerosis, there are a multitude of genes that play a role in the development of the disease. Some particularly important genes regulate inflammation, proliferation, differentiation, and other processes that contribute to plaque formation, progression, and rupture. Genes that code for endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), estrogen receptor-alpha (ERα), estrogen receptor-beta (ERβ), fibroblast growth factor-2 (FGF2), hypoxia-induced factor 1-alpha (HIF-1α), extracellular superoxide dismutase, nuclear factor κ-B (NFκB), cyclooxygenase-2 (Cox-2), and eotaxin are all genes that are implicated in atherosclerotic development as well as impacted by *in utero* exposures. eNOS and iNOS are both implicated in NO production, which is necessary for blood pressure regulation, vascular tone, inhibition of platelet aggregation and leukocyte adhesion, and prevention of SMC

proliferation[59]. Without proper NO levels, vascular health is severely altered; however, excessive NO production can result in oxidative stress from peroxynitrite formation[23,60,61]. Estrogens (natural antioxidants), mediated by estrogen receptors such as ERα and ERβ (receptors that could induce genes that combat oxidation), tend to protect vascular tissue against oxidative stress[62]. FGF is a growth factor that is implicated in development, angiogenesis, and adipogenesis as well as cell growth, proliferation, migration, and differentiation of different cell types[63]. HIF is a transcription factor that regulates many genes involved in angiogenesis and is also associated with inflammatory pathways, such as those that involve macrophages and neutrophils[64]. NFκB is a pro-inflammatory transcription factor that has been implicated in atherosclerotic development. NFκB has been shown to play a role in the initiation, organization, amplification, and progression of the inflammatory response. It also initiates the necessary cascade for chronic inflammation.[65,66] Specifically, NFκB can mediate induction of inflammatory genes such as TNF-α and adhesion molecules such as ICAM-1 and VCAM-1. Both ICAM-1 and VCAM-1 aid in leukocyte transmigration, monocyte accumulation, and SMC proliferation; all vital processes in the development of atherosclerotic plaques.[67] Cox-2 is another inflammatory molecule involved in the atherosclerosis process[68]. Cox-2 regulates prostanoid production, where prostanoids impact cardiovascular function via vascular tone regulation, vascular inflammatory response, leukocyte-endothelial cell adhesion, and platelet aggregation[69,70]. Expression of Cox-2 can be induced by TNF-α stimulation[71]. Eotaxin is an eosinophil chemoattractant that plays a vital role in inflammatory responses and has been associated with atherosclerosis[72].

Atherosclerosis and Diesel Exhaust

There have been multiple prior studies that have aimed to elucidate the mechanisms behind diesel exhaust exposure and atherosclerosis. Because of the complexity of diesel exhaust, it is difficult to decipher which component of the exhaust is causal for atherosclerosis; however, PM is often credited with atherosclerosis due to its ability to stimulate cytokines that can translocate to the systemic circulation, thus causing a system-wide inflammatory response and contribute to ongoing inflammation. Previous studies have found cause and effect between exposure to PM_{10} and accelerated progression of atherosclerosis in rabbits prone to atherosclerosis development[73] and ApoE knockout mice[23], whereas an association has been found between PM_{10} exposure and atherosclerosis in human carotid arteries[74,75].

Previous animal studies of diesel exhaust exposure and atherosclerosis have indicated various mechanistic pathways by which DE might alter normal physiology and leave the body susceptible to atherosclerosis. In a study conducted by Yin *et al.*, it was found that in ApoE -/- mice, DE altered the antioxidant properties of high-density lipoproteins (HDLs) as well as the anti-inflammatory properties that protect against atherosclerosis. DE was also found to increase lipid peroxidation in the bronchoalveolar compartment and systemic tissues and to induce the 5-lipoxygenase pathway in the liver, which would contribute to the oxidation of LDLs and further progress plaque development.[76]

Another study conducted by Bai *et al.* detected a relationship between DE exposure, iNOS upregulation, and atherosclerosis in mice. After DE exposure, iNOS expression was increased in thoracic aorta and heart tissue. It was also found that iNOS levels were increased in blood vessels, which consequentially resulted in reduced contractility. There was also a positive correlation between iNOS expression and foam cell formation, a possible effect of increased

oxidized LDLs due to oxidative stress by increased NO concentrations, and the number of SMCs in the aortic lesions.[77] Cox-2 expression in the thoracic aorta and aortic root, both in whole protein and in mRNA, was also increased after DE exposure. It was also found that Cox-2 expression was positively correlated with aortic smooth muscle cell content after DE exposure.[78] On a cellular level, DE exposure increased the levels of phagocytizing and non-phagocytizing alveolar macrophages, plaque cellularity, foam cell formation, lipid accumulation, and SMC content[79].

Fetal Programming and Epigenetics

The effects of DE and PM exposure on atherosclerosis in adults have been well studied, however, little is known about the effects and mechanisms behind *in utero* exposures. A phenomenon referred to as "fetal programming" captures the concern of maternal exposures adversely altering fetal development. Fetal programming involves the "programming" of fetal structural and physiological processes in response to chronic maternal exposures that result in environmental stressors to the fetus[80]. Examples of intrauterine stressors could range from transplacental passage of particulates to oxidized fatty acids to pro-inflammatory factors[81], and exposure to these intrauterine stressors could contribute to the development of adult morbidities[82–86]. Previous studies have positively shown that adverse intrauterine stressors have affected birthweight and subsequently resulted in adult mortalities and morbidities. For example, reduced birthweight was found to be associated with increased adult risk of cardiovascular mortality and increased ventricular hypertrophy and hypertension[87–89]. Another example of fetal programming includes fetal exposure to arsenic and increased and accelerated onset of adulthood atherosclerosis[90]. Weldy *et al.* observed that *in utero* DE exposure resulted in adult weight gain, altered blood pressure, and increased susceptibility to pressure overload-induced heart failure, indicating that exposure to DE during development yields long-term effects on adult myocardial susceptibility to injury[91].

Environmental stressors that cause the mother to release inflammatory mediators can result in fetal exposure to increased levels of cytokines, chemokines, and lipid mediators through the circulation[92,93]. Because both exposure to DE and atherosclerosis development are known to result in and from inflammatory response and oxidative stress, it is plausible that maternal exposure to DE could expose the fetus to maternal inflammatory mediators and thus elicit

cardiovascular fetal programming. This response could result in fetal cardiovascular alterations and possibly increase the risk of atherosclerotic development in adulthood.

Programming can present itself in three forms: developmental disruption, which is a direct response to suboptimum fetal conditions and interferes with normal fetal development; immediate adaptation, which allows the fetus to alter normal fetal functions in order to conserve insufficient resources for self-preservation; and predictive adaptation, which is a change in the developing fetus that does not appear to have any immediate value but prepares the fetus for future adverse environments[86]. Fetal programming can also result in epigenetic changes such as DNA methylation, histone modification, and microRNA expression that could contribute to disease onset in adulthood[94].

DNA Modification

The most studied epigenetic alteration involves direct DNA modification, specifically DNA methylation through the action of DNA methyltransferases (DNMTs). There are three primary DNMTs: DNMT1, DNMT3a, and DNMT3b. DNMTs utilize S-adenosylmethionine as a universal methyl donor for DNA methylation.[67] Because DNA replication generally copies methylated bases, DNA methylation is considered inheritable[95]. DNA methylation impacts gene transcription by impeding access of DNA transcription factors to the DNA; therefore, hypermethylation is generally indicative of gene silencing, and hypomethylation is generally associated with increased gene transcription[96]. Covalent binding of methyl groups further impacts gene expression by recruiting methyl-CpG binding proteins. Methyl-CpG proteins induce transcriptional silencing by directly blocking transcription factor binding and also by recruiting transcriptional co-repressors and histone-modifying complexes.[67] DNA methylation

also plays a substantial role in maintaining genomic stability as well as chromatin structure[97,98]. DNA methylation impacting gene transcription generally occurs on 5' cytosines. Approximately 1-4% of cytosines are methylated in healthy human somatic cells, of which 70-90% of the methylated cytosines occur in CpG dinucleotides (CpG islands). CpG islands in 5' regulatory areas are generally unmethylated, whereas CpG islands in intronic and repetitive DNA elements are often methylated.[99–101]

As previously mentioned, DNA methylation is considered heritable due to the nature of DNA replication. Patterns in DNA methylation are mainly established during embryogenesis and early postnatal life. Under normal circumstances, DNA methylation is a necessary cellular process for long-term gene silencing, proper development, X chromosome inactivation, and genomic imprinting. Early embryos contain DNA that is hypomethylated, but as tissues and organs differentiate, DNA becomes progressively more methylated based on environmental signals.[102–108] In epidemiology studies associating maternal diet with fetal susceptibility in adulthood to metabolic disorders like obesity, glucose intolerance, hypercholesterolemia, and type II diabetes, it was also shown that these susceptibilities could be permanently passed trans-generationally from mother (F0) to F1 and F2 progeny[109,110].

Prior studies of epigenetics and atherosclerosis have identified several possible genes involved in atherosclerosis development and progression that could be impacted by DNA methylation. Atherosclerosis can be impacted by either increased or decreased methylation, depending on the specific gene and its function in atherosclerosis. In general, atherosclerosis is associated with global hypomethylation in SMCs sampled from human atherosclerotic lesions and aortic samples[62,111,112]. Hypomethylation of extracellular superoxide dismutase is characteristic of atherosclerotic lesions[113], whereas hypermethylation of eNOS, nNOS,[114]

iNOS[115], FGF2[116], ERα[117,118], and ERβ[119] is associated with atherosclerosis. Hypomethylation has also been associated with the promoter region of glucocorticoid receptors and PPARα, both key transcription factors for regulatory metabolic pathways implicated in atherosclerosis development[120–122]. The gene encoding for 15-lipoxygenase (ALOX15), a lipid peroxidating enzyme, is shown to be hypomethylated in the promoter region in samples taken from atherosclerotic plaques[100,123], explaining the high levels of ALOX15 expression in atherosclerotic plaques.

Histone Modification

Histone modification is another main method of epigenetic alteration. Histones have N-terminal tails that can be modified through various methods and consist of four core histones: H2A, H2B, H3, and H4[124]. Histone modification includes acetylation, methylation, ubiquitination, and SUMOylation of lysine residues, phosphorylation of serine residues, and methylation of arginines. The most common form of histone modification involves histone acetylation, in which acetyl groups are added to the ε-portion of lysine residues. Histone acetylation is accomplished by histone acetyltransferases (HATs), however, histones can also be deacetylated by histone deacetylases (HDACs).[72] Transcription activators and repressors regulate transcription via HAT or HDAC recruitment[124]. Because there is action by both acetyltransferases and deacetylases, histone acetylation can be considered reversible. Acetylation tends to relax chromatin, and in general, relaxed chromatin results in increased gene transcription; whereas tightly bound chromatin results in decreased gene transcription due to the inability of transcription factors and RNA polymerase to reach binding sites on DNA. Consequentially, HATs are known to induce transcription whereas HDACs are known to inhibit

transcription. Acetylation is also generally associated with hypomethylation of CpG islands in DNA, therefore, non-acetylated histones are associated with hypermethylated CpG islands in DNA.[72]

As with DNA methylation, there have been numerous previous studies that identify histone acetylation as playing an important role in atherosclerosis development. HDACs and HATs have both been associated with regulating expression of inflammatory genes, which would impact the initiation of atherosclerotic development[125]. Specific inflammatory genes involved in atherosclerosis development that have been shown to be impacted by histone acetylation include nuclear factor kappa B (NFκB), granulocyte macrophage-colony stimulating factor (GM-CSF), eotaxin, cyclooxygenase-2 (Cox-2), and glucocorticoid receptors[72]. NFκB activation has been associated with HATs and HDACs: HATs generally activate NFκB via H3 and H4 acetylation at the NFκB binding promoter regions whereas HDACs tend to repress NFκB[67,126]. GM-CSF has been shown to prevent progression of atherosclerosis[127], and in response to NFκB-mediated inflammation, GM-CSF expression is increased by hyperacetylation[128–130]. Eotaxin transcription is reduced by inhibiting histone acetylation[72]. Cox-2 is induced when HDACs are inhibited[131]. The glucocorticoid receptors have been shown to have increased acetylation of H3K9 and H4K9 in association with atherosclerosis[132]. In a study analyzing histone modification in endothelial cells of atherosclerotic plaques, it was shown that the eNOS promoter region had increased acetylation of H3K9 and H4K12[133].

Differentiation, proliferation, and migration of vascular smooth muscle cells have also been associated with histone acetylation. SMC differentiation and migration occur via hyperacetylation, whereas deacetylation inhibits SMC differentiation and migration.[134–136] However, SMC proliferation appears to be inhibited by histone acetylation[137]. Serum response

factor and cell-cycle regulators are specific factors of vascular SMC proliferation that are impacted by acetylation.

As with DNA methylation, there is controversy about acetylation's role in atherosclerosis, due to variations in the physiological response to acetylation depending on the gene acetylated and its role in atherosclerosis. It has been shown in atherosclerosis-prone mice that neointimal lesions were worsened by HDAC inhibitors, leading to the assumption that atherosclerosis development and progression is generally modulated by histone acetylation[138]. In contrast, studies performed by Findeisen *et al.* found that histone acetylation was inhibitory of cell proliferation, and HDAC inhibition actually reduced neointima formation[139].

Specific Aims

The goal of this study is to examine the relationship between *in utero* and postnatal exposure to DE on development of atherosclerosis in adulthood. This study hypothesizes that *in utero* exposure of hyperlipidemic apolipoprotein E-deficient mice to DE will accelerate formation of atherosclerotic lesions in adulthood, and epigenetic changes induced *in utero* will be associated with the acceleration of atherosclerosis. Specific epigenetic changes include DNA methylation and histone acetylation that could alter patterns of gene expression associated with atherogenesis.

The hypotheses of this study will be tested using three specific aims, as follows:

Aim 1: Determine the effects of *in utero* exposure or *in utero* plus postnatal exposure to diesel exhaust on atherosclerotic lesion development

Aim 2: Determine whether *in utero* or *in utero* plus postnatal exposure to diesel exhaust leads to epigenetic reprogramming in the aorta

Aim 3: Identify transcriptional pathways in the aorta that were altered by *in utero* and postnatal exposure to diesel exhaust.

These specific aims will elucidate the relationship between *in utero* and postnatal exposure to diesel exhaust and their impact on the development of atherosclerosis in adulthood.

Materials and Methods

Experimental Design

The study consisted of three treatment groups: a control group of pups and mothers only exposed to filtered air (FA/FA); a group of pups and mothers exposed to DE during pregnancy and immediately switched to FA after birth (DE/FA); and a group of pups and mothers exposed to DE until weaning, with the pups then switched to FA for the remainder of the study (DE/DE).

The primary factors assessed were aortic sinus lesion area and global RNA in the aorta.

Study Setting

Timed matings of Apo E -/- mice took place in the Northlake Diesel Facility (NLD) at the University of Washington (Seattle, WA). Mice were monitored at the NLD until weaning, at which point they were transported to the South Lake Union (SLU) Brotman vivarium. Mice were sacrificed in the Rosenfeld Laboratory at SLU. Mice were cared for in the centralized SPF facilities of the NLD and the Brotman vivarium according to the University of Washington's Department of Comparative Medicine's guidelines. All procedures were approved by the University of Washington Institutional Animal Care and Use Committee prior to study initiation. Tissues from sacrificed animals were analyzed in the Rosenfeld Laboratory at SLU.

Mouse Breeding

Apo E-/- breeder mice on a C57BL/6 background were taken from the Rosenfeld colony at the University of Washington South Lake Union Brotman vivarium. Breeders used were between the ages of 8 weeks and 36 weeks. Actual matings were performed at the University of Washington NLD. Apo E -/- mice underwent timed matings in which two-thirds of the matings

were performed in DE and one-third in FA. Males were placed in a cage with one female and were removed after a vaginal plug was observed (generally by 48 hours post male introduction to the female cage). DE was exposed at a concentration of 250 μg $PM_{2.5}/m^3$.

Weekly weights were collected in order to assess the differences in week 1 weight, final weight, and weight-gain over time between the different treatment groups. Apo E -/- mice were weaned at four weeks of age; at which time all treatment groups were transported back to the SLU vivarium and placed in FA for the remainder of the study.

Mice were eligible for study inclusion if they survived until 4 weeks (RNA/DNA analysis) or 16 weeks (histological analysis) of age without any serious medical issues (i.e. deformities, illness, etc.). All breeders and weaned mice were fed standard mouse chow with free access to water. All animal housings were temperature- and humidity-controlled on a 12-hour light/dark cycle.

Diesel Exposures

The diesel engine used for exposures was a Yanmar model YDG5500EV-6EI (Osaka, Japan). The fuel used was ultra low-sulfur road diesel. The engine was powered and produced exhaust for 6 hours per day, five days per week with an average $PM_{2.5}$ concentration of 250 $\mu g/m^3$. $PM_{2.5}$ within the cage was measured using a nephelometer. The hourly time-weighted average of $PM_{2.5}$ was 45 $\mu g/m^3$/hr.

RNA Sequencing

RNA analysis was only performed on male mice. Male Apo E -/- mice were anesthetized at weaning using isoflurane vapor. The DE/DE, DE/FA, and FA/FA groups consisted of 4, 3, and 5 males, respectively. Whole blood was collected via retro orbital bleeds prior to sacrifice, separated into plasma and cells by centrifugation at 16,300 x *g* for 10 minutes, and plasma was collected and stored in -80 °C for possible lipid profiling tests. Animals were then euthanized with 50 μL of 1:1 mixture of ketamine and xylazine and dissected. Prior to tissue extraction, animals were perfused with 20 mL of sterile PBS to clear the remaining blood. The thoracic aorta was then extracted, flash-frozen in liquid nitrogen, and stored in -80 °C until RNA and DNA extraction and purification were performed by individuals in the Farin and Bammler labs at the University of Washington. Hearts, livers, kidneys, spleens, and lungs were also collected during dissection and stored in neutral buffered formalin at room temperature for possible future studies.

RNA Profiling[140]

RNA profiling was conducted at the Center for Ecogenetics and Environmental Health CEEH) Function Genomics and Proteomics Core at the University of Washington. RNA analysis was conducted according to the CEEH Functional Genomics and Proteomics Core protocol. An ion proton platform (Life Technologies, Grand Island, NY) was used to sequence the RNA samples. The sequences were then aligned to a mm10 mouse genome using a subred aligner[141]. A Bioconductor Rsubread package was used to generate counts per gene. Each read from the Ion Proton was then compared to the mm10 genome to determine where the read aligned, after which all the reads that aligned to each gene were summed. The relative

abundance of a gene's transcript can be estimated by the number of reads that align to that gene. These counts were then used to test for differential abundance of a transcript between the three different treatments. Three comparisons were made between treatment groups: genes that were altered in the DE/DE group compared to the FA/FA group, genes that were altered in the DE/FA group compared to the FA/FA group, and genes that were altered in the DE/DE group compared to the DE/FA group. Because a gene model was used and not a transcript model, it is assumed that each read aligns to a particular gene, disregarding the fact that some genes can have multiple transcript variants. This simplifies the analysis and increases power, but decreases the ability to find genes that make different transcripts. Significant changes in gene expression were filtered based on a fold change of 1.5 and a $p < 0.05$. A false discovery rate (FDR) of 5% was established to determine the number of significantly altered genes across treatment groups.

Ingenuity Pathway Analysis (IPA) software (QIAGEN, Venlo, Limburg) was used to associate altered genes to certain transcriptional pathways. RNA sequencing data was filtered in IPA based on a fold change of 1.5 and a $p < 0.05$, but a FDR was not set. Using IPA, significant pathways were determined by identifying those pathways that were associated with the highest number of altered genes due to exposure.

Histological Analysis

Tissue Extraction

Mice used for the histological analysis were sacrificed at 16 weeks of age. Animals were anesthetized with isoflurane vapor. Blood was then collected via retro orbital blood draws. Whole blood was separated into cells and plasma by centrifugation at 16,3000 x *g* for 10 minutes. Plasma was collected and stored at -80 °C. After blood collection, the animals were then

euthanized with 50 μL of a 1:1 mixture of ketamine and xylazine. Euthanized animals were perfused with 20 mL of sterile PBS and 10 mL of formalin to clear the remaining blood before tissue extraction. Hearts and aortas were extracted and fixed in neutral buffered formalin at room temperature for at least 24 hours before processing.

Tissue Processing

Hearts were bisected to separate the aortic sinus from the ventricles after fixation. The heart was bisected such that the plane was precisely perpendicular to the angle at which the aorta exits the top of the heart. The aortic hemisphere of the bisected heart was then placed in biopsy cassettes and processed in a Shandon Citadel automatic tissue processor (ThermoFisher Scientific, Waltham, MA). The ventricle hemispheres of the hearts were placed back in formalin at room temperature. Paraplast Plus (Sigma-Aldrich, St. Louis, MO) paraffin wax was used for tissue infiltration during processing. Prior to infiltration, tissue was dehydrated with a graded ethanol series. Tissue was placed in 75% ethanol for 25 minutes, 95% ethanol for two sets of 25 minutes, and 100% ethanol for three sets of 30 minutes. After dehydration, the tissues were placed in the automatic tissue processor following a protocol of fresh Slide Brite (ThermoFisher Scientific, Waltham, MA) for three sets of 35 minutes each, paraffin for 60 minutes, and fresh paraffin for 90 minutes. Paraffin-infiltrated tissue was stored at room temperature until embedding.

Tissue Embedding

Stainless steel embedding molds were filled with molten paraffin and the paraffin-infiltrated aortic bisections were centered on the bottom of the molds such that the aortic valve

was visible through the aorta under a dissecting microscope. Embedding molds were then placed on a cooling tray and allowed to solidify overnight. Embedded blocks were removed from the embedding molds and stored at room temperature after complete cooling and solidification of the paraffin.

Tissue Sectioning

Hearts were sectioned into 5 μm sections using a Spencer Model 820 rotary microtome (American Optical, Buffalo, NY). Negatively-charged slides (Tanner Scientific, Sarasota, FL) were used to collect 3 sections of tissue at a time. Tissues were collected when the aortic sinus started to form and collection was ceased when the aortic sinus was surpassed. Slides were placed into staining racks to dry at room temperature overnight and were then transferred to an oven set to 60 °C to bake overnight. After baking was complete, slides were stored at room temperature in a slide cabinet.

Tissue Staining

Every other slide collected was chosen for Movat's pentachrome stain. Movat's pentachrome stain was chosen to decipher between various components of the stained cardiovascular tissue: nuclei (which appear blue after staining), elastic fibers (which appear black after staining), collagen and reticular fibers (which appear yellow after staining), proteoglycans (which appear light blue after staining), plasma proteins (which appears bright red after staining), and muscle (which appears red after staining).

Slides were coverslipped using Richard-Allan Scientific Cytoseal XYL (ThermoFisher Scientific, Waltham, MA) and Corning coverglass (Corning, Corning, NY) after staining was

completed. Once coverslipped, slides were stored at room temperature in a slide cabinet until imaging was performed.

Tissue Imaging

Stained tissues were imaged using a Nikon Eclipse E400 microscope coupled with a Nikon DS-1 camera (Nikon, Chiyoda, Tokyo). Tissues were imaged at 4X magnification using the Nikon Plan Fluor objective. NIS Elements F Package was used to view and save the images for further analysis.

Tissue Analysis

Tissue analysis was done in a blinded fashion: analysis of the tissue was conducted without knowledge of the treatment group of the tissue. The cross-sectional area of atherosclerotic lesions was determined by analyzing the Movat-stained tissues using computer-assisted morphometric analysis (Image Pro, Media Cybernetics, Silver Spring, MD). Only the middle section of stained slides was analyzed for lesion area. This equates to tissues being analyzed every 30 μm, or approximately 17% of total tissue sectioned.

Lipid Profiling

Approximately 150 μL of whole blood were collected from all mice used for histology at weeks 8, 12, and 16 for lipid profiling. Whole blood was centrifuged at 16,300 x *g* for 10 minutes. Plasma was collected and stored at -80 °C until profiling could be conducted.

Plasma lipoproteins were separated by high-resolution size-exclusion chromatography (Superose 6 column) on an AKTA FPLC instrument (GE Healthcare). A 120-μL aliquot of

plasma pooled from each group was separated at a flow rate of 0.2 mL/min with PBS. Aliquots (100 μL) from each 0.5-mL fraction were used for cholesterol and triglyceride determinations. Total cholesterol levels were determined colorimetrically (Sigma #401-25P) with cholesterol standards (Sigma-Aldrich Corp., St. Louis, MO). Plasma triglyceride levels were determined colorimetrically after removal of free glycerol (WAKO L-Type Triglyceride-M and Multi Calibrator Lipids).

Statistic Analysis

All collected data were expressed as the mean ± standard deviation. Statistically-significant differences between treatment groups were determined by the Student's T test. Data were considered statistically significant at a p-value <0.05.

Study Power

No power calculations were needed for this study because the study was proposed as a pilot study, in which it is required that there be 6 animals per group. Sample size was directly correlated to the success of breeding. The data acquired from the completion of the pilot study will be used to calculate power for the scaled-up study.

Results

Breeding Success and Sample Sizes

A total of 13 pups in the DE/DE group: one litter of 6 and one litter of 7. Of these pups, one litter (n = 6) was sacrificed for RNA analysis and only 4 pups from the second litter survived until 16 weeks (n = 7). This resulted in a total of 4 pups available for the 16-week histological analysis: three males and one female.

Fourteen pups were available for the total DE/FA group: two litters of 7 pups each. Three pups from the first litter were sacrificed for RNA analysis at 4 weeks, and only two of the remaining pups survived until week 16. All pups from the second litter survived until the 16 week time point, resulting in 9 total mice for histological analysis: 3 males and 6 females.

Twenty-one pups were bred under FA/FA conditions. There were 5 litters that produced viable pups with litter sizes of 3, 3, 7, 2, and 6. Six of the pups from the n=7 litter were sacrificed for RNA analysis. Both of the pups from the n=2 litter and one of the pups from the n=6 litter died at 4 weeks of age. All other pups survived until 16 weeks and were viable for the histological analysis, resulting in 12 total pups for FA/FA: 7 males and 5 females.

Premature pup death per week across treatment groups is displayed in Figure 1. Premature deaths were only observed through week 4. The DE/DE group had one death per week starting at week 2 until week four, the DE/FA group had one death per week starting at week 3 until week four, and the FA/FA group had three deaths during week 4.

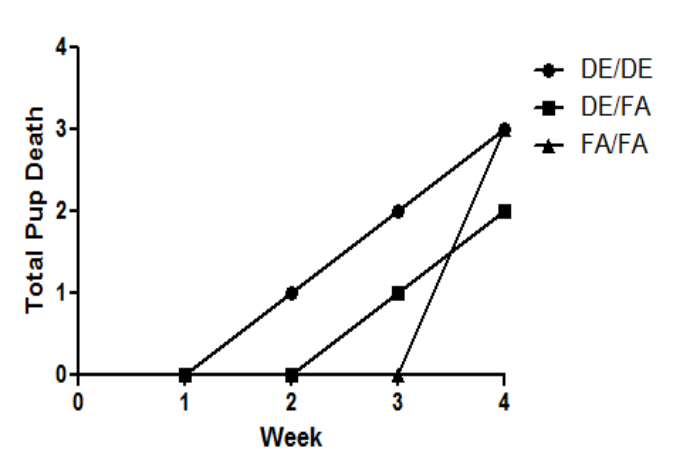


Figure 1: Total premature pup death across treatments

Body Weight

Body weights were analyzed for week 1, week 16, and overall weight gain. There was a significant difference in week 1 weights between the DE/DE group and both of the other treatments. Significance could be determined for the overall treatment and for males only because power was not strong enough for a female-only analysis. The sample size for DE/DE was $n_{total} = 13$, $n_{male} = 3$, $n_{female} = 1$, DE/FA was $n_{total} = 14$, $n_{male} = 3$, $n_{female} = 6$, and FA/FA was $n_{total} = 18$, $n_{male} = 12$, $n_{female} = 5$. The average week 1 weights for total (both male and female) DE/DE, DE/FA, and FA/FA were 3.45±0.60 g, 2.74±0.48 g, and 2.93±0.49 g, respectively. Male-only week 1 weights for DE/DE, DE/FA, and FA/FA were 4.2±0.10 g, 2.73±0.15 g, and 2.96±0.40 g, respectively. Categories that reached statistical significance ($p < 0.05$) were total DE/DE vs. DE/FA ($p = 0.003$), total DE/DE vs. FA/FA ($p = 0.007$), male DE/DE vs. male DE/FA ($p = 0.0002$), and male DE/DE vs. male FA/FA ($p = 1.2E-07$). Results from the week 1 body weights for all subjects, males only, and females only are displayed in Figures 2, 3, and 4, respectively.

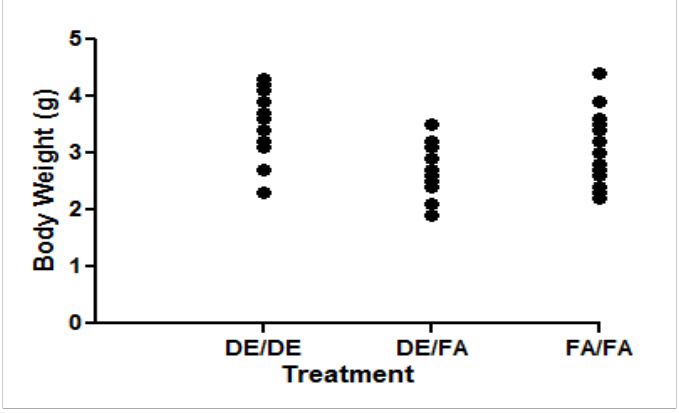


Figure 2: Week 1 Body Weight Distribution for All Subjects

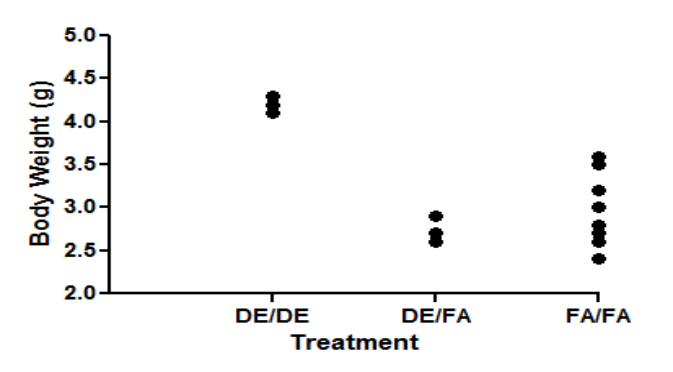


Figure 3: Week 1 Body Weight Distribution for Males Only

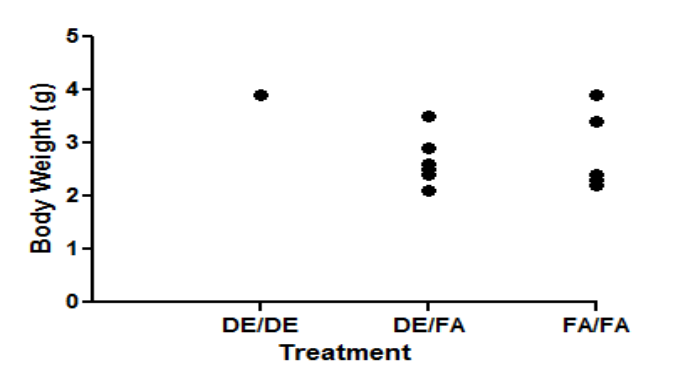


Figure 4: Week 1 Body Weight Distribution for Females Only

Statistical significance was not reached for any of the other analyses. Sample sizes for week 16 and overall weight gain DE/DE was $n_{total} = 4$, $n_{male} = 3$, $n_{female} = 1$, DE/FA was $n_{total} = 9$, $n_{male} = 3$, $n_{female} = 6$, and FA/FA was $n_{total} = 12$, $n_{male} = 7$, $n_{female} = 5$. For the week 16 weights, the total (both male and female) DE/DE, DE/FA, and FA/FA weights were 25.5±4.0 g, 23.1±3.4 g, and 26.0±4.4 g, respectively. Male-only week 16 weights for DE/DE, DE/FA, and FA/FA were 27.3±2.1 g, 26.7±3.2 g, and 29.3±2.0 g, respectively. Overall weight gain was 21.6±4.8 g, 20.4±3.3 g, and 22.9±4.0 g for total DE/DE, DE/FA, and FA/FA, respectively. For males only, weight gain was 23.1±2.2 g, 23.9±3.6 g, and 26±1.4 g for DE/DE, DE/FA, and FA/FA, respectively. Results from the week 16 body weights for all subjects, males only, and females only are displayed in Appendix 2; A, B, and C, respectively. Results for overall body weight

gain for all subjects, males only, and females only are displayed in Appendix 3; A, B, and C, respectively.

Lipid Profiles

Triglyceride and total cholesterol summaries from the lipid profiling are displayed in Table 1. Graphical representation of the triglyceride and cholesterol fractions is displayed in Figures 5 and 6, respectively. Fractions 14-18 represent VLDL, 18-27 represent LDL, and 28-33 represent HDL.

Table 1: Triglyceride and total cholesterol summaries from week 8, week 12, and week 16 lipid profiles

	Triglycerides (mg/dL)	**Total Cholesterol (mg/dL)**
DE/DE Week 8	102	336
DE/FA Week 8	69	378
FA/FA Week 8	96	345
DE/DE Week 12	48	432
DE/FA Week 12	42	465
FA/FA Week 12	57	477
DE/DE Week 16	45	531
DE/FA Week 16	38	492
FA/FA Week 16	53	531

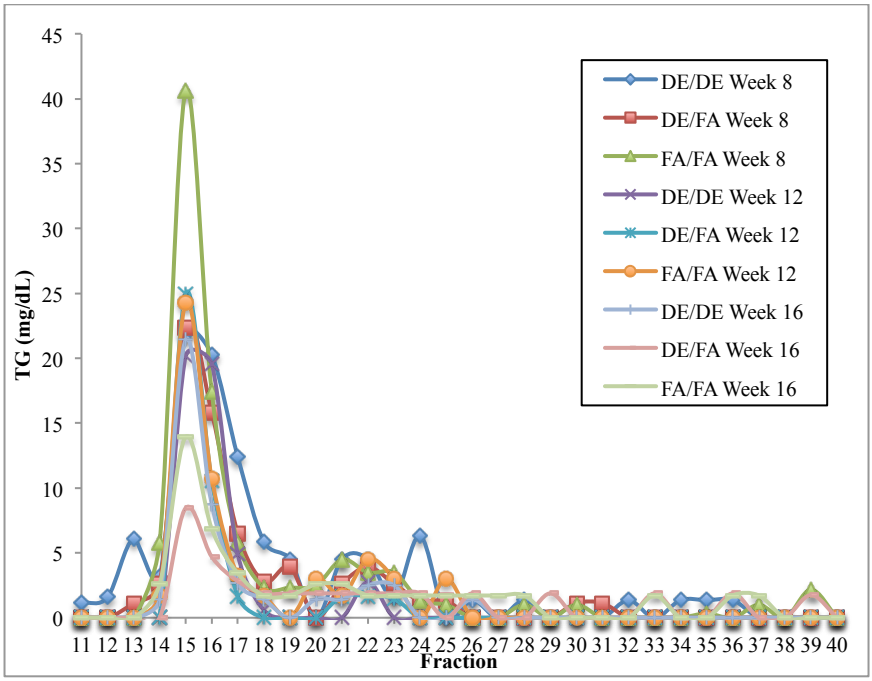


Figure 5: Fractional distribution of triglycerides for DE/DE, DE/FA, and FA/FA samples at weeks 8, 12, and 16.

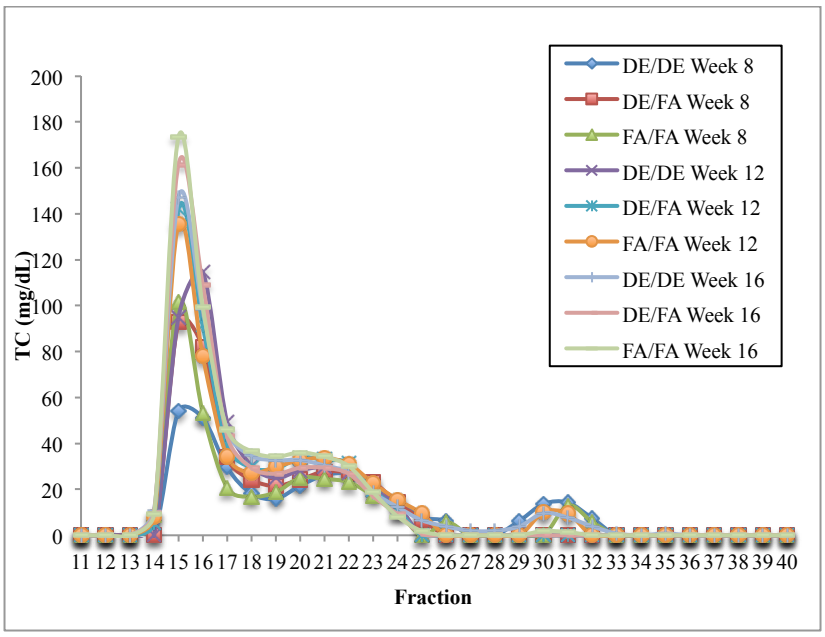


Figure 6: Fractional distribution of total cholesterol for DE/DE, DE/FA, and FA/FA samples at weeks 8, 12, and 16.

There was no significant difference between gross triglycerides and total cholesterol across treatments and time points. However, there were significant differences between treatments and time points when analyzing between VLDL, LDL, and HDL. It was found that HDLs were significantly increased in the DE/DE group when compared to the DE/FA group at both weeks 8 ($p = 0.02$) and 16 ($p = 0.01$) and the FA/FA group at week 16 ($p = 0.02$). The fractions representing HDLs from Figure 6 are displayed in Figure 7.

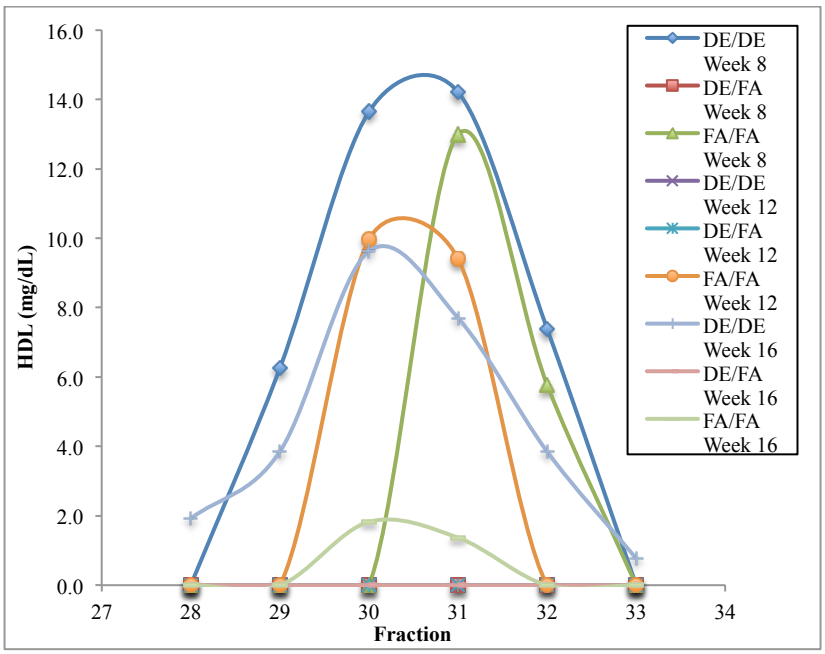


Figure 7: Fractional distribution of HDL for DE/DE, DE/FA, and FA/FA samples at weeks 8, 12, and 16.

Histology

Statistical significance ($p < 0.05$) was not reached for any of the treatment groups with respect to lesion area in the aortic sinus. The total average lesion areas for DE/DE, DE/FA, and FA/FA were 35,459±37,264 μm^2, 54,431±37,626 μm^2, and 31,123±16,484 μm^2, respectively. Because of the lack of power with respect to the DE/DE females (n=1), comparative analysis could only be conducted on the males. The male average lesion areas for DE/DE, DE/FA, and FA/FA were 16,958±5413 μm^2, 19,652±13,584 μm^2, and 24,302±13,650 μm^2, respectively. The distribution of atherosclerotic lesion area for all subjects, males only, and females only are displayed in Figures 7, 8, and 9, respectively.

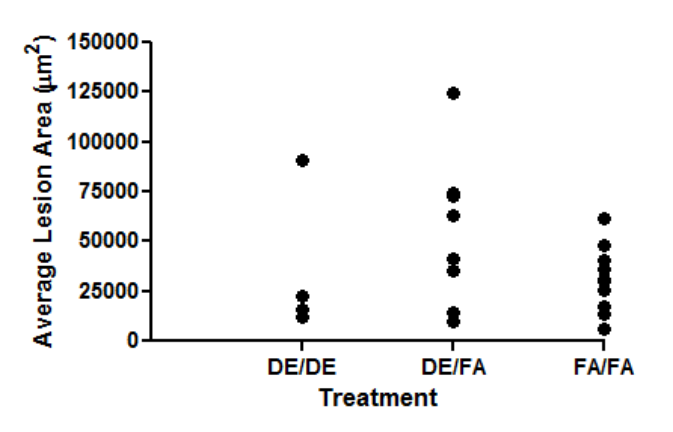


Figure 8: Atherosclerotic Lesion Area Distribution for All Subjects

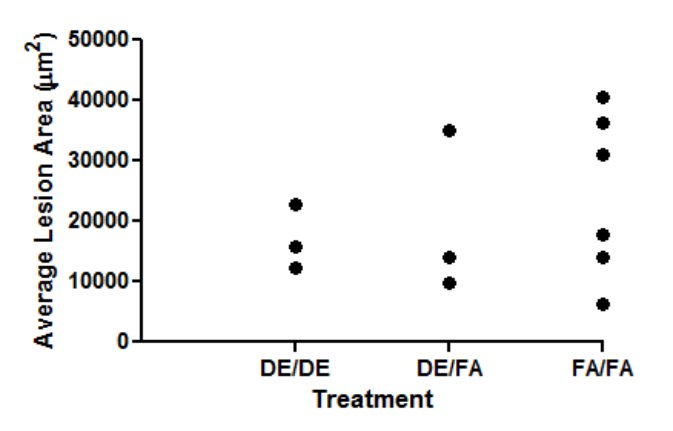


Figure 9: Atherosclerotic Lesion Area Distribution for Males Only

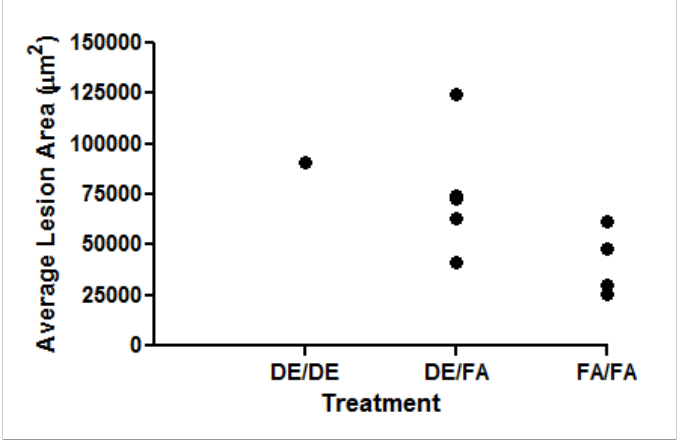


Figure 10: Atherosclerotic Lesion Area Distribution for Females Only

RNA Sequencing

The global RNA sequencing analysis resulted in 860, 1393, and 2049 unadjusted genes that were altered between DE/DE vs. FA/FA, DE/FA vs. FA/FA, and DE/DE vs. DE/FA, respectively, using filter criteria of $p < 0.05$ and a fold change of 1.5. After applying a filter for false discovery rate of less than 5%, a total of 2, 21, and 182 genes were altered between DE/DE vs. FA/FA, DE/FA vs. FA/FA, and DE/DE vs. DE/FA, respectively. The two genes that were significantly altered due to DE/DE exposure when compared to the FA/FA group using the FDR were *CYP2b10* and *mrps23*. It is interesting to note that *CYP2b10* was downregulated in both the DE/DE and DE/FA group when comparing both to the FA/FA group.

An analysis of genes purely altered by *in utero* exposure was conducted by comparing the genes altered in both the DE/DE group and the DE/FA group when compared to the FA/FA group. Using the criteria of a $p < 0.05$ and a fold change of 1.5, there were 179 genes that were upregulated in the DE/DE group compared to the FA/FA group, 679 genes upregulated in the DE/FA group compared to the FA/FA group, and 15 genes that were upregulated in both the DE/DE and DE/FA groups when compared to FA/FA. Under the same filtering criteria, there were 529 genes that were downregulated in the DE/DE group compared to the FA/FA group, 562 genes downregulated in the DE/FA group compared to the FA/FA group, and 137 genes that were downregulated in both the DE/DE and DE/FA groups when compared to FA/FA. Figures 10 and 11 display Venn diagrams that show the number of genes that were upregulated and downregulated, respectively, in both the DE/DE group and the DE/FA group when compared to the FA/FA control group.

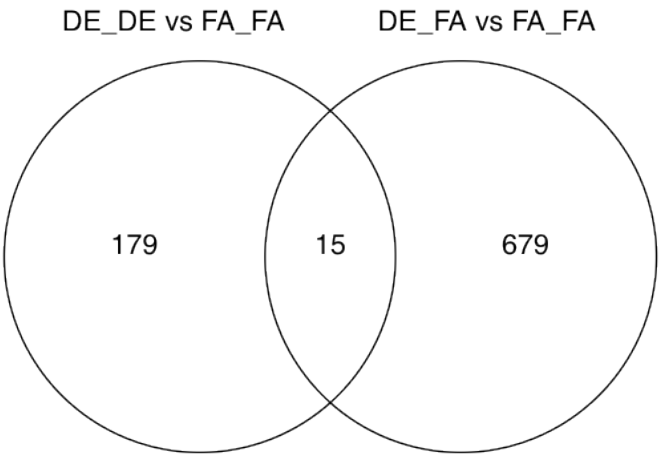


Figure 11: Upregulated genes in both the DE/DE vs. FA/FA comparison and the DE/FA vs. FA/FA comparison

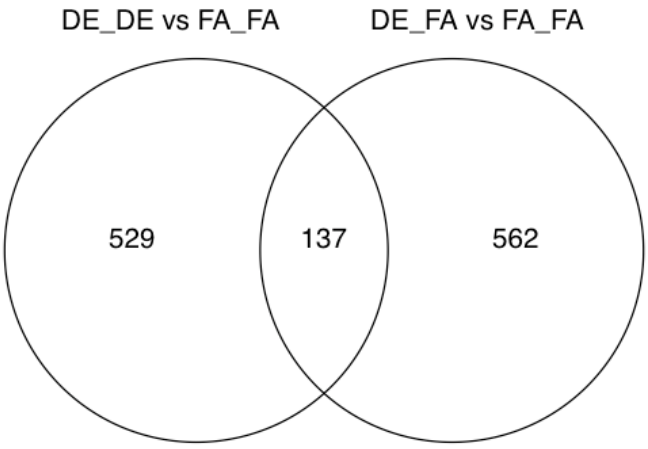


Figure 12: Downregulated genes in both the DE/DE vs. FA/FA comparison and the DE/FA vs. FA/FA comparison

Analysis using the IPA software produced 5 pathways for each treatment comparison that contained the most altered genes. Results in parentheses are displayed as the *p*-value and the number of genes that were found altered by the exposure out of the total number of genes considered a part of the named pathway. Five pathways that were most changed from DE/DE to FA/FA were mitochondrial dysfunction (p = 3.98E-20, 27/171 genes), oxidative phosphorylation (p = 2.48E-15, 19/109 genes), TCA cycle II (p = 8.75E-10, 8/23 genes), glutaryl-CoA degradation (p = 3.01E-07, 5/11 genes), and tryptophan degradation III (p = 3.34E-07, 6/20

genes). Pathways changed between DE/FA and FA/FA were RhoGDI signaling (p = 6.77E-04, 11/173 genes), hepatic fibrosis/hepatic stellate cell activation (p = 1.94E-03, 11/197 genes), regulation of actin-based motility by Rho (p = 2.18E-03, 7/91 genes), axonal guidance signaling (p = 2.53E-03, 18/432 genes), and VDR/RXR activation (p = 4.46E-03, 6/78). Five pathways that were most changed from DE/DE to DE/FA were hepatic fibrosis/hepatic stellate cell activation (p = 3.07E-18, 42/197 genes), epithelial adherens junction signaling (p = 3.45E-12, 29/146 genes), actin cytoskeleton signaling (p = 2.18E-10, 33/217 genes), axonal guidance signaling (p = 1.45E-09, 48/432 genes), and cellular effects of sildenafil (p = 2.70E-08, 22/129 genes).

The DE/DE vs. FA/FA results were the main focus in this study because this comparison had the most severe difference in exposure. When filtering pathways by "molecular and cellular functions," energy production (5.60E-14<p<1.49E-02, 53 altered genes), lipid metabolism (5.60E-14<p<1.49E-02, 74 altered genes), and molecular transport (4.40E-13<p<1.49E-02, 76 altered genes) were among the top five most dramatically impacted by DE exposure. There were 18 total functions that were altered within "energy production," with oxidation of lipids (p = 5.60E-14, 24 total genes), oxidation of fatty acids (p = 1.59E-11, 19 total genes), and beta-oxidation of lipids (p = 2.94E-11, 13 total genes) being the top three contributors. It was found that most genes within "energy production" were downregulated after DE exposure. There were 67 total functions that were altered within "lipid metabolism," with oxidation of lipids (p = 5.60E-14, 24 genes), concentration of fatty acids (p = 4.40E-13, 27 genes), and oxidation of fatty acids (p = 1.59E-11, 19 genes) being the top three contributors. The majority of altered genes within "lipid metabolism" were also downregulated after DE exposure. Molecular transport contained a total of 37 functions that were altered by DE exposure, the top three being

concentration of fatty acids (p = 4.40E-13, 27 genes), concentration of lipids (p = 3.84E-09, 42 genes), and quantity of reactive oxygen species (p = 2.12E-08, 16 genes). The majority of genes within "concentration of fatty acids and concentration of lipids" were downregulated; however, there was a noticeable group of genes that were upregulated within "quantity of reactive oxygen species."

It was also found that the top two most altered pathways when filtering for "developmental disorder" were fatty acid oxidation disorder (p = 2.99E-08, 7 genes) and mitochondrial respiratory chain deficiency (p = 4.12E-07, 8 genes). This filter was chosen in order to elucidate specific developmental processes that would have been impacted by DE exposure *in utero*, a period of time when the body is rapidly growing and is especially susceptible to developmental toxicants.

Discussion

Breeding Success

There was premature pup death in all treatment groups prior to weaning, however, there were different patterns of death. The premature pup death plot in Figure 1 shows that there was a constant rate of death beginning at week 2 in the DE/DE group whereas there was a steep increase in pup death at week 4 for the FA/FA group. The constant rate of death in the DE/DE group could have been due to adverse conditions created by the DE exposure; however, post-death examination of the pups is necessary to determine the exact nature of death.

Body Weight

The observed trend among body weights showed a significant increase in week 1 weight for the DE/DE group over the DE/FA and FA/FA group. This result contradicts expectations because it was expected that early body weight would be decreased in the DE/DE group compared to the FA/FA group, due to adverse *in utero* conditions resulting in fewer necessary nutrients to reach a healthy body weight.

A possible explanation for the difference in results could be that the current study substituted week 1 body weight for birth weight. This was done due to low sample size and the fear of maternal rejection if the pups were handled at birth. It could be that pups exposed to DE were in fact born with a lower birth weight than FA exposed, but quickly gained weight in the first week due to an abundance of nutrient availability.

It was also expected that week 16 body weight and overall weight gain would be significantly greater in DE exposed over FA exposed subjects, as seen in the Weldy *et al.* results, where significant weight gain was observed over time after *in utero* DE exposure[91]. This pattern

was not observed in the current study, however, it is possible that it could have been observed in a study with larger sample sizes. It could also be that the study was not conducted long enough (i.e. until 32 weeks) for sufficient changes in body weight to be observed. Using ApoE-deficient mice might have attenuated differences in weight gain across treatment groups and thus shadowed any subtle effects of the diesel exhaust exposure. Tibia length was also not measured so the possibility that weight was due simply to bigger bodies rather than excess fat storage cannot be excluded.

Lipid Profiles

It was expected that lipid profiles would be significantly altered between groups, with the DE/DE group having higher values of triglycerides and total cholesterol, but more specifically, higher values of VLDL and LDL and lower values of HDL. Such results would support the hypothesis that DE exposure *in utero* created an environment that increased susceptibility to cardiovascular disease long term. Although this study's results contradict what was expected, they correlate with the body weight findings. It would be expected that subjects with higher total triglycerides would have higher body weights due to overall larger stores of fat within the body. However, neither body weight nor triglyceride levels were positively correlated to *in utero* DE exposure. It is possible that using a Western diet (i.e. high fat content) over the standard chow diet could have increased the overall triglyceride and total cholesterol levels across treatments and perhaps amplified any effects of the DE exposure *in utero*.

Another factor that could have impacted the lipid profiles was the pooling of the samples. Male and female samples were pooled for each treatment. Due to small sample size, separating the lipid profiles based on sex was not an option. It is possible that males and females had

inherently different lipid profiles that would have hidden an underlying relationship between DE exposure and total triglyceride and cholesterol levels.

In previous studies, it has been shown that exercise is associated with both increases in oxidative stress and plasma HDL levels[142]. Using this platform, it could be theorized that exposure to DE increased oxidative stress and thus stimulated production of HDLs to counteract the increase in oxidative stress. In order to prove this theory, concurrent testing of both oxidative stress markers and HDLs levels would need to be conducted throughout the study.

Histology

Although there was no statistically significant effect of DE exposure *in utero*, there was a trend towards protection of atherosclerotic development in males when exposed to DE *in utero*. This result aligns with a previous study conducted in the Rosenfeld lab in which it was observed that *in utero* DE exposure also trended towards smaller atherosclerotic plaques in male mice at 16 weeks of age (results not shown). However, both of these studies had small sample sizes that made it difficult to decipher a clear relationship between *in utero* DE exposure and atherosclerotic plaque development.

An interesting finding was the trend towards protection against atherosclerotic development in males when exposed to DE *in utero*. The DE/DE group for the lipid profiles was strongly influenced by males ($n_{male} = 3$, $n_{female} = 1$). If DE exposure in fact increased the levels of HDLs in males, it could explain why progression of atherosclerotic development trended towards decreased lesion areas in males. If DE did not alter the antioxidant properties of HDLs as observed in the Yin *et al*. study[76], then higher levels of HDLs could have protected against the lipid oxidation that highly contributes to atherosclerotic lesion development.

A possible explanation for the lack of results, besides small sample size, could be the short time frame. It is likely that significant trends of atherosclerotic progression could not be deciphered at 16 weeks because 16 weeks is approximately the earliest time point at which significant atherosclerotic lesion are observable. Because of the early time point, some subjects had miniscule lesions whereas others had large plaques already. Thirty-two weeks, the time point in atherosclerotic development in mice where atheroma is prominent, could have reduced the variability in plaque size within individual treatments. DE exposure also might not impact early stages of atherosclerotic development as significantly as later stages. A study of 32 weeks could perhaps more easily elucidate a significant difference in lesion area if *in utero* DE exposure increases the progression of later stages of atherosclerotic development.

RNA Sequencing

There were two genes that were significantly altered by DE/DE exposure when compared to FA/FA samples. There is no evidence of either of these two genes being directly associated with atherosclerotic development. As previously mentioned, *CYP2b10* was downregulated in both DE/DE and DE/FA when compared to FA/FA. This leads to the conclusion that *CYP2b10* regulation could be significantly altered due purely to *in utero* exposure.

When comparing the Venn diagrams in Figures 10 and 11, it is apparent that there are 152 genes that were either upregulated or downregulated in both the DE/DE and DE/FA group when compared to the FA/FA group (15 upregulated, 137 downregulated). Although these genes are not filtered based on a false discovery rate, the data points towards genes that are impacted due to *in utero* exposure to DE only. Because these genes were altered in both groups, it adds weight, to the results despite the lack of a false discovery rate filter.

This analysis only focused on differences between the DE/DE and FA/FA group because these groups were most different with respect to exposure. Although the results from the IPA analysis indicate pathways that could be potentially impacted by *in utero* DE exposure, results of the IPA analysis are highly speculative and supplemental studies must be conducted to either confirm or deny the findings of this study. The genes determined "significant" for the IPA analysis did not include an FDR filter, therefore, there could be a substantial number of genes that falsely appeared significantly altered between treatment groups. A strict FDR filter (less than 5%) would show which genes and transcription pathways are most confidently impacted by DE exposure *in utero*. If these genes and pathways were associated with atherosclerotic progression, it could support the hypothesis that *in utero* exposure to DE does in fact alter fetal programming, such that exposed fetuses are susceptible to lesion formation. It would also be interesting to see how the associated genes and pathways change as the FDR becomes less and less stringent. If the top genes and pathways with a strict FDR remain the most altered genes and pathways as the FDR becomes less stringent, it would instill confidence on the validity and significance of *in utero* DE exposure on genetic fetal programming.

Limitations

The main limitations of this study were sample size and a short time frame. The first round of breedings resulted in zero viable pups due to dam cannibalism. The loss of these litters may be due to outside stressors (i.e. construction disturbances). Due to the shortened time frame, only a second round of breedings was conducted. These breedings did not result in as many DE/DE pups as desired, which severely limited study power for the DE/DE groups. A minimum of 36 total pups (18 males and 18 females) should have been produced to determine statistical significance for both treatment effects and sex effects. This would have allowed for 3 animals from each sex to be analyzed for each treatment group, including 3 animals per sex per treatment to be used for RNA sequencing. Ideally, 72 pups would have been produced, resulting in 6 animals per treatment per sex, including the RNA analysis. A total of 41 offspring were produced; however, distribution of these animals was such that the FA/FA group had a much larger sample size than DE/DE. Post-birth deaths also decreased overall sample sizes, with 3 deaths in the DE/DE group, 2 deaths in the DE/FA group, and 3 deaths in the FA/FA group. As a result of the breeding distributions across treatment groups, only 4 total animals from DE/DE were available for histological analysis. The lack of sufficient DE/DE animals made small variations appear large and left no possibility for an analysis on sex effects in response to DE exposure.

This study was also limited in the overall time available to finish the project. The histological analysis was only done at 16 weeks of age, whereas a longer time point (i.e. 32 weeks) would have been more ideal. A longer time point would have allowed a more substantial amount of time for atherosclerotic lesions to form. At 16 weeks of age, it is possible that atherosclerotic lesions might not yet have formed in animals that would have formed them at 32

weeks. Therefore, there could be more significant variation between subjects at 16 weeks than at 32 weeks. It is also possible that the shortened time frame shadowed effects of DE on quickening atherosclerotic progression in later stages of disease development. Decreased study time also resulted in the inability to conduct targeted DNA bisulfite sequencing and sufficient follow-up studies on RNA results. This limits the validity of the RNA results; however, the results may function as a base for future studies.

Another limitation of the proposed project includes the lack of a dose-response. It would be beneficial to include another dose of diesel exhaust in order to analyze the lesion area in response to increasing doses; however, time and resources did not permit the use of multiple doses in the current study.

The nature of exposure of DE itself may not have been representative of real-world experience. DE exposure was characterized by $PM_{2.5}$ concentration. When time-weighted for hourly $PM_{2.5}$ exposure, the exposure level in the study (45 $\mu g/m^3$) is still higher than EPA regulated levels of atmospheric $PM_{2.5}$ (35 $\mu g/m^3$). It is unlikely that the average pregnant woman would be exposed to $PM_{2.5}$ levels this high on a daily basis, unless participating in a pollution-prone occupation such as mining or working in loading-dock environments. On an annual exposure level, the experimental exposure concentration is much higher than the average real-world exposure level, excluding high-pollution areas like Eastern Asia.

A limitation to the initial design of the study may have been the lack of a FA/DE group. Many of the significant changes were observed between the DE/DE and DE/FA group, indicating that there are important changes occurring postnatally. Because there was no group that was only exposed to DE postnatally, it is impossible to discern effects of postnatal exposure to DE (either by direct inhalation of DE or by exposure to factors within maternal milk).

Suggestions for Future Studies

The most crucial follow-up studies involve validating the RNA results. These studies could include DNA methylation, histone acetylation, and qPCR of targeted genes from the RNA results. If DNA methylation and histone acetylation studies show that genes found altered in the RNA sequencing study were also altered via methylation or acetylation, then more specific hypotheses can be formed regarding the epigenetic mechanisms of *in utero* DE exposure on adulthood susceptibility to disease. qPCR of the most altered genes (either by upregulation or downregulation) should also be conducted to determine the overall abundance of the gene in relation to treatment group.

Another suggestion to validate the genetic analysis would include analyzing the levels of oxidative stress throughout the study. A thiobarbituric acid reactive substances (TBARs) or 3-nitrotyrosine (3-NT) test for lipid peroxidation would be especially pertinent for this study. A TBARs or 3-NT test showing higher levels of lipid oxidation for the DE/DE group over the FA/FA group could bring validation to the hypothesis that DE exhaust *in utero* predisposes the adult to increased oxidative stress. HDL levels should also be measured at time points correlating to the oxidative stress tests to investigate the hypothesis that HDL production is stimulated by increased ROS levels. To examine the viability of the HDL (i.e. the antioxidant properties), a 2',7'-dichlorofluorescein (DCF) fluorescence test could be conducted. Specifically, the DCF test estimates the ability of HDL to inhibit low-density lipoprotein oxidation. HDL validity assays could also be coupled with RNA sequencing data conducted on liver tissue and specific to HDL synthesis. If genes and pathways associated with HDL synthesis are significantly upregulated in the DE/DE group, the results would further support the hypothesis that HDLs are in fact increased due to DE exposure.

To further correlate epigenetic changes with an adverse intrauterine environment, maternal blood should be collected during pregnancy to analyze for possible cytokines, chemokines, and other circulating proinflammatory factors. If there are significant differences between control and DE-exposed circulating proinflammatory factors, it could be hypothesized that these factors either directly or indirectly create an adverse intrauterine environment, thus resulting in possible epigenetic and adulthood histological alterations in the *in utero*-exposed pups.

An *in vitro* study to examine cell-types specifically affected by DEP exposure could also provide mechanistic information as to how DE exposure actually impacts atherosclerotic development at a cellular level. For instance, if leukocytes happen to be directly impacted by DEP, DEP exposure could hypothetically increase their ability to adhere to vessel walls, thus increasing the likelihood of monocyte infiltration and subsequent formation of the fatty streak due to foam cell formation.

References

1. Young PEG, Editor A. Bureau of Transportation Statistics United States Department of Transportation Journal of Transportation and Statistics and Statistics. *J Transp Stat Stat*. 2003;6.

2. Brook RD, Rajagopalan S, Pope CA, et al. Particulate matter air pollution and cardiovascular disease: An update to the scientific statement from the American Heart Association. *Circulation*. 2010;121(21):2331-78. doi:10.1161/CIR.0b013e3181dbece1.

3. Brook RD, Franklin B, Cascio W, et al. Air pollution and cardiovascular disease: a statement for healthcare professionals from the Expert Panel on Population and Prevention Science of the American Heart Association. In: *Circulation*.Vol 109.; 2004:2655-2671. doi:10.1161/01.CIR.0000128587.30041.C8.

4. Agency USEP. *Health Assessment Document For Diesel Engine Exhaust*.; 2002:1-669. Available at: http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=29060#area.

5. Weldy CS, Wilkerson HW, Larson TV, Stewart JA, Kavanagh TJ. DIESEL particulate exposed macrophages alter endothelial cell expression of eNOS, iNOS, MCP1, and glutathione synthesis genes. *Toxicol Vitr*. 2011;25:2064-2073. doi:10.1016/j.tiv.2011.08.008.

6. EPA US. National Ambient Air Quality Standards (NAAQS). 2012. Available at: http://www.epa.gov/air/criteria.html. Accessed April 28, 2014.

7. Pope CA, Dockery DW. Health effects of fine particulate air pollution: lines that connect. *J Air Waste Manag Assoc*. 2006;56:709-742.

8. Simkhovich BZ, Kleinman MT, Kloner RA. Air Pollution and Cardiovascular Injury. Epidemiology, Toxicology, and Mechanisms. *J Am Coll Cardiol*. 2008;52:719-726. doi:10.1016/j.jacc.2008.05.029.

9. Lewtas J. Air pollution combustion emissions: Characterization of causative agents and mechanisms associated with cancer, reproductive, and cardiovascular effects. *Mutat Res - Rev Mutat Res*. 2007;636:95-133. doi:10.1016/j.mrrev.2007.08.003.

10. S. Lim S, Vos T, Flaxman AD, et al. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990–2010: a systematic analysis for. *Lancet*. 2013;380:2224-2260. doi:10.1016/S0140-6736(12)61766-8.

11. Hesterberg TW, Long CM, Bunn WB, Lapin CA, McClellan RO, Valberg PA. Health effects research and regulation of diesel exhaust: an historical overview focused on lung cancer risk. *Inhal Toxicol*. 2012;24:1-45. doi:10.3109/08958378.2012.691913.

12. Guo X, Liu ESL, Ko JKS, et al. Protective role of cyclooxygenase inhibitors in the adverse action of passive cigarette smoking on the initiation of experimental colitis in rats. *Eur J Pharmacol*. 2001;411:193-203. doi:10.1016/S0014-2999(00)00914-6.

13. Brook RD, Rajagopalan S, Pope CA, et al. Particulate matter air pollution and cardiovascular disease: An update to the scientific statement from the american heart association. *Circulation*. 2010;121:2331-2378. doi:10.1161/CIR.0b013e3181dbece1.

14. Kittelson DB. Engines and nanoparticles: A review. *J Aerosol Sci*. 1998;29:575-588. doi:10.1016/S0021-8502(97)10037-4.

15. Agency USEP. National-scale air toxics assessment for 1999: Estimated emissions, concentration and risk. *Natl Air Toxics Assess*. 2006. Available at: www.epa.gov/ttn/atw/nata1999/natafinalfact.html . Accessed April 15, 2014.

16. Maier KL, Alessandrini F, Beck-Speier I, et al. Health effects of ambient particulate matter--biological mechanisms and inflammatory responses to in vitro and in vivo particle exposures. *Inhal Toxicol*. 2008;20:319-337. doi:10.1080/08958370701866313.

17. Sawyer K, Mundandhara S, Ghio AJ, Madden MC. The effects of ambient particulate matter on human alveolar macrophage oxidative and inflammatory responses. *J Toxicol Environ Health A*. 2010;73:41-57. doi:10.1080/15287390903248901.

18. Mudway IS, Stenfors N, Duggan ST, et al. An in vitro and in vivo investigation of the effects of diesel exhaust on human airway lining fluid antioxidants. *Arch Biochem Biophys*. 2004;423:200-212. doi:10.1016/j.abb.2003.12.018.

19. Pourazar J, Mudway IS, Samet JM, et al. Diesel exhaust activates redox-sensitive transcription factors and kinases in human airways. *Am J Physiol Lung Cell Mol Physiol*. 2005;289:L724-L730. doi:10.1152/ajplung.00055.2005.

20. Nordenhall C, Pourazar J, Blomberg A, Levin JO, Sandstrom T, Adelroth E. Airway inflammation following exposure to diesel exhaust: a study of time kinetics using induced sputum. *Eur Respir J*. 2000;15:1046-1051. Available at: <Go to ISI>://000087792000012.

21. Salvi S, Blomberg A, Rudell B, et al. Acute inflammatory responses in the airways and peripheral blood after short-term exposure to diesel exhaust in healthy human volunteers. *Am J Respir Crit Care Med*. 1999;159:702-709. doi:10.1164/ajrccm.159.3.9709083.

22. Stenfors N, Nordenhäll C, Salvi SS, et al. *Different Airway Inflammatory Responses in Asthmatic and Healthy Humans Exposed to Diesel.*; 2004:82-86. doi:10.1183/09031936.03.00004603.

23. Sun Q, Wang A, Jin X, et al. Long-term air pollution exposure and acceleration of atherosclerosis and vascular inflammation in an animal model. *JAMA*. 2005;294:3003-3010. doi:10.1001/jama.294.23.3003.

24. Sun Q, Yue P, Ying Z, et al. Air pollution exposure potentiates hypertension through reactive oxygen species-mediated activation of Rho/ROCK. *Arterioscler Thromb Vasc Biol*. 2008;28:1760-1766. doi:10.1161/ATVBAHA.108.166967.

25. Törnqvist H, Mills NL, Gonzalez M, et al. Persistent endothelial dysfunction in humans after diesel exhaust inhalation. *Am J Respir Crit Care Med*. 2007;176:395-400. doi:10.1164/rccm.200606-872OC.

26. Nemmar A, Vanbilloen H, Hoylaerts MF, Hoet PH, Verbruggen A, Nemery B. Passage of intratracheally instilled ultrafine particles from the lung into the systemic circulation in hamster. *Am J Respir Crit Care Med*. 2001;164:1665-1668. doi:10.1164/rccm2101036.

27. Nemmar A, Hoet PHM, Vanquickenborne B, et al. Passage of inhaled particles into the blood circulation in humans. *Circulation*. 2002;105:411-414. doi:10.1161/hc0402.104118.

28. Nemmar A, Hoylaerts MF, Hoet PHM, Nemery B. Possible mechanisms of the cardiovascular effects of inhaled particles: Systemic translocation and prothrombotic effects. In: *Toxicology Letters*.Vol 149.; 2004:243-253. doi:10.1016/j.toxlet.2003.12.061.

29. Bruchas RR, de Las Fuentes L, Carney RM, et al. The St. Louis African American health-heart study: methodology for the study of cardiovascular disease and depression in young-old African Americans. *BMC Cardiovasc Disord*. 2013;13. doi:10.1186/1471-2261-13-66.

30. Stary HC, Blankenhorn DH, Chandler AB, et al. A definition of the intima of human arteries and of its atherosclerosis-prone regions. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler Thromb Vasc Biol*. 1992;12:120-134.

31. Dzau VJ, Braun-Dullaeus RC, Sedding DG. Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. *Nat Med*. 2002;8:1249-1256. doi:10.1038/nm1102-1249.

32. Rosenfeld ME. An overview of the evolution of the atherosclerotic plaque: from fatty streak to plaque rupture and thrombosis. *Z Kardiol*. 2000;89 Suppl 7:2-6. doi:10.1007/s003920070045.

33. Glagov S, Zarins C, Giddens DP, Ku DN. Hemodynamics and atherosclerosis. Insights and perspectives gained from studies of human arteries. *Arch Pathol Lab Med*. 1988;112:1018-1031.

34. Rosenfeld ME. Oxidized LDL Affects Multiple Atherogenic Cellular Responses. *Circulation*. 1991;83:2137-2140.

35. Nakashima Y, Raines EW, Plump AS, Breslow JL, Ross R. Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse. *Arterioscler Thromb Vasc Biol*. 1998;18:842-851. doi:10.1161/01.ATV.18.5.842.

36. Iiyama K, Hajra L, Iiyama M, et al. Patterns of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 expression in rabbit and mouse atherosclerotic lesions and at sites predisposed to lesion formation. *Circ Res*. 1999;85:199-207. doi:10.1161/01.RES.85.2.199.

37. Wang GP, Deng ZD, Ni J, Qu ZL. Oxidized low density lipoprotein and very low density lipoprotein enhance expression of monocyte chemoattractant protein-1 in rabbit peritoneal exudate macrophages. *Atherosclerosis*. 1997;133:31-36. doi:10.1016/S0021-9150(97)00109-3.

38. Amberger A, Maczek C, Jürgens G, et al. Co-expression of ICAM-1, VCAM-1, ELAM-1 and Hsp60 in human arterial and venous endothelial cells in response to cytokines and oxidized low-density lipoproteins. *Cell Stress Chaperones*. 1997;2:94-103. doi:10.1379/1466-1268(1997)002<0094:CEOIVE>2.3.CO;2.

39. Frostegaard J, Ulfgren AK, Nyberg P, et al. Cytokine expression in advanced human atherosclerotic plaques: Dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis*. 1999;145:33-43. doi:10.1016/S0021-9150(99)00011-8.

40. Rosenfeld ME. Inflammation, lipids, and free radicals: lessons learned from the atherogenic process. *Semin Reprod Endocrinol*. 1998;16:249-261. doi:10.1055/s-2007-1016285.

41. Stary HC, Chandler AB, Glagov S, et al. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation*. 1994;89:2462-2478. doi:10.1161/01.CIR.89.5.2462.

42. Newby AC, Zaltsman AB. Fibrous cap formation or destruction - The critical importance of vascular smooth muscle cell proliferation, migration and matrix formation. *Cardiovasc Res*. 1999;41:345-360. doi:10.1016/S0008-6363(98)00286-7.

43. Mombouli J V, Vanhoutte PM. Endothelial dysfunction: from physiology to therapy. *J Mol Cell Cardiol*. 1999;31:61-74. doi:10.1006/jmcc.1998.0844.

44. Rosenfeld ME, Averill MM, Bennett BJ, Schwartz SM. Progression and disruption of advanced atherosclerotic plaques in murine models. *Curr Drug Targets*. 2008;9:210-216. doi:10.2174/138945008783755575.

45. Glagov S, Weisenberg E, Zarins CK, Stankunavicius R, Kolettis GJ. Compensatory enlargement of human atherosclerotic coronary arteries. *N Engl J Med*. 1987;316:1371-1375. doi:10.1056/NEJM198705283162204.

46. Kockx MM. Apoptosis in the atherosclerotic plaque: quantitative and qualitative aspects. *Arterioscler Thromb Vasc Biol*. 1998;18:1519-1522. doi:10.1161/01.ATV.18.10.1519.

47. Falk E. Why do plaques rupture? *Circulation*. 1992;86:III30-I42.

48. Lendon CL, Davies MJ, Born G V, Richardson PD. Atherosclerotic plaque caps are locally weakened when macrophages density is increased. *Atherosclerosis*. 1991;87:87-90. doi:10.1016/0021-9150(91)90235-U.

49. Taubman MB, Fallon JT, Schecter AD, et al. Tissue factor in the pathogenesis of atherosclerosis. *Thromb Haemost*. 1997;78:200-204.

50. Aviram M. Modified forms of low density lipoprotein affect platelet aggregation in vitro. *Thromb Res*. 1989;53:561-567. doi:10.1016/0049-3848(89)90145-X.

51. Ardlie NG, Selley ML, Simons LA. Platelet activation by oxidatively modified low density lipoproteins. *Atherosclerosis*. 1989;76:117-124. doi:10.1016/0021-9150(89)90094-4.

52. Rosenfeld ME. Cellular mechanisms in the development of atherosclerosis. *Diabetes Res Clin Pract*. 1996;30 Suppl:1-11.

53. Tsukada T, Rosenfeld ME, Ross R, Gown AM. Immunocytochemical analysis of cellular components in atherosclerotic lesions. Use of monoclonal antibodies with the Watanabe and fat-fed rabbits. *Arteriosclerosis*. 1986;6:601-613.

54. Rosenfeld ME, Tsukada T, Gown AM, Ross R. Fatty streak initiation in WHHL and comparably hypercholesterolemic fat-fed rabbits. *Arteriosclerosis*. 1987;7:9-23.

55. Flaherty JT, Pierce JE, Ferrans VJ, Patel DJ, Tucker WK, Fry DL. Endothelial nuclear patterns in the canine arterial tree with particular reference to hemodynamic events. *Circ Res*. 1972;30:23-33. doi:10.1161/01.RES.30.1.23.

56. Levesque MJ, Nerem RM. The elongation and orientation of cultured endothelial cells in response to shear stress. *J Biomech Eng*. 1985;107:341-347. doi:10.1115/1.3138567.

57. Jaffe EA. Cell biology of endothelial cells. *Hum Pathol*. 1987;18:234-239. doi:10.1016/S0046-8177(87)80005-9.

58. Doran AC, Meller N, McNamara CA. Role of smooth muscle cells in the initiation and early progression of atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2008;28:812-819. doi:10.1161/ATVBAHA.107.159327.

59. Jones SP, Bolli R. The ubiquitous role of nitric oxide in cardioprotection. *J Mol Cell Cardiol*. 2006;40:16-23. doi:10.1016/j.yjmcc.2005.09.011.

60. Ito T, Ikeda M, Yamasaki H, Sagai M, Tomita T. Peroxynitrite formation by diesel exhaust particles in alveolar cells: Links to pulmonary inflammation. *Environ Toxicol Pharmacol*. 2000;9:1-8. doi:10.1016/S1382-6689(00)00053-3.

61. White CR, Brock TA, Chang LY, et al. Superoxide and peroxynitrite in atherosclerosis. *Proc Natl Acad Sci U S A*. 1994;91:1044-1048. doi:10.1073/pnas.91.3.1044.

62. Kim GH, Ryan JJ, Archer SL. The role of redox signaling in epigenetics and cardiovascular disease. *Antioxid Redox Signal*. 2013;18:1920-36. doi:10.1089/ars.2012.4926.

63. Raju R, Palapetta S, Sandhya V, et al. A Network Map of FGF-1/FGFR Signaling System. *J Signal Transduct*. 2014.

64. Scholz CC, Taylor CT. Targeting the HIF pathway in inflammation and immunity. *Curr Opin Pharmacol*. 2013;13:646-653. doi:10.1016/j.coph.2013.04.009.

65. Barnes PJ, Adcock IM. Transcription factors and asthma. *Eur Respir J Off J Eur Soc Clin Respir Physiol*. 1998;12:221-234. doi:10.1183/09031936.98.12010221.

66. Wright JG, Christman JW. The role of nuclear factor kappa B in the pathogenesis of pulmonary diseases: implications for therapy. *Am J Respir Med*. 2003;2:211-219.

67. Baccarelli A, Ghosh S. Environmental exposures, epigenetics and cardiovascular disease. *Curr Opin Clin Nutr Metab Care*. 2012;15:323-329. doi:10.1097/MCO.0b013e328354bf5c.

68. Cipollone F, Rocca B, Patrono C. Cyclooxygenase-2 Expression and Inhibition in Atherothrombosis. *Arterioscler Thromb Vasc Biol*. 2004;24:246-255. doi:10.1161/01.ATV.0000104005.92603.f2.

69. Kawabe J, Ushikubi F, Hasebe N. Prostacyclin in vascular diseases. - Recent insights and future perspectives -. *Circ J*. 2010;74:836-843.

70. Dubois RN, Abramson SB, Crofford L, et al. Cyclooxygenase in biology and disease. *FASEB J*. 1998;12:1063-1073. doi:9737710.

71. Cipollone F, Prontera C, Pini B, et al. Overexpression of functionally coupled cyclooxygenase-2 and prostaglandin E synthase in symptomatic atherosclerotic plaques as a basis of prostaglandin E(2)-dependent plaque instability. *Circulation*. 2001;104:921-927. doi:10.1161/hc3401.093152.

72. Pons D, de Vries FR, van den Elsen PJ, Heijmans BT, Quax PHA, Jukema JW. Epigenetic histone acetylation modifiers in vascular remodelling: new targets for therapy in cardiovascular disease. *Eur Heart J*. 2009;30:266-277. doi:10.1007/BF03086114.

73. Suwa T, Hogg JC, Quinlan KB, Ohgami A, Vincent R, Van Eeden SF. Particulate air pollution induces progression of atherosclerosis. *J Am Coll Cardiol*. 2002;39:935-942. doi:10.1016/S0735-1097(02)01715-1.

74. Künzli N, Jerrett M, Mack WJ, et al. Ambient air pollution and atherosclerosis in Los Angeles. *Environ Health Perspect*. 2005;113:201-206. doi:10.1289/ehp.7523.

75. Künzli N, Jerrett M, Garcia-Esteban R, et al. Ambient air pollution and the progression of atherosclerosis in adults. *PLoS One*. 2010;5. doi:10.1371/journal.pone.0009096.

76. Yin F, Lawal A, Ricks J, et al. Diesel exhaust induces systemic lipid peroxidation and development of dysfunctional pro-oxidant and pro-inflammatory high-density lipoprotein. *Arterioscler Thromb Vasc Biol*. 2013;33:1153-1161. doi:10.1161/ATVBAHA.112.300552.

77. Bai N, Kido T, Kavanagh TJ, et al. Exposure to diesel exhaust up-regulates iNOS expression in ApoE knockout mice. *Toxicol Appl Pharmacol*. 2011;255:184-192. doi:10.1016/j.taap.2011.06.013.

78. Bai N, Tranfield EM, Kavanagh TJ, Kaufman JD, Rosenfeld ME, Van Eeden SF. Exposure to diesel exhaust upregulates COX-2 expression in ApoE knockout mice. *Inhal Toxicol*. 2012;24(8):518-527.

79. Bai N, Kido T, Suzuki H, et al. Changes in atherosclerotic plaques induced by inhalation of diesel exhaust. *Atherosclerosis*. 2011;216:299-306. doi:10.1016/j.atherosclerosis.2011.02.019.

80. Rogers L, Velten M. Maternal Inflammation, Growth Retardation, and Preterm Birth: Insights into Adult Cardiovascular Disease. *Life Sci*. 2007;18(13-14):417-429.

81. Liguori A, D'Armiento FP, Palagiano A, et al. Effect of gestational hypercholesterolaemia on omental vasoreactivity, placental enzyme activity and transplacental passage of normal and oxidised fatty acids. *BJOG An Int J Obstet Gynaecol*. 2007;114:1547-1556. doi:10.1111/j.1471-0528.2007.01510.x.

82. Barker DJP. The Developmental Origins of Adult Disease. *J Am Coll Nutr*. 2004;23:588S-595. Available at: http://www.jacn.org/cgi/content/abstract/23/suppl_6/588S.

83. Barker DJP. Developmental origins of adult health and disease. *J Epidemiol Community Heal*. 2004;58:114-115. doi:10.1136/jech.58.2.114.

84. Gluckman PD, Cutfield W, Hofman P, Hanson MA. The fetal, neonatal, and infant environments-the long-term consequences for disease risk. In: *Early Human Development*.Vol 81.; 2005:51-59. doi:10.1016/j.earlhumdev.2004.10.003.

85. Godfrey KM, Barker DJ. Fetal programming and adult health. *Public Health Nutr*. 2007;4:611-624. doi:10.1079/PHN2001145.

86. Hanson MA, Gluckman PD. Developmental processes and the induction of cardiovascular function: conceptual aspects. *J Physiol*. 2005;565:27-34. doi:10.1113/jphysiol.2004.082339.

87. Barker DJ, Osmond C, Golding J, Kuh D, Wadsworth ME. Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *BMJ*. 1989;298:564-567. doi:10.1136/bmj.298.6673.564.

88. Vijayakumar M, Fall CH, Osmond C, Barker DJ. Birth weight, weight at one year, and left ventricular mass in adult life. *Br Heart J*. 1995;73:363-367. doi:10.1136/hrt.73.4.363.

89. Martyn CN, Barker DJ, Jespersen S, Greenwald S, Osmond C, Berry C. Growth in utero, adult blood pressure, and arterial compliance. *Br Heart J*. 1995;73:116-121. doi:10.1136/hrt.73.2.116.

90. Srivastava S, D'Souza SE, Sen U, States JC. In utero arsenic exposure induces early onset of atherosclerosis in ApoE-/- mice. *Reprod Toxicol*. 2007;23:449-456. doi:10.1016/j.reprotox.2007.01.005.

91. Weldy CS, Liu Y, Liggitt HD, Chin MT. In utero exposure to diesel exhaust air pollution promotes adverse intrauterine conditions, resulting in weight gain, altered blood pressure, and increased susceptibility to heart failure in adult mice. *PLoS One*. 2014;9:e88582. doi:10.1371/journal.pone.0088582.

92. Epstein FH, Goldenberg RL, Hauth JC, Andrews WW. Intrauterine Infection and Preterm Delivery. *N Engl J Med*. 2000;342:1500-1507. doi:10.1056/NEJM200005183422007.

93. Romero R, Espinoza J, Gonsalves LF, Kusanovic JP, Friel LA, Nien JK. Inflammation in preterm and term labour and delivery. *Semin Fetal Neonatal Med*. 2006;11:317-326. doi:10.1016/j.siny.2006.05.001.

94. Ordovás J, Smith C. Epigenetics and Cardiovascular Disease. *Nat Rev Cardiol*. 2010;7(9):510-519.

95. Feinberg AP, Tycko B. The history of cancer epigenetics. *Nat Rev Cancer*. 2004;4:143-153. doi:10.1038/nrc1279.

96. Turunen MP, Aavik E, Yl??-Herttuala S. Epigenetics and atherosclerosis. *Biochim Biophys Acta - Gen Subj*. 2009;1790:886-891. doi:10.1016/j.bbagen.2009.02.008.

97. Jakubowski H. Pathophysiological consequences of homocysteine excess. *J Nutr*. 2006;136:1741S-1749S.

98. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet*. 2003;33 Suppl:245-254. doi:10.1038/ng1089.

99. Ehrlich M, Gama-Sosa MA, Huang LH, et al. Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells. *Nucleic Acids Res*. 1982;10:2709-2721. doi:10.1093/nar/10.8.2709.

100. Hiltunen MO, Turunen MP, Häkkinen TP, et al. DNA hypomethylation and methyltransferase expression in atherosclerotic lesions. *Vasc Med*. 2002;7:5-11. doi:10.1191/1358863x02vm418oa.

101. Miranda TB, Jones PA. DNA methylation: the nuts and bolts of repression. *J Cell Physiol*. 2007;213:384-390. doi:10.1002/jcp.21224.

102. Klose RJ, Bird AP. Genomic DNA methylation: The mark and its mediators. *Trends Biochem Sci*. 2006;31:89-97. doi:10.1016/j.tibs.2005.12.008.

103. Hoffman AR, Hu JF. Directing DNA methylation to inhibit gene expression. *Cell Mol Neurobiol*. 2006;26:425-438.

104. Hellman A, Chess A. Gene body-specific methylation on the active X chromosome. *Science*. 2007;315:1141-1143. doi:10.1126/science.1136352.

105. Sapienza C, Peterson AC, Rossant J, Al E. Degree of methylation of transgenes is dependent on gamete of origin. *Nature*. 1987;328:251-254.

106. Reik W, Howlett SK, Surani MA. Imprinting by DNA methylation: from transgenes to endogenous gene sequences. *Dev Suppl*. 1990:99-106.

107. Reik W, Howlett SK, Surani MA. Genomic imprinting determines methylation of parental alleles in transgenic mice. *Nature*. 1987;328:248-251.

108. Bird A. DNA methylation patterns and epigenetic memory. *Genes Dev*. 2002;16:6-21. doi:10.1101/gad.947102.

109. Bertram C, Khan O, Ohri S, Phillips DI, Matthews SG, Hanson MA. Transgenerational effects of prenatal nutrient restriction on cardiovascular and hypothalamic-pituitary-adrenal function. *J Physiol*. 2008;586:2217-2229. doi:10.1113/jphysiol.2007.147967.

110. Bygren LO, Kaati G, Edvinsson S. Longevity determined by paternal ancestors' nutrition during their slow growth period. *Acta Biotheor*. 2001;49:53-59. doi:10.1023/A:1010241825519.

111. Ehrlich M. Cancer-linked DNA hypomethylation and its relationship to hypermethylation. *Curr Top Microbiol Immunol*. 2006;310:251-274.

112. Lund G, Andersson L, Lauria M, et al. DNA methylation polymorphisms precede any histological sign of atherosclerosis in mice lacking apolipoprotein E. *J Biol Chem*. 2004;279:29147-29154. doi:10.1074/jbc.M403618200.

113. Laukkanen MO, Mannermaa S, Hiltunen MO, et al. Local hypomethylation in atherosclerosis found in rabbit ec-sod gene. *Arterioscler Thromb Vasc Biol*. 1999;19:2171-2178. doi:10.1161/01.ATV.19.9.2171.

114. Wilcox JN, Subramanian RR, Sundell CL, et al. Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels. *Arterioscler Thromb Vasc Biol*. 1997;17:2479-2488. doi:10.1161/01.ATV.17.11.2479.

115. Jiang Y, Zhang J, Xiong J, Cao J, Li G, Wang S. Ligands of peroxisome proliferator-activated receptor inhibit homocysteine-induced DNA methylation of inducible nitric oxide synthase gene. *Acta Biochim Biophys Sin*. 2007;39:366-376. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17492134.

116. Chang P-Y, Lu S-C, Lee C-M, et al. Homocysteine inhibits arterial endothelial cell growth through transcriptional downregulation of fibroblast growth factor-2 involving G protein and DNA methylation. *Circ Res*. 2008;102:933-941. doi:10.1161/CIRCRESAHA.108.171082.

117. Post WS, Goldschmidt-Clermont PJ, Wilhide CC, et al. Methylation of the estrogen receptor gene is associated with aging and atherosclerosis in the cardiovascular system. *Cardiovasc Res*. 1999;43:985-991. doi:10.1016/S0008-6363(99)00153-4.

118. Ying AK, Hassanain HH, Roos CM, et al. Methylation of the estrogen receptor-alpha gene promoter is selectively increased in proliferating human aortic smooth muscle cells. *Cardiovasc Res*. 2000;46:172-179.

119. Kim J, Kim JY, Song KS, et al. Epigenetic changes in estrogen receptor ?? gene in atherosclerotic cardiovascular tissues and in-vitro vascular senescence. *Biochim Biophys Acta - Mol Basis Dis*. 2007;1772:72-80. doi:10.1016/j.bbadis.2006.10.004.

120. Lillycrop KA, Phillips ES, Torrens C, Hanson MA, Jackson AA, Burdge GC. Feeding pregnant rats a protein-restricted diet persistently alters the methylation of specific cytosines in the hepatic PPAR alpha promoter of the offspring. *Br J Nutr*. 2008;100:278-282. doi:10.1017/S0007114507894438.

121. Das SK, Chakrabarti R. Role of PPAR in cardiovascular diseases. *Recent Pat Cardiovasc Drug Discov*. 2006;1:193-209. doi:10.2174/157489006777442441.

122. Walker BR. Glucocorticoids and cardiovascular disease. *Eur J Endocrinol*. 2007;157:545-559. doi:10.1530/EJE-07-0455.

123. Udali S, Guarini P, Moruzzi S, Choi SW, Friso S. Cardiovascular epigenetics: From DNA methylation to microRNAs. *Mol Aspects Med*. 2013;34:883-901. doi:10.1016/j.mam.2012.08.001.

124. Wang Y, Miao X, Liu Y, et al. Dysregulation of histone acetyltransferases and deacetylases in cardiovascular diseases. *Oxid Med Cell Longev*. 2014;2014. doi:10.1155/2014/641979.

125. Kuo MH, Allis CD. Roles of histone acetyltransferases and deacetylases in gene regulation. *Bioessays*. 1998;20:615-626. doi:10.1002/(SICI)1521-1878(199808)20:8<615::AID-BIES4>3.0.CO;2-H.

126. Ito K, Hanazawa T, Tomita K, Barnes PJ, Adcock IM. Oxidative stress reduces histone deacetylase 2 activity and enhances IL-8 gene expression: Role of tyrosine nitration. *Biochem Biophys Res Commun*. 2004;315:240-245. doi:10.1016/j.bbrc.2004.01.046.

127. Shindo J, Ishibashi T, Yokoyama K, et al. Granulocyte-macrophage colony-stimulating factor prevents the progression of atherosclerosis via changes in the cellular and extracellular composition of atherosclerotic lesions in watanabe heritable hyperlipidemic rabbits. *Circulation*. 1999;99:2150-2156. doi:10.1161/01.CIR.99.16.2150.

128. Ito K, Barnes PJ, Adcock IM. Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1beta-induced histone H4 acetylation on lysines 8 and 12. *Mol Cell Biol*. 2000;20:6891-6903. doi:10.1128/MCB.20.18.6891-6903.2000.

129. Cosio BG, Tsaprouni L, Ito K, Jazrawi E, Adcock IM, Barnes PJ. Theophylline restores histone deacetylase activity and steroid responses in COPD macrophages. *J Exp Med*. 2004;200:689-695. doi:10.1084/jem.20040416.

130. Tomita K, Barnes PJ, Adcock IM. The effect of oxidative stress on histone acetylation and IL-8 release. *Biochem Biophys Res Commun*. 2003;301:572-577. doi:10.1016/S0006-291X(02)03029-2.

131. Tong X, Yin L, Giardina C. Butyrate suppresses Cox-2 activation in colon cancer cells through HDAC inhibition. *Biochem Biophys Res Commun*. 2004;317:463-471. doi:10.1016/j.bbrc.2004.03.066.

132. Vinci MC, Polvani G, Pesce M. Epigenetic Programming and Risk: The Birthplace of Cardiovascular Disease? *Stem Cell Rev Reports*. 2013;9:241-253. doi:10.1007/s12015-012-9398-z.

133. Fish JE, Matouk CC, Rachlis A, et al. The expression of endothelial nitric-oxide synthase is controlled by a cell-specific histone code. *J Biol Chem*. 2005;280:24824-24838. doi:10.1074/jbc.M502115200.

134. Cao D, Wang Z, Zhang C-L, et al. Modulation of smooth muscle gene expression by association of histone acetyltransferases and deacetylases with myocardin. *Mol Cell Biol*. 2005;25:364-376. doi:10.1128/MCB.25.1.364-376.2005.

135. Qiu P, Li L. Histone acetylation and recruitment of serum responsive factor and CREB-binding protein onto SM22 promoter during SM22 gene expression. *Circ Res*. 2002;90:858-865. doi:10.1161/01.RES.0000016504.08608.B9.

136. Yan Z-Q, Yao Q-P, Zhang M-L, et al. Histone deacetylases modulate vascular smooth muscle cell migration induced by cyclic mechanical strain. *J Biomech*. 2009;42:945-948. doi:10.1016/j.jbiomech.2009.01.012.

137. Findeisen HM, Kahles FK, Bruemmer D. Epigenetic Regulation of Vascular Smooth Muscle Cell Function in Atherosclerosis. *Current Atherosclerosis Reports*. 2013:1-8.

138. Choi J-H, Nam K-H, Kim J, et al. Trichostatin A exacerbates atherosclerosis in low density lipoprotein receptor-deficient mice. *Arterioscler Thromb Vasc Biol*. 2005;25:2404-2409. doi:10.1161/01.ATV.0000184758.07257.88.

139. Findeisen HM, Gizard F, Zhao Y, et al. Epigenetic regulation of vascular smooth muscle cell proliferation and neointima formation by histone deacetylase inhibition. *Arterioscler Thromb Vasc Biol*. 2011;31:851-860. doi:10.1161/ATVBAHA.110.221952.

140. MacDonald JW. *Statistical Analysis of Mice Exposed to Diesel Exhaust*.; 2014.

141. Liao Y, Smyth GK, Shi W. The Subread aligner: Fast, accurate and scalable read mapping by seed-and-vote. *Nucleic Acids Res*. 2013;41. doi:10.1093/nar/gkt214.

142. Galán AI, Palacios E, Ruiz F, et al. Exercise, oxidative stress and risk of cardiovascular disease in the elderly. Protective role of antioxidant functional foods. *BioFactors*. 2006;27:167-83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17012773.

143. Janssen BG, Munters E, Pieters N, et al. Placental Mitochondrial DNA Content and Particulate Air Pollution during in Utero Life. *Environ Health Perspect*. 2012;120(09).

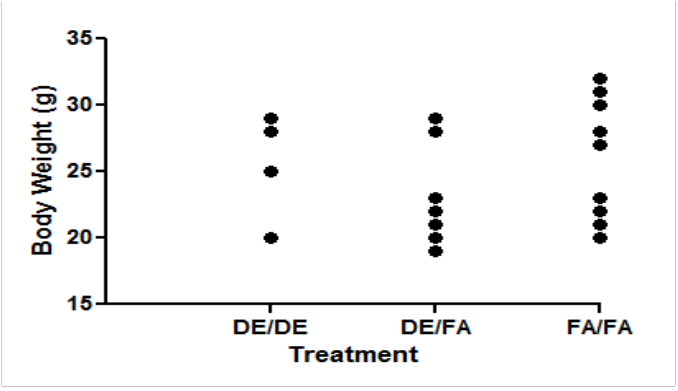
Appendix 1

National Ambient Air Quality Standards (NAAQS) as of October 2011[6]

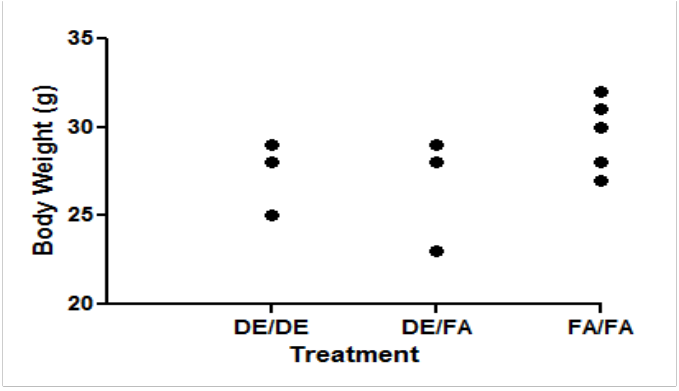
Pollutant		Primary/Secondary	Averaging Time	Level	Form
Carbon Monoxide		Primary	8-hour	9 ppm	Not to be exceeded more than once per year
			1-hour	35 ppm	
Lead		Primary and Secondary	Rolling 3 month average	0.15 $\mu g/m^3$	Not to be exceeded
Nitrogen Dioxide		Primary	1-hour	100 ppb	98^{th} percentile, averaged over 3 years
		Primary and Secondary	Annual	53 ppb	Annual Mean
Ozone		Primary and Secondary	8-hour	0.075 ppm	Annual fourth-highest daily maximum 8-hour concentration, averaged over 3 years
Particle Pollution	$PM_{2.5}$	Primary	Annual	12 $\mu g/m^3$	Annual mean, averaged over 3 years
		Secondary	Annual	15 $\mu g/m^3$	Annual mean, averaged over 3 years
		Primary and secondary	24-hour	35 $\mu g/m^3$	98^{th} percentile, averaged over 3 years
	PM_{10}	Primary and Secondary	24-hour	150 $\mu g/m^3$	Not to be exceeded more than once per year on average over 3 years
Sulfur Dioxide		Primary	1-hour	75 ppb	99^{th} percentile of 1-hour daily maximum concentrations, averaged over 3 years
		Secondary	3-hour	0.5 ppm	Not to be exceeded more than once per year

Appendix 2

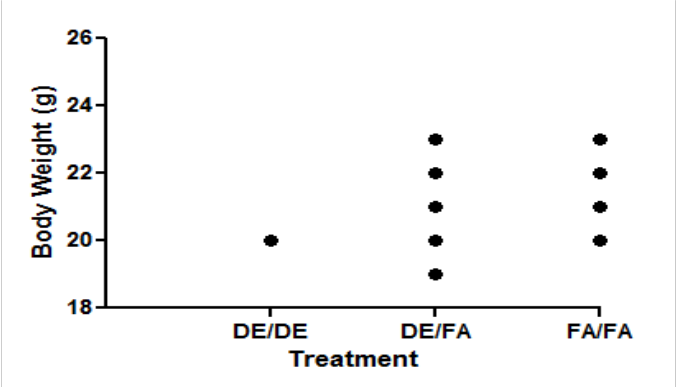
Week 16 Body Weight Distributions for All Subjects, Males Only, and Females Only



2.A: Week 16 Body Weight Distribution for All Subjects



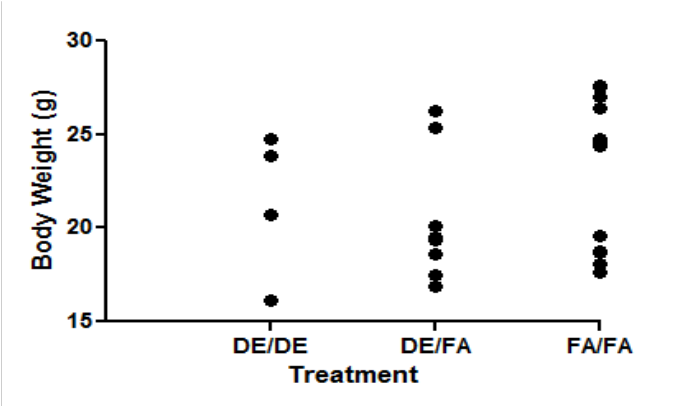
2.B: Week 16 Body Weight Distribution for Males Only



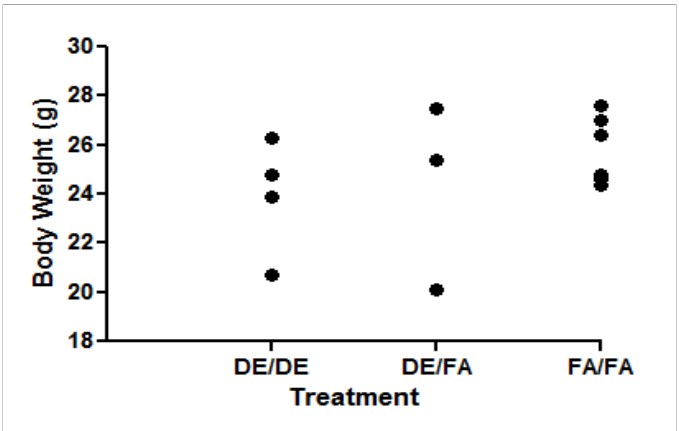
2.C: Week 16 Body Weight Distribution for Females Only

Appendix 3

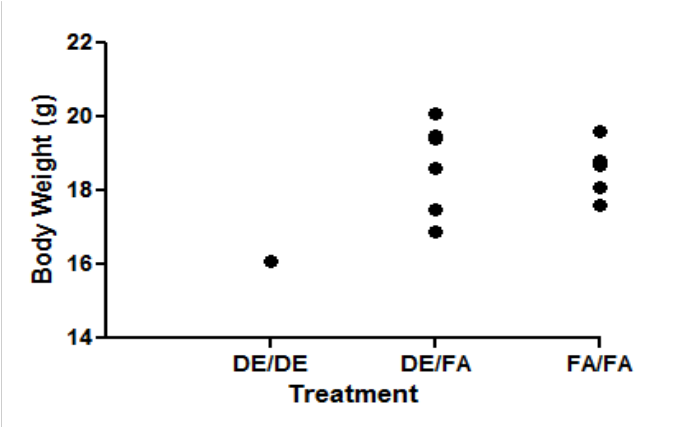
Overall Body Weight Gain Distributions for All Subjects, Males Only, and Females Only



3.A: Overall Body Weight Gain Distribution for All Subjects



3.B: Overall Body Weight Gain Distribution for Males Only



3.C: Overall Body Weight Gain Distribution for Females Only