Association between *in-utero* exposure to diesel exhaust and N-acetyl-cysteine supplementation in hyperlipidemic pregnant mice and development of atherosclerosis at multiple vascular sites in the offspring

Divya Ravi

A thesis
submitted in partial fulfillment of the
requirements for the degree of

Master of Public Health

University of Washington
2016

Committee:
Michael Rosenfeld, Chair
Terrance Kavanagh

Program Authorized to Offer Degree:
Environmental and Occupational Health Sciences

University of Washington

Abstract

Association between *in-utero* exposure to diesel exhaust and N-acetyl-cysteine supplementation in hyperlipidemic pregnant mice and development of atherosclerosis at multiple vascular sites in the offspring

Divya Ravi

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

Background: Ischemic heart disease (IHD) is the single largest cause of death worldwide, accounting for about 17.3 million annual deaths. In addition to the traditional risk factors for IHD, recent evidence points towards the role of environmental factors such as diesel exhaust (DE) emissions in the pathogenesis of the disease. Chemically, DE consists of a mixture of toxic gases and diesel particulate matter (DPM). Several mechanisms have been proposed for the toxicity of DPM in the body, such as its ability to cause oxidative stress, impair immunity, stimulate an inflammatory response and cause thromboischemic changes. Furthermore, there is ongoing research that supports the association between impaired *in-*

utero growth and the pathogenesis of adult onset diseases including coronary heart disease. This study was designed to evaluate the effect of *in-utero* exposure to diesel exhaust in the development of atherosclerosis in the offspring later in life, with a focus on the histological changes.

Methodology: In this study, pregnant hyperlipidemic apolipoproteinE (*apoE -/-)* deficient mice were randomized into one of four exposure groups:

1) Diesel exhaust and N-acetyl cysteine (NAC), referred to as DN
2) diesel exhaust and control water, referred to as DC
3) filtered air and NAC and, referred to as FN
4) filtered air and control water, referred to as FC.

The exposures were restricted to the prenatal period and were discontinued after birth. The offspring born to these dams were nurtured in a controlled environment until they were 16 weeks of age, at which point they were sacrificed. Various tissue specimens were isolated, including the innominate arteries (IA) which were examined microscopically for the presence of atherosclerotic lesions and vascular remodeling. In specific, the atherosclerotic lesion areas and medial expansion areas were quantified and differences between the study groups were statistically analyzed.

Results: Offspring born to diesel plus NAC (DN) dams exhibited the larger mean IA atherosclerotic lesion areas and medial thickening. The prevalence of peri-vascular adipose tissue (PVAT) in the DE exposed groups combined (diesel plus control water [DC] and diesel plus NAC [DN]) was 1.49 times (95% CI 1.02-1.54) that of the prevalence of PVAT in the filtered air groups (filtered air control [FC] and filtered air plus NAC [FN]). First litter pups recorded a significantly higher prevalence (p=0.011) of PVAT than their second litter

counterparts. No correlation was seen between lesion development in the IA versus lesion development in the aortic sinus for a given group. Lastly, there was an increase in cumulative mortality between the 12th and 16th week for the DC group compared to the other groups.

Conclusion: The results of this study suggest that *in-utero* DE exposure and NAC supplementation is associated with PVAT, but is largely not associated with vascular remodeling and atherosclerotic progression. More research is needed to further understand the inflammatory response to environmental toxins and the role of protective agents in the disease process.

Acknowledgements

I would like to thank Dr. Michael Rosenfeld for his guidance and support through the length of this study. I would like to acknowledge Dr. Terrance Kavanagh for his valuable input and mentorship at every step of this project. Thank you to Jenna Harrigan for involving me in this study and to Orvalio Augusto, for helping with the data organization and statistical analysis. I would also like to acknowledge members of the Rosenfeld lab- Jerry Ricks and Luz Wigzell for assisting with the morphometry and data collection.

Table of Contents

Introduction……. 10

Hypothesis and Specific Aims……. 15

Methods……... 17

Ethics Statement

Overall Design

Approach to Statistical Analysis

Results …… 20

Innominate Artery Histology

Litter Order

Differential Lesion Development

Cumulative Mortality

Discussion ……. 23

Innominate Artery Histology

Litter Order

Differential Lesion Development

Cumulative Mortality

Limitations

Conclusion …… 29

Tables…… 30

Table 1. *Study groups based on exposure status*

Table 2. *General Characteristics of Mouse Innominate Arteries*

Table 3*. Prevalence Ratios of Innominate Lesions stratified by treatment group and litter order*

Table 4. *Innominate Lesion Areas (μm^2) stratified by treatment group and litter order*

Table 5. *Innominate Medial Expansion Areas (μm^2) stratified by treatment group and litter order*

Table 6. *Prevalence of Peri-vascular adipose tissue stratified by treatment group and litter order*

Table 7. *Aortic Lesion Areas (μm^2) stratified by treatment group and litter order*

Figures …… 36

Figure 1. *Anatomic location of the innominate artery and aortic sinus*

Figure 2. *Movat stained section of innominate artery depicting artery lumen, lesion, medial thickening, peri-vascular adipose tissue*

Figure 3. *Magnified Movat stained section of innominate artery depicting atherosclerotic plaque and measures areas*

Figure 4. *Box plot representing Innominate Lesion Areas (μm2) among treatment groups on a logarithmic scale*

Figure 5*. Bar graph denoting prevalence of innominate artery lesions among treatment groups*

Figure 6. *Bar graph denoting prevalence of peri-vascular adipose tissue among treatment groups*

Figure 7. *Scatter plots denoting correlation between average aortic lesion area and average innominate lesion areas (in μm^2) stratified according to treatment group*

Figure 8. *Scatter plots denoting correlation between average aortic lesion area and average innominate medial expansion areas (in μm^2) stratified according to treatment group*

Figure 9. *Cumulative Mortality from birth to 16 weeks, stratified according to treatment group*

Appendices ……44

Appendix I. *Cardiac Tissue Collection and Processing*

Appendix II. *Histological Analysis*

References

List of Abbreviations	
IHD	Ischemic Heart Disease
IA	Innominate Artery
ApoE (-/-)	Apolipoprotein E knockout
DE	Diesel Exhaust
DPM	Diesel Particulate Matter
$PM_{2.5}$	Particulate Matter sized 2.5 μm
NAC	N-Acetyl-Cysteine
DC	Diesel exhaust and Control water
DN	Diesel exhaust and N-acetyl cysteine
FC	Filtered air and Control water
FN	Filtered air and N-acetyl-cysteine
GSH	Glutathione
CIMT	Carotid Intimal Medial Thickness
PVAT	Peri-vascular Adipose Tissue
LG	Licking/Grooming

Introduction

Cardiovascular disease is a leading cause of mortality in the world, accounting for about 17.3 million deaths annually (WHO 2015 factsheet). Of these, about 7.4 million deaths were attributed to coronary heart disease and about 6.7 million were attributed to stroke. Traditional risk factors associated with Ischemic Heart Disease (IHD) include hypertension, dyslipidemia, hyperglycemia, central obesity, age and smoking. More recent epidemiological studies indicate the role of environmental factors, such as particulate air pollution in the pathogenesis of the disease. Another area of research that has gained attention in recent times is epigenetics and the development of chronic adult diseases. However, there is less known about the association between prenatal exposure to environmental pollutants and the onset of IHD in adulthood. This study provides insight into the pathogenesis of atherosclerosis later in life following *in-utero* exposure to diesel exhaust (DE). The following paragraphs provide necessary background for this thesis.

Several human and animal based studies (Hesterberg et al. 2009, Lucking et al. 2008, Villeneuve et al. 2015) have demonstrated the ischemic effects of particulate air pollution on blood vessels. Villeneuve and colleagues denoted a positive association between particulate matter ($PM_{2.5}$) exposure and cardiovascular mortality in a large prospective cohort study on women. A crossover study by Lucking et al (2008) demonstrated an increase in thrombus formation following exposure to DE. Likewise, studies have closely examined the physiological effect of $PM_{2.5}$ on cardiovascular function and neuroendocrine response. One such study (Chen et al, 2012) on the short term effects of air pollution found a reduction in pulse pressure with exposure to industrial emissions, whereas Chan and colleagues (2015)

examined the association between DE exposure and blood pressure as a part of the NIEHS Sister Study. Their cross sectional study revealed a rise in systolic blood pressure of 1.4mmHg, following a 10 μg/m^3 increase in $PM_{2.5.}$ Background on the chemical composition of DE and its effects at the cellular level may help explain the changes observed in cardiopulmonary function arising post DE exposure.

Diesel exhaust is the end product of combustion of diesel fuel and is a mixture of gases such as carbon monoxide (CO), nitric oxides (NO, NO_2), sulphur dioxide (SO_2), and fine particulate matter called Diesel Particulate Matter (DPM). This DPM consists of an elemental carbon core with organic compounds such as polyaromatic hydrocarbons (PAH) adhered to the surface of the particle (Wichmann, 2007). Broadly, the mechanisms involved in cardiovascular toxicity following DPM exposure can be categorized as indirect and direct (Nelin et al, 2012). The indirect effect of $PM_{2.5}$ entails the stimulation of pulmonary cytokine release and subsequent systemic inflammation which causes downstream adverse cardiovascular effects. Following inhalation, DPM can get deposited along the respiratory tract and impair mucociliary clearance (Duan et al, 2013). It can also get internalized by alveolar macrophages and potentially disrupt the normal immunological functioning of these cells (Gualtieri et al, 2011). Evidence suggests that $PM_{2.5}$ can trigger an inflammatory response in the pulmonary parenchyma. Several other studies (Feng et al, 2016 and Davel et al, 2012) observed a surge in the level of systemic cytokines post DE exposure, while Budinger et al (2011) highlighted the ability of $PM_{2.5}$ to stimulate coagulation pathways. The indirect pathway also involves an impairment in cardiac autonomic function such as a decline in heart rate variability in rats following exposure to $PM_{2.5}$ (Wang et al, 2012) and

promote a diabetic state by reducing insulin sensitivity (Brook et al, 2013). On the other hand, the direct route of toxicity is less understood and involves the passage of fine particles ($PM_{2.5}$ and smaller) directly into circulation possibly through gaps between the alveolar epithelial cells. (Shimada et al. 2006, Wold et al. 2006)

Studies that examine the molecular mechanisms, suggest that concentrated ambient particles cause oxidative stress. This is evinced by an increase in anti-oxidant enzymes such as superoxide dismutase (SOD) and catalase in the rat lung post exposure (Gurgueira et al, 2002). Likewise, Davel et al (2012) observed inflammatory changes characterized by a decrease in endothelial nitric oxide synthase (eNOS) protein expression and an increase in tumor necrosis factor (TNF-α) in rat pulmonary arteries following $PM_{2.5}$ exposures. cDNA microarray studies on alveolar macrophages exposed to organic extracts of diesel exhaust particles (OE-DEP) and urban fine particles (UFP) noted an up-regulation of the genes responsible for the transcription of enzymes such as heme-oxygenase (HO-1 and HO-2), thioredoxin peroxidase-2 (TDPX-2), glutathione S-transferase P-subunit (GST-P), nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH dehydrogenase), and proliferating cell nuclear antigen (PCNA) (Koike et al, 2002). These enzymes are known to play a role in anti-oxidation and cell proliferation. Interestingly, Hirano et al described an increase in the viability of OE-DEP and OE-UFP-exposed cells on exposure to *N*-acetyl-l-cysteine (NAC), a potent anti-oxidant and glutathione (GSH) precursor. Moreover, the transcription level of HO-1 was also reduced in OE-DEP- and OE-UFP-exposed cells on exposure to NAC (Hirano et al, 2003). This suggests a protective role of NAC and perhaps GSH in minimizing the oxidative stress caused by DE. One objective of this study is to

determine the role of maternal NAC supplementation in the pathogenesis of atherosclerosis in the offspring.

As iterated earlier, there is new and ongoing research supporting the Barker hypothesis which alludes to the association between impaired *in-utero* growth and complex adult onset disease (Dover 2009). The association between gestational diabetes and obesity in the adult offspring was studied by Whitaker et al (1997). Raanan and associates (2015) described the role of organophosphorus pesticide exposure amongst pregnant agricultural workers and the potentiation of asthma in the offspring during adolescence. Palinski and Napoli carried out several animal and human-based studies to highlight the association between maternal hypercholesterolemia and the formation of fatty streak in fetal arteries, possibly by the trans-placental passage of normal and oxidized fatty acids (2011). More recently, they indicated maternal C-reactive protein level as a surrogate for childhood aortic atherosclerosis (2008). Closely linked to this thesis is evidence that relates *in-utero* arsenic exposure and atherosclerosis in the offspring of *ApoE (-/-)* mice (Srivastava et al, 2008). A study by States et al (2012), suggested an epigenetic effect of trans-placental arsenic. They noted a change in genes involved in lipid metabolism and increase in heat shock proteins (HSP-70) following *in-utero* arsenic exposure.

Epigenetic mechanisms such as DNA methylation, histone modification, microRNA have been connected to adult onset cardiovascular disease. The phenotypic changes that occur are perpetuated through subsequent cell divisions and result in pathophysiological changes (Wadhwa et al, 2009). Lund et al (2004) analyzed epigenetic alterations in *ApoE (-/-)* mice

and suggested that atherosclerosis is associated with and possibly succeeds genomic DNA methylation (both hypomethylation and hypermethylation). DNA methylation changes at CpG sites of genes involved in inflammatory response were observed following DE exposure in asthmatics (Jiang et al, 2014). Taken together, the above observations may help explain the epigenetic mechanism (in addition to oxidative stress) in the developmental programming of adult atherosclerosis in the offspring of dams exposed in this current study.

With the growing burden of ischemic heart disease, researchers have tried to describe different morphological processes that can be considered as sentinels of underlying coronary heart disease. Iacobellis et al (2005) used echocardiographs to describe epicardial fat as a reflection of intra-abdominal fat and underlying insulin resistance. Carotid intimal-medial thickness (CIMT) has been described as a surrogate of coronary atherosclerotic progression (Kolodgie et al, 2007), while Simon et al (2010) concluded that CIMT was an independent but modest predictor of coronary heart disease. Furthermore, the traditional 'inside-out' model of plaque development with endothelial injury leading to foam cell inflammation (Maiellaro et al, 2007) has given way to the 'outside-in' model with peri vascular adipose tissue (PVAT) playing a vital role in plaque formation (Omar et al. 2014). This view is further supported by the works of Lehman et al (2010) on the participants in the Framingham Heart Study offspring cohort. Weintraub and associates (2014) performed numerous studies on mice and human subjects to describe the pro-inflammatory and paracrine role of PVAT in vascular disease and its tendency to co-localize with an atherosclerotic plaque. They also discussed the secretion of pro-inflammatory cytokines such as Leptin and anti-inflammatory cytokines such as adiponectin from PVAT (Weintraub

et al, 2010). Building on what is known about the pathogenesis of atherosclerosis, this study was designed to examine the salient histological changes associated with plaque formation in innominate mouse arteries.

Lastly, while DE has been classified as a Group I carcinogen by the International Agency for Research on Cancer (IARC-Press release 2013), there is no current permissible exposure limit set by OSHA (Occupational Safety and Health Administration) for DE emissions. With the growing recognition that diesel-fuel technology has the potential to reduce greenhouse-gas emission, the increasing acceptance of diesel engines poses several health threats, warranting research in this area. Focused studies like this thesis could provide insight into the health effects of DE in vulnerable populations (such as pregnant women, children and elderly). This could further lay the foundation for setting standards by regulatory bodies and influence health policies in human exposure.

Hypothesis and Specific Aims

The primary goal of this study is to understand the effect of *in-utero* exposure to DE in the pathogenesis of atherosclerotic plaque in the offspring. The long term goal is to extrapolate the significant results of this study to humans and understand the effect of exposing a pregnant woman to diesel emissions on the development of cardiovascular morbidity in her child later in life, in addition to understanding the role of anti-oxidant supplements such as NAC as a protective factor. The specific objective of this thesis is to study the association between prenatal DE exposure and NAC supplementation in hyperlipidemic apolipoprotein E (*apoE -/-)* deficient pregnant mice and the development of atherosclerotic plaque in their

offspring by quantifying different components of the transverse arterial section such as lesion area, medial thickening and peri-vascular adipose tissue (PVAT) in the IAs.

Aim 1: To study the association between *in-utero* DE exposure +/- maternal NAC supplementation and the pathogenesis of atherosclerosis in the IA of offspring by quantifying the various morphological components (atherosclerotic lesion area, medial expansion area and PVAT prevalence) of the transverse arterial section after differential histological staining.

The null hypothesis for this aim was that DE exposure is not associated with any histopathological change in the IAs of the offspring.

Aim 2: To determine whether amongst those exposed to DE, does supplementation of maternal drinking water with NAC concurrently affect the plaque formation in the offspring during adulthood.

This study tested the hypothesis that there does not exist any difference in the average cross sectional area of the atherosclerotic plaque amongst those exposed to DE stratified according to NAC exposure.

Aim 3: To determine the presence of differential lesion development with respect to anatomical location when comparing average lesion areas between aortic sinus and IA for a given animal following DE ± NAC exposure.

This study tested the null hypothesis that there is no association between average lesion areas recorded at the aortic sinus and IA for the same animal within each group.

Methods

Ethics Statement

This study was carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health recommendations. All animal experiments were approved by the University of Washington Institutional Animal Care and Use Committee (IACUC protocol no. 2650-08).

Overall design

The study commenced in June 2014, designed as a randomized experimental study using Apolipoprotein-E (*apoE -/-*) knockout mice on a C57BL/6 genetic background. The research facility where the exposure arm of the study was carried out was specifically designed to provide a controlled inhalation exposure to diluted and mixed DE; with particulate matter ranging between 250-300 mcg/m^3. In addition, the particulate carbon content and other gaseous constituents of the DE had been standardized to be consistent and replicable.

The mice were confined to this facility for a period of about one week to allow for acclimatization and subsequent mating. Once occurrence of mating was confirmed, each *ApoE (-/-)* female mouse was randomly assigned to one of the four study groups: 1) Diesel exhaust plus NAC drinking water (DN), 2) Diesel exhaust plus Control water (DC), 3) Filtered air plus NAC drinking water (FN), 4) Filtered air plus Control water (FC) (Refer to Table1 for study groups). The final sample size attained after recurrent mating was 190, with each exposure group having 39-59 mice. The exposure to DE was carried out for 6 hours per day

for 5 days a week from the estimated time of mating until birth of the litter which is approximately 19-21 days given the gestation period of these mice.

A total of 165 mice survived until 16 weeks of age, at which time they were sacrificed. The hearts with the aorta *in situ* were dissected out. (Refer to Appendix I for procedure details). The IA were subsequently excised from the heart specimens and processed into paraffin blocks (Refer to Figure 1 for anatomic location of IA and aortic sinus). These blocks were then sectioned using a microtome into 5μm thick sections and mounted onto glass microscope slides. These slides were serially numbered and every fifth slide was selected for differential staining using Modified Movat Pentachrome stain (as followed by Russell Jr. 1972) and viewed microscopically. (Refer to Appendix II for the protocol followed for staining).

The surface area of the lesion from each cross section of the IA was defined as the difference between the area enclosed by the internal elastic lamina and the area of the arterial lumen and was computed using Image Pro Plus software (*Media Cybernetics,* Rockville, MD). The average innominate cross sectional lesion area of each animal was estimated as the mean of the lesion areas, obtained from the multiple slides that were selected and stained for each individual animal. In addition, the medial expansion area was defined as the sum area of the parts of the innominate tunica media that had visibly hypertrophied in the cross-section. The average medial expansion area was calculated as the mean of the medial expansion areas obtained from the multiple slides stained for each animal. The PVAT was a qualitative indicator of the presence of adventitial fat tissue circumferential to the artery in the section

(Refer to Figure 2 and 3 for Movat stained section of IA depicting areas measured). The histology data obtained was tabulated in an Excel spread sheet for further statistical analysis.

Approach to statistical analysis

A 2 sided hypothesis was adopted for the primary objective of this study. According to this model μ_{dec} and μ_{fac} are the mean areas for the atherosclerotic plaque lesion for the DE exposure and filter control groups respectively. The null hypothesis for Aim 1 is H_o: $\mu_{dec} = \mu_{fac}$, i.e. the mean innominate lesion areas are the same for the DE exposure and filter control groups, and the alternative hypothesis is H_a: $\mu_{dec} \neq \mu_{fac}$, i.e. the mean innominate lesion areas are not the same for the DE exposure and filter control groups or there is an association between *in-utero* exposure to DE emission and development of atherosclerotic plaque in mouse IAs. The t-test statistic was used to test the p-value under the null hypothesis at a 5% level of significance (α criterion set at 5% to obtain a 95% confidence interval).

The null hypothesis for Aim 2 is H_o: $\mu_{den} = \mu_{dec}$, i.e. the mean innominate lesion areas are the same for the DE (+) NAC exposure and DE (+) filtered water control groups, and the alternative hypothesis is H_a: $\mu_{den} \neq \mu_{dec}$, i.e. the mean innominate lesion areas are not the same for the DE (+) NAC exposure and DE (+) filtered water control groups, or NAC can affect the association between diesel exhaust exposure and atherosclerotic plaque. The t-test statistic was used to test the p-value under the null hypothesis at a 5% level of significance (α criterion set at 5% to obtain a 95% confidence interval).

In addition to the main objective of the study, the role of litter order in the development of plaque was also analyzed. For this, the association between DE exposure and mean lesion areas was stratified according to litter order: 1st if the pup belonged to the first pregnancy litter or 2nd if the pup belonged to the second pregnancy litter and so on (the mother would have been exposed to DE during each gestation). Statistical analysis of data was done using STATA 13 software package.

Results

1) Innominate Artery Histology:

A total of 75 IAs were isolated and analyzed in this study. The distribution of arteries between the four treatment groups ranged between 12-27. 53.3% of the arteries belonged to male offspring while 46.7% were from female offspring. Specific details on gender representation and size range of the four treatment groups can be found in Table 2.

Statistical significance ($p<0.05$) was not attained for the lesion area differences amongst the treatment groups, however several interesting trends were observed. The prevalence of atherosclerotic lesions in the IA was the highest in the DC group with 58.8% and lowest in the FC group with 29.6%. Figure 4 displays box plots that compare lesion areas between the four groups on a logarithmic scale. The means of innominate lesion areas for the four treatment groups were as follows- 1753.3 μm^2 in FC ,1981.7 μm^2 in DC ,2146.9 μm^2 in FN, and 3203.9 μm^2 in the DN group. Table 3 provides details on prevalence ratios of lesion areas and Table 4 has the mean innominate lesion areas with standard deviation for the four groups. It is worth noting that the absence of lesion in several mouse IAs resulted in wide standard deviations about the means.

In regard to medial expansion areas in the innominate sections, statistical significance was not obtained when treatment groups were compared. However, the trend in mean areas was similar to atherosclerotic innominate lesions. The mean innominate medial expansion area was the highest in the DN group with 6367.5 μm^2, and was 5954.3 μm^2, 3847.0 $\mu m^2$and 3779.2 μm^2, respectively in the DC, FC and FN groups. When comparing the medial expansion of diesel groups vs. filtered air, the mean ratio was 1.61 (95% CI of 0.83-3.09) with p=0.106. Table 5 provides details on innominate medial expansion areas stratified according to exposure status.

PVAT was observed in nearly 57% of the IAs. In specific, the prevalence of PVAT amongst FC, FN, DC and DN arteries was observed to be 51.9%, 33.3%, 58.8% and 73.7% respectively. Figure 6 is a bar graph representing prevalence ratios of PVAT for the treatment groups. When comparing prevalence of PVAT between groups that were and were not exposed to DE, statistical significance was attained ($p<0.035$). Prevalence of PVAT in diesel exposed groups combined (DC and DN) was 1.49 times (95% CI 1.02-1.54) that of prevalence of PVAT in the filtered air groups (FC and FN), as shown in Table 6.

2) Litter Order

The prevalence of PVAT was significantly different (p=0.011) between the offspring of the first litter (dams who were exposed for the first time) and those of the second litter (dams who had been previously exposed in a prior pregnancy). The prevalence of PVAT amongst second order pups was lower than the prevalence in the first order pups (prevalence ratio = 0.46, 95% CI 0.25- 0.84). However, there was no apparent difference in the mean innominate

lesion areas and medial thickening areas between pups belonging to different litter orders. Area values (innominate lesion, medial expansion and aortic lesion) stratified according to litter order can be found in the respective tables.

3) Differential lesion characteristics

In an alternative course of this study, aortic sinus lesion areas were measured in the hearts of 148 mice that survived until 16 weeks (17 mouse hearts sustained unintended damage during the course of tissue processing). Although statistical significance for average aortic lesion area was not obtained between the treatment groups, correlation between the average aortic lesion areas and the average innominate lesion areas was analyzed for the 75 animals included in this study. The mean aortic lesion areas for FC, FN, DC and DN were 11216.6 μm^2, 12226.2 μm^2, 15914.8 $\mu m^2$and 13668.2 μm^2 respectively as shown in Table 7. For a given treatment group, there was no association between the development of aortic lesion and of innominate lesion, with correlation coefficients (r) ranging between -0.198 and 0.425. Similar trends were observed, when average aortic lesion areas were compared with average innominate medial expansion areas for animals within a given treatment group. Figures 7 & 8 are scatter plots representing the above findings.

4) Cumulative Mortality

A total of 190 pups were born over the course of this study. Of these 104 were male, 84 were female and 2 were undetermined or unsexed prior to death. The distribution of pups according to *in-utero* exposure status was 59 of FC, 39 of FN, 41 of DC and 51 of DN. Of the 190 pups born, only 165 pups survived through the entire study period of 16 weeks. This

offspring survival was analyzed in terms of cumulative mortality and although statistically non-significant, varied between treatment groups as weeks progressed. The cumulative mortality at 4 and 8 weeks followed a similar pattern with FN group recording the lowest mortality and DC having the highest mortality rate. Until 12 weeks of age, the difference in cumulative mortality between groups is within a 5-7% range. However, between 12 and 16 weeks, the rate of deaths in the DC group relatively increased and at 16 weeks of age, the cumulative mortality of DC was 22%, DN was 14% while both FC and FN were at 10%. Figure 9 depicts the cumulative mortality at various time points for the four groups from birth until 16 weeks.

Discussion:

Innominate Artery Histology

The observed pattern of higher prevalence of atherosclerotic changes in diesel exposed groups (DC and DN) align with our predictive outcome, and with observations made by Miller and colleagues (2013). In particular, the prevalence of innominate lesions was highest in the DC group which could be a result of oxidative stress and other inflammatory changes arising from $PM_{2.5}$ exposures (Feng et al 2016). In a similar study of *ApoE (-/-)* mice undertaken by Campen et al (2009), diesel emissions had a significant impact on vascular pathology characterized by vascular remodeling and upregulation of transcription factors and induction of lipid peroxides. However, the findings reported by Campen and coworkers were statistically distinguishable which may be attributed to the potent PM concentrations

that were administered postnatally in their study as opposed to the exposure protocol (prenatal only) adopted in this study.

When the quantitative mean of the average lesion areas was stratified according to specific exposure status, the unexpected observation was that the DN group recorded a value higher than the DC group. While NAC is an established glutathione(GSH) prodrug, its anti-oxidative ability may have been overwhelmed in the presence of DE, a potent inflammatory stressor. A similar trend was noted by Childs et al (2001), who observed an increase in oxidative stress following concurrent Vitamin C and NAC supplementation in an injured muscle. At the molecular level, they observed an increase in lactate dehydrogenase enzyme (LDH), creatinine kinase (CK), superoxide dismutase (SOD) and glutathione peroxidase levels in the Vitamin C + NAC group compared to the placebo group. Although the underlying mechanism was not completely understood, it may explain the trend observed in this thesis. Another explanation for the higher mean lesion areas in the DN group, maybe an unintended selection bias, analogous to the healthy worker survivor effect. With a 22% mortality in the DC group (explained in greater detail subsequently), it is possible that the healthier mice, with smaller lesions survived until 16 weeks. In retrospect, necropsy of the mice that died prior to 16 weeks might have provided insight into the cause of premature death.

It has been proposed that CIMT is a well-known surrogate of atherosclerosis (Simon et al 2009, Finn et al 2010) and coronary heart disease, however there is less known about the extended significance of innominate medial thickening. An interesting, albeit statistically non-significant trend was observed for medial expansion areas of IAs in this study. The average medial expansion areas were higher amongst the DE exposed groups, with DN

recording the highest mean areas. The tendency for NAC supplemented groups to record larger mean areas (innominate lesion and medial expansion) underlines the need to closely examine the effect of NAC at the molecular level. This thesis did not measure indicators of oxidative stress. Analyzing levels of enzymes such as HMOX1, NADPH, SOD and glutathione peroxidase as done in a few studies cited earlier (Koike et al, 2003; Childs et al, 2001) could provide insight into the extent of oxidative stress following $PM_{2.5}$ + NAC supplementation at the molecular level.

Research suggests that PVAT tissue correlates with coronary plaque formation. Cumulative evidence supports the role of PVAT in the pathogenesis of atherosclerosis centripetally. Keeping with the 'outside-in' model, Omar et al (2014) proposed that PVAT abuts the adventitial layer of larger blood vessels and facilitates local inflammation. In this study, the prevalence of PVAT was associated with DE exposure ($p= 0.035$) and aligned with the expected outcome. While meaningful differences between diesel exposed and non-exposed groups were derived, the presence of macrophage infiltration or inflammatory changes in the adipose tissue, as done in a study by Bolton et al (2014), was not noted. Additionally, groups that developed larger innominate lesions and medial thickening also recorded higher PVAT prevalence. However, the temporality of these morphological changes in the artery was not analyzed. Previous studies have demonstrated adventitial remodeling prior to functional changes in the endothelium (Herrmann, 2001), but the exact mechanism that drives adventitial inflammation is yet to be determined. Multiple studies have examined the effect of transplanted PVAT in mice. Takaoka et al (2009) recorded an enhanced neointimal response to injury in the absence of PVAT in a vessel, suggesting a protective role

of PVAT. Manka et al (2014), on the other hand reported enhanced adventitial inflammation in hyperlipidemic mice following PVAT transplantation.

Litter Order Influence

Offspring belonging to second order litters were expected to have larger lesion areas and higher prevalence of PVAT. These predictions were based on the presumptive additive effect following DE exposure in dams in successive pregnancies. However, in this study, first litter mice recorded significantly higher PVAT prevalence than their second litter counterparts. Although, statistically non-significant, the mean average lesion area (innominate and aortic sinus) for every treatment group was also observed to be lower in the second litter animals. Analogous to a sensitized allergic reaction, it is possible that maternal anti-oxidant response had amplified between successive pregnancies. Change in maternal behavior has also been implicated in altering fetal epigenetic programming. Weaver et al (2004) reported differences in DNA methylation of offspring born to high-licking/grooming (LG) mothers. Caldji and associates (2000) observed differences in maternal care-giving behavior between first litter and subsequent ones and noted that offspring born to high LG nursing dams showed reduced corticosterone response to stress. It can be theorized that postpartum behavior of dams in this thesis varied between subsequent litters and influenced first litter offspring to develop larger lesions.

Differential Lesion Characteristics

As mentioned above, several epidemiological studies have described CIMT as a surrogate for coronary artery disease. (Finn, 2010; Kolodgie, 2007). However, in this study there was no correlation drawn between the development of lesion in the IA and aortic sinus although the hierarchy of lesion development at both sites was similar between the treatment groups. It is possible that the premature sacrifice of the offspring at 16 weeks of age, and small sample sizes may have deterred the visualization of more distinguishable lesions.

Cumulative mortality

The pattern of cumulative mortality amongst the treatment groups (although not statistically significant) was in accordance with our predictive outcomes. The DC group recorded the highest number of deaths over the course of 16 weeks. Interestingly, there was a spike in the mortality of the DC group between week 12 and week 16. It can be theorized that the pups born to the DC dams experienced increased oxidative stress in the absence of NAC supplementation. Another explanation for the higher offspring mortality amongst the DE groups can be a difference in maternal postpartum behavior. As mentioned earlier (Caldi, 2000), decreased maternal care-giving behavior like pup licking/grooming may have influenced offspring health outcomes in this study. However, exact causes for the premature deaths in this study were not looked into, restricting the reproducibility of these outcomes.

Limitations

While exposure to DE and NAC was maintained during the entire pre-natal period allowing time for inflammatory effects of $PM_{2.5}$ to develop, the mice were relatively young at the time of sacrifice. Although, it can be theorized that age-related physiological changes in vascular structures were minimized in this study, atherosclerotic lesions may not have matured sufficiently in 16 weeks. Secondly, as mentioned earlier, the cause of death in the mice that did not survive until 16 weeks was not established and possibility of survival bias remains.

Furthermore, inflammatory response in the dams following PM exposure was not looked into. Immunohistochemistry of maternal tissue like placenta, lung and liver could have helped establish the extent of maternal oxidative stress and possibility of trans-placental passage of inflammatory mediators (Weldy et al, 2014). Similarly, measuring maternal biomarkers during pregnancy would have informed of any influence NAC supplementation had in the early phase of oxidative stress. Lastly, the challenge in isolating the IA from the preserved mediastinal structures lead to a reduced sample size in this silo of the study. As a result, extrapolation of these findings to a larger study population and beyond is skewed by reduced power, attrition bias and possible under-representation of some exposure groups. Future studies could overcome these limitations and look closely at changes in biomarkers as well as temporality of the changes observed in this thesis.

Conclusion:

The results obtained in this study suggest that *in-utero* DE exposure is associated with PVAT ($p<0.05$), but is largely not associated with vascular remodeling and atherosclerotic progression in mice. Furthermore, offspring born to the first litters recorded greater PVAT prevalence than their second litter siblings ($p<0.05$).

Thus, more research is needed to further understand the inflammatory response to environmental toxin exposure and role of protective agents in the disease progression

Tables and Figures

Table 1. *Study groups based on exposure status*

Exposure to		Diesel Exhaust	
		YES	NO
N-Acetyl Cysteine	YES	**DN** (Diesel Exhaust and NAC)	**FN** (Filtered Air and NAC)
	NO	**DC** (Diesel Exhaust and Control Water)	**FC** (Filtered Air and Control Water)

Table 2: *General Characteristics of Mouse Innominate Arteries*

	FC		FN		DC		DN		Total	
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
Total mice	27	36	12	16	17	22.7	19	25.3	75	100
Gender										
Female	10	37.0	8	66.7	8	47.1	9	47.4	35	46.7
Male	17	63.0	4	33.3	9	52.9	10	52.6	40	53.3
Litter Order										
1	19	70.4	7	58.3	12	70.6	12	63.2	50	66.7
2	8	29.6	5	41.7	5	29.4	6	31.6	24	32.0
3	0	0.0	0	0.0	0	0.0	1	5.3	1	1.3

***Table 3**: Prevalence Ratios of Innominate Lesions stratified by treatment group and litter order*

Group of exposure	Yes		No		Prevalence	Prevalence Ratio (PR)	
	N	%	N	%	%	PR (95% CI)	p
Total	41		34		54.7	-	
DE Exposure							0.115
Yes	21	51.2	15	44.1	58.3	1.70 (0.88 - 3.31)	
No	13	31.7	26	76.5	33.3	1	
NAC Exposure							0.714
Yes	16	39.0	15	44.1	51.6	1.12 (0.62 - 2.03)	
No	18	43.9	26	76.5	40.9	1	
DE±NAC Exposure							0.438
FC	8	19.5	19	55.9	29.6	1	
FN	5	12.2	7	20.6	41.7	1.33 (0.43 - 4.10)	
DC	10	24.4	7	20.6	58.8	1.96 (0.80 - 4.77)	
DN	11	26.8	8	23.5	57.9	1.87 (0.80 - 4.40)	
Litter Order							0.202
1	27	65.9	23	67.6	54.0	1	
2	7	17.1	18	52.9	28.0	0.61 (0.29 - 1.30)	

Table 4: *Innominate Lesion Areas (μm²) stratified by treatment group and litter order*

Group of exposure	Innominate Lesion Area (μm²)			
	Mean	SD	Mean Ratio (PR)	
			MR (95% CI)	p
All subjects	2235.6	3986.71	-	
DE Exposure				0.421
Yes	2626.8	3922.39	1.40 (0.62 - 3.19)	
No	1874.4	4062.26	1	
NAC Exposure				0.308
Yes	2794.7	4503.81	1.52 (0.68 - 3.38)	
No	1841.6	3579.97	1	
DE±NAC Exposure				0.620
FC	1753.3	4304.92	1	
FN	2146.9	3617.46	1.22 (0.32 - 4.64)	
DC	1981.7	2072.32	1.13 (0.32 - 3.99)	
DN	3203.9	5035.25	1.83 (0.67 - 5.00)	
Litter Order				0.180
1	2940.9	4647.95	1	
2	825.0	1337.88	0.28 (0.04 - 1.80)	

Table 5: *Innominate Medial Expansion Areas (μm²) stratified by treatment group and litter order*

Group of exposure	Innominate Medial Expansion Lesion Area (μm²)			
	Mean	SD	Mean Ratio (PR)	
			MR (95% CI)	p
All subjects	4962.1	6888.40	-	
DE Exposure				0.106
Yes	6172.4	6575.07	1.61 (0.83- 3.09)	
No	3844.8	7065.08	1	
NAC Exposure				0.767
Yes	5365.6	7643.87	1.11 (0.56 - 2.21)	
No	4677.7	6379.17	1	
DE ± NAC Exposure				0.562
FC	3874.0	6897.23	1	
FN	3779.2	7745.03	0.98 (0.29- 3.25)	
DC	5954.3	5407.30	1.54 (0.66 - 3.59)	
DN	6367.5	7614.87	1.64 (0.73 - 3.70)	
Litter Order				0.059
1	6191.2	7746.78	1	
2	2503.8	3786.31	0.38 (0.14 - 1.04)	

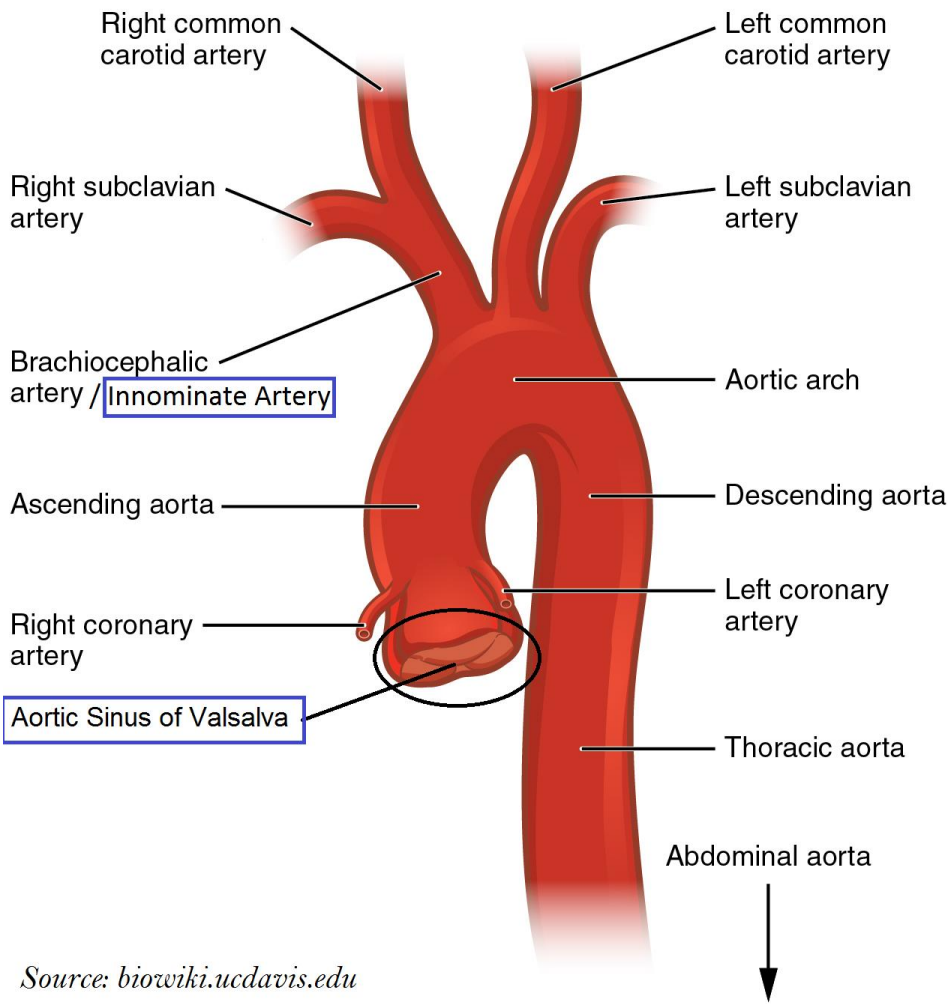
Table 6: *Prevalence of Peri-vascular adipose tissue stratified by treatment group and litter order*

Group of exposure	Yes		No		Prevalence	Prevalence Ratio (PR)	
	N	%	N	%	%	PR (95% CI)	p
Total	42		32		56.8	-	
DE Exposure							0.035
Yes	24	57.1	18	56.3	57.1	1.49 (1.03 - 2.15)	
No	11	26.2	21	65.6	34.4	1	
NAC Exposure							0.843
Yes	18	42.9	24	75.0	42.9	1.04 (0.70 - 1.54)	
No	13	31.0	19	59.4	40.6	1	
DE±NAC Exposure							0.202
FC	14	33.3	13	40.6	51.9	1	
FN	4	9.5	8	25.0	33.3	0.64 (0.27 - 1.55)	
DC	10	23.8	6	18.8	62.5	1.21 (0.72 - 2.01)	
DN	14	33.3	5	15.6	73.7	1.42 (0.90 - 2.24)	
Litter Order							0.011
1	34	81.0	15	46.9	69.4	1	
2	8	19.0	17	53.1	32.0	0.46 (0.25 - 0.84)	

Table 7: *Aortic Lesion Areas (μm²) stratified by treatment group and litter order*

Group of exposure	Aortic Lesion Area (μm²)			
	Mean	SD	Mean Ratio (PR)	
			MR (95% CI)	p
All subjects	13162.9		-	
Diesel Exposure				0.106
Yes	14725.4	14312.78	1.65 (0.90 - 3.05)	
No	11553.1	10354.32	1	
NAC Exposure				0.988
Yes	13121.2	11341.37	0.99 (0.53 - 1.87)	
No	13194.7	13514.66	1	
DE ± NAC Exposure				0.446
FC	11216.6	10690.02	1	
FN	12226.2	10116.81	1.06 (0.42- 2.67)	
DC	15914.8	16640.58	1.74 (0.79 - 3.83)	
DN	13668.2	12280.67	1.63 (0.69 - 3.87)	
Litter Order				0.588
1	13684.5	13917.19	1	
2	12020.4	8954.47	0.87 (0.52 - 1.45)	

Figure 1: *Anatomic location of the innominate artery and aortic sinus*



Source: biowiki.ucdavis.edu

Figure 2: *Movat stained section of innominate artery.*
Key features include: (A)Artery Lumen, (B) Lesion, (C) Medial thickening, (D) Peri vascular adipose tissue

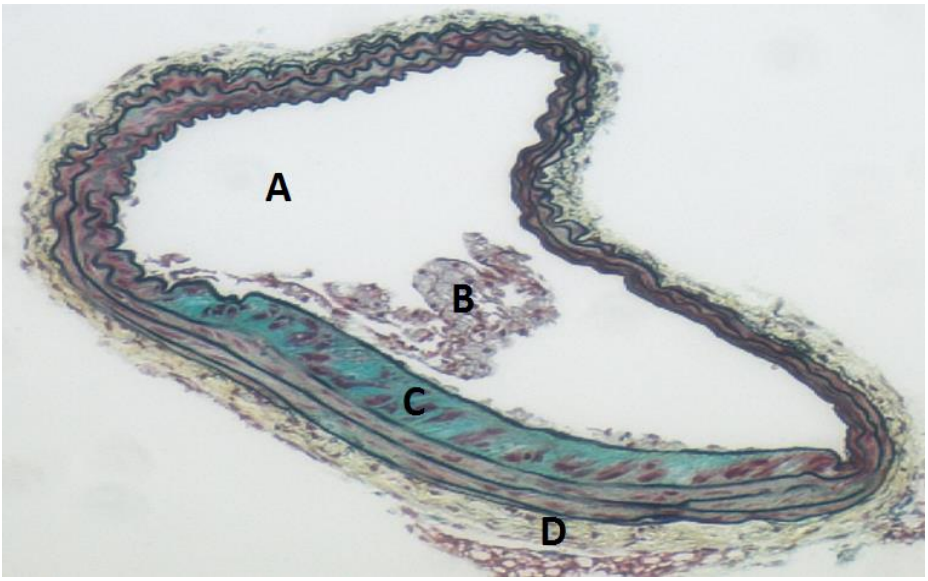


Figure 3: *Magnified Movat stained section of innominate artery depicting atherosclerotic plaque. Following parameters were measured and analyzed:*

i. *Average Innominate Lesion Area (μm^2)*
ii. *Average Innominate Medial Expansion Area (μm^2)*
iii. *Average Aortic Sinus Lesion Area (μm^2); (not shown in adjoining figure)*
iv. *Peri-vascular adipose tissue prevalence*

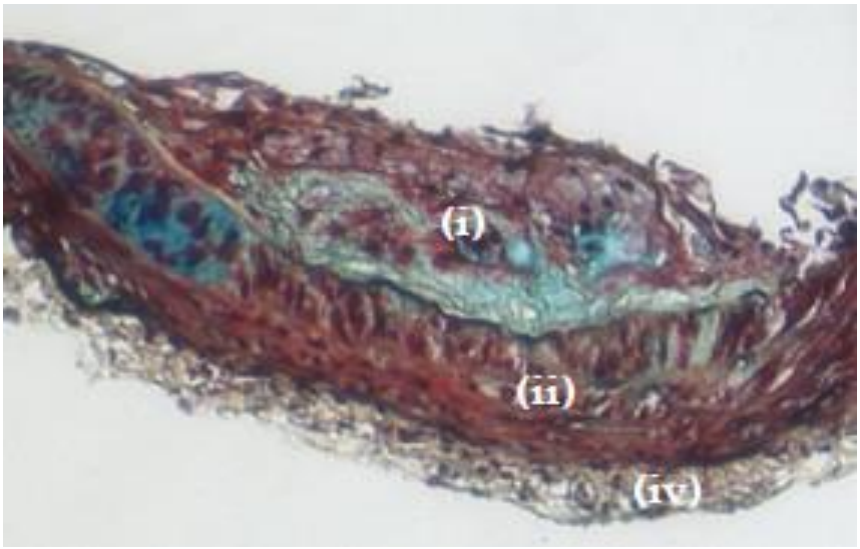


Figure 4: *Box plot representing Innominate Lesion Areas (µm2) among treatment groups on a logarithmic scale*

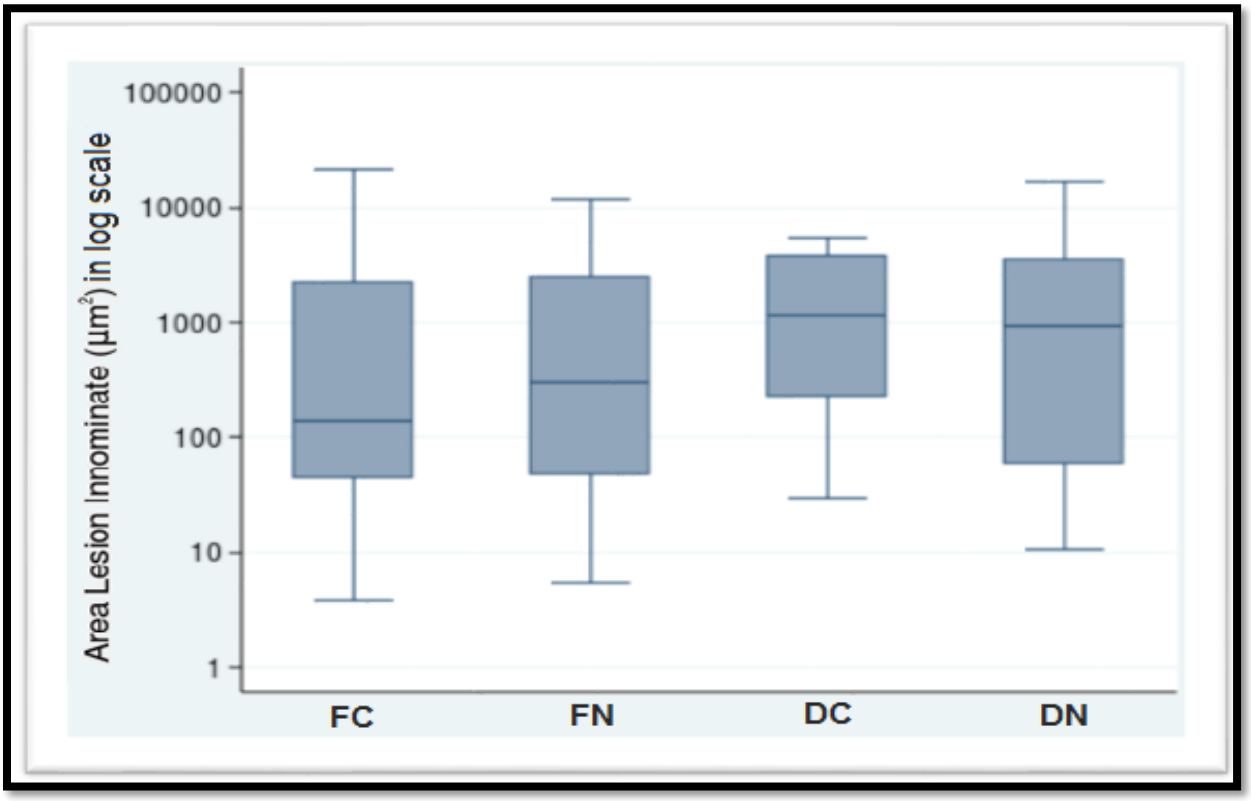


***Figure 5**: Bar graph denoting prevalence of innominate artery lesions among treatment groups*

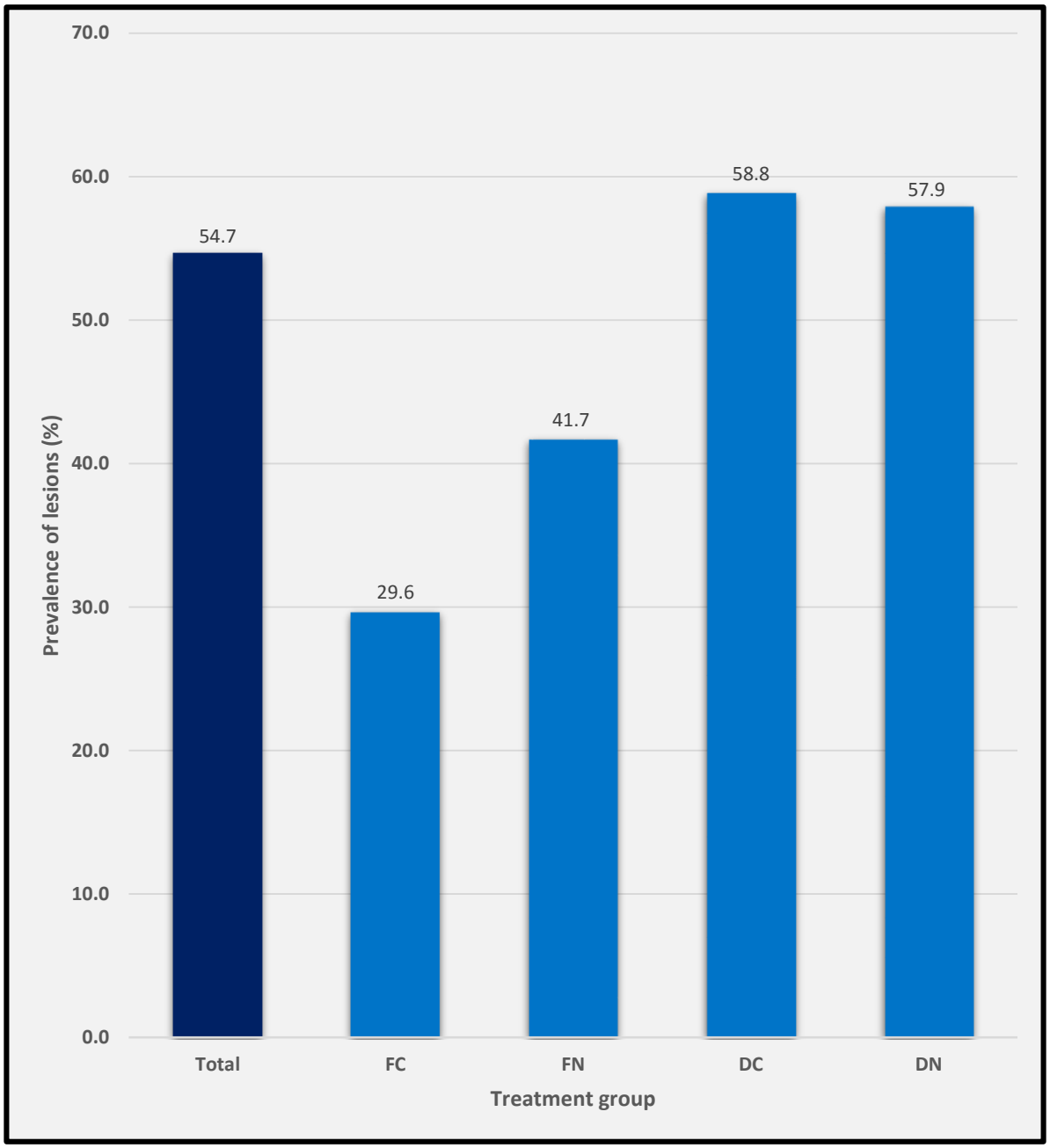


***Figure 6**: Bar graph denoting prevalence of peri-vascular adipose tissue among treatment groups*

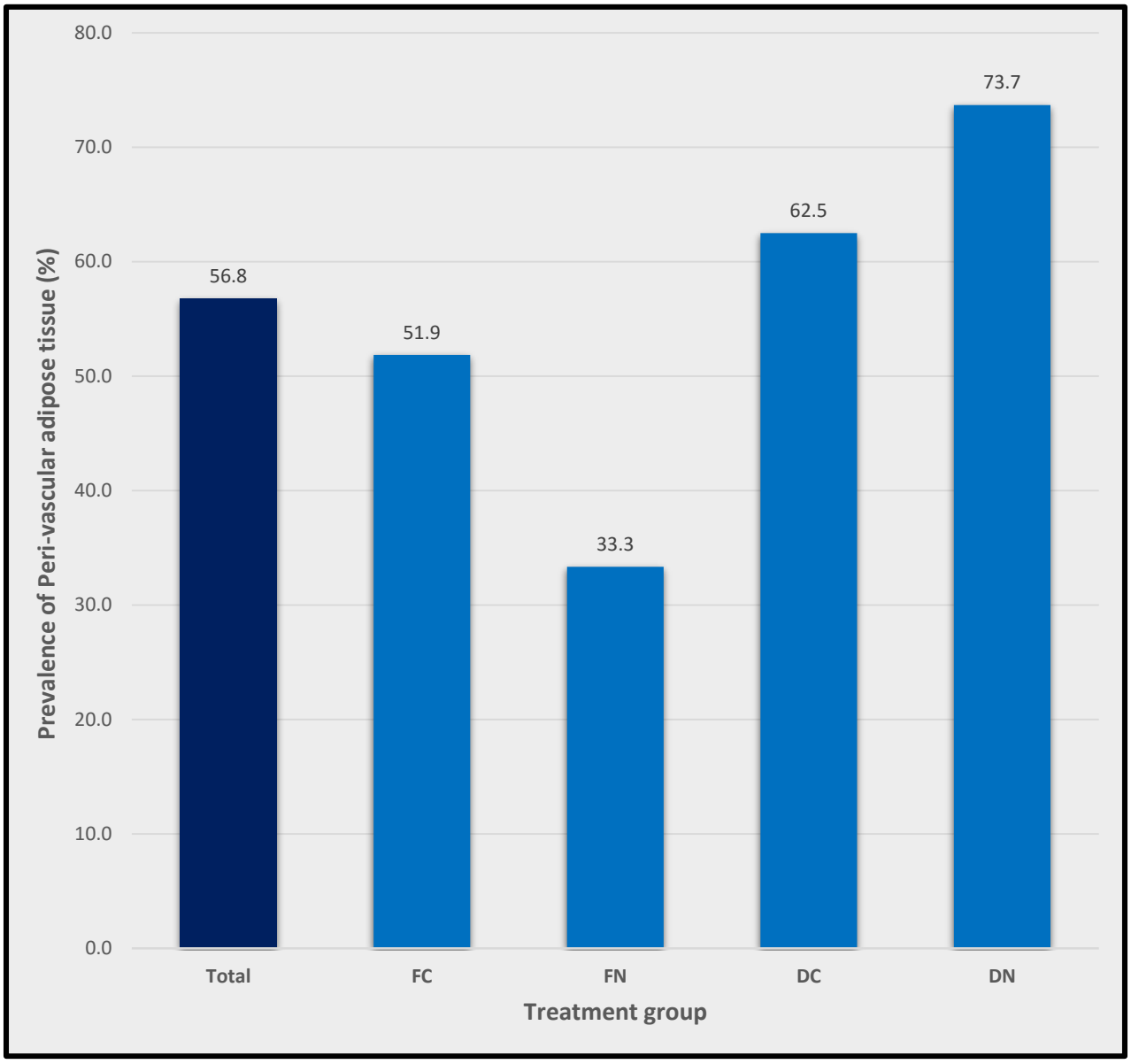


Figure 7*: Scatter plots denoting correlation between average aortic lesion area and average innominate lesion areas (in μm²) stratified according to treatment group.*

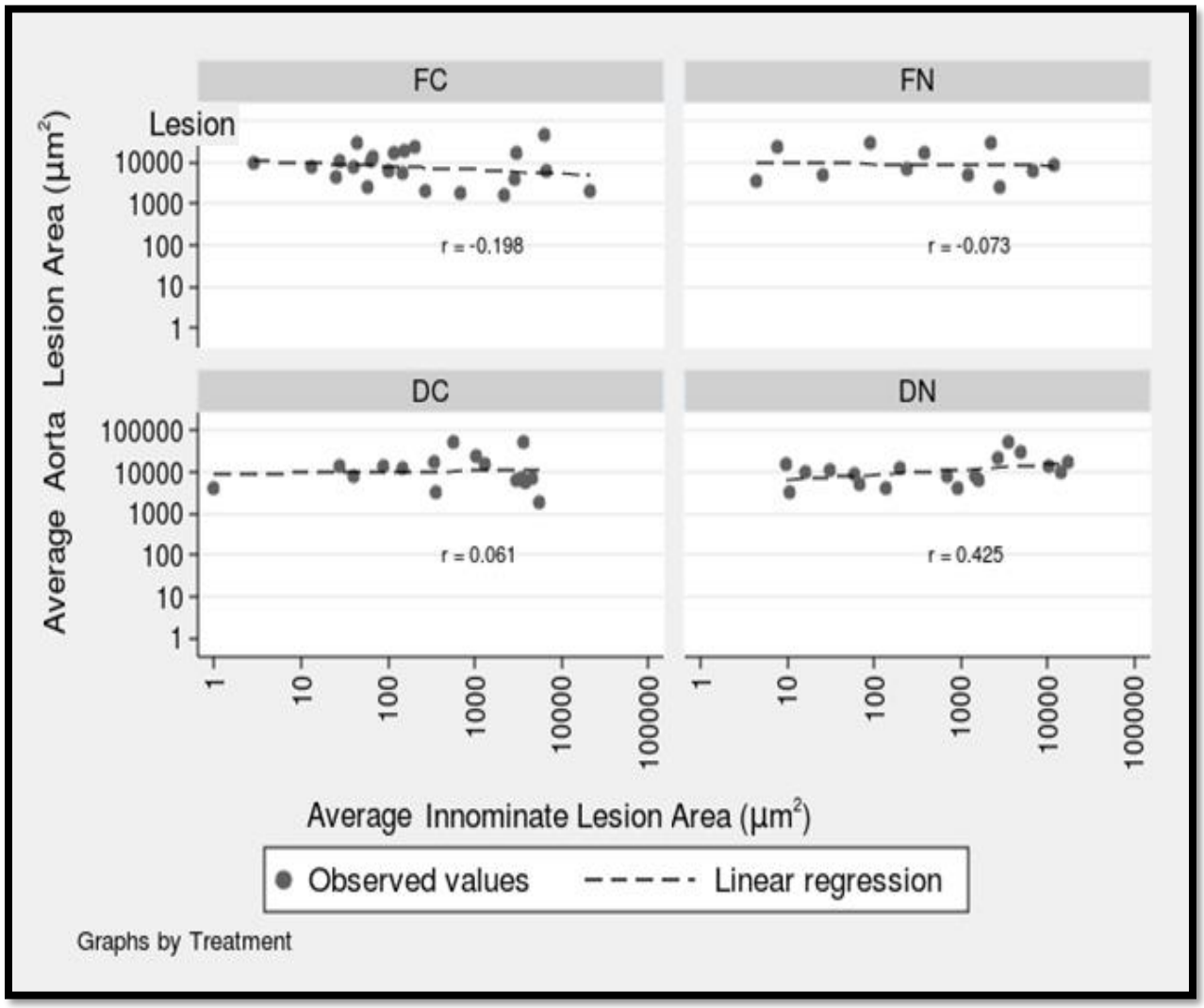


Figure 8: *Scatter plots denoting correlation between average aortic lesion area and average innominate medial expansion areas (in μm²) stratified according to treatment group.*

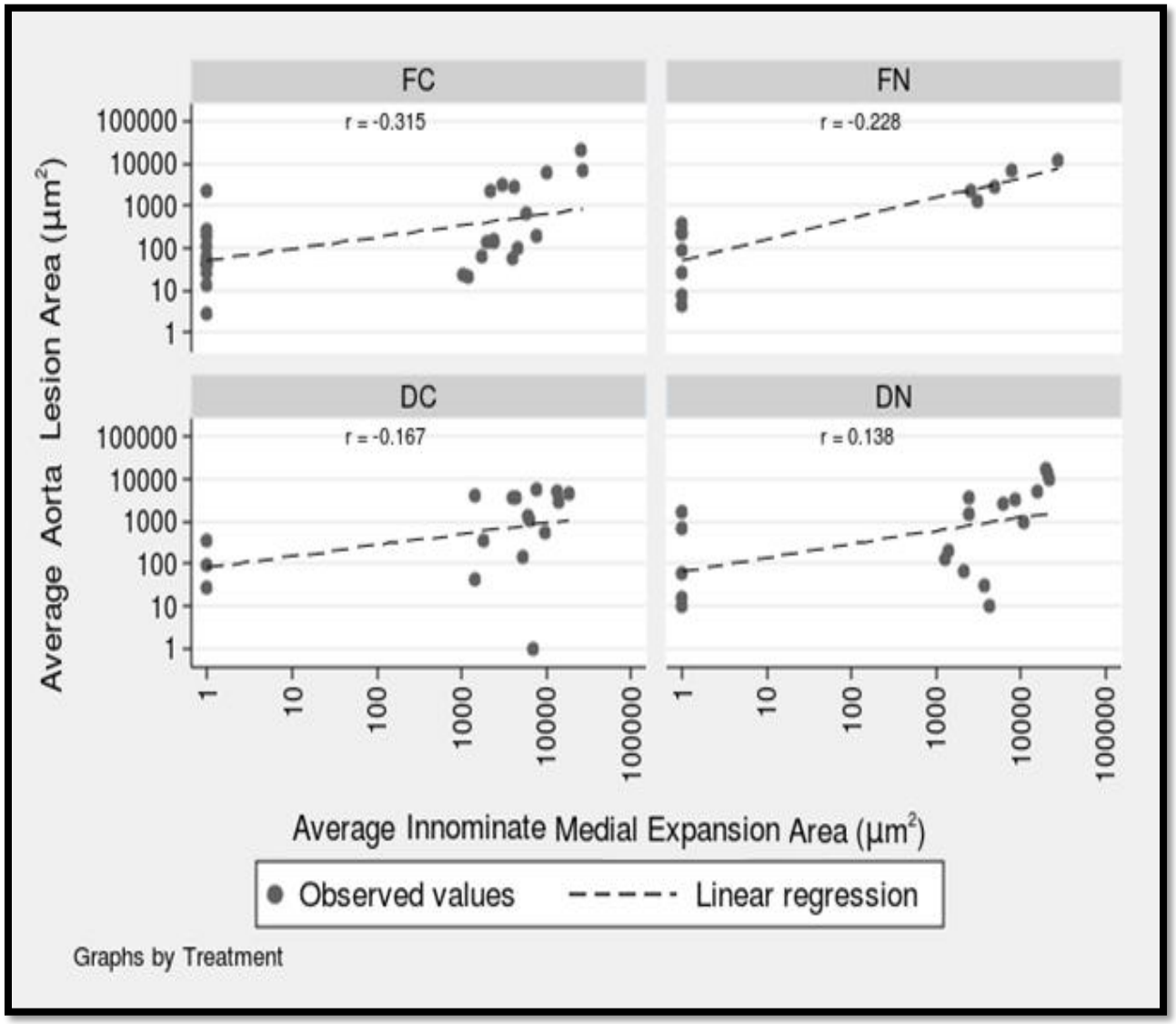
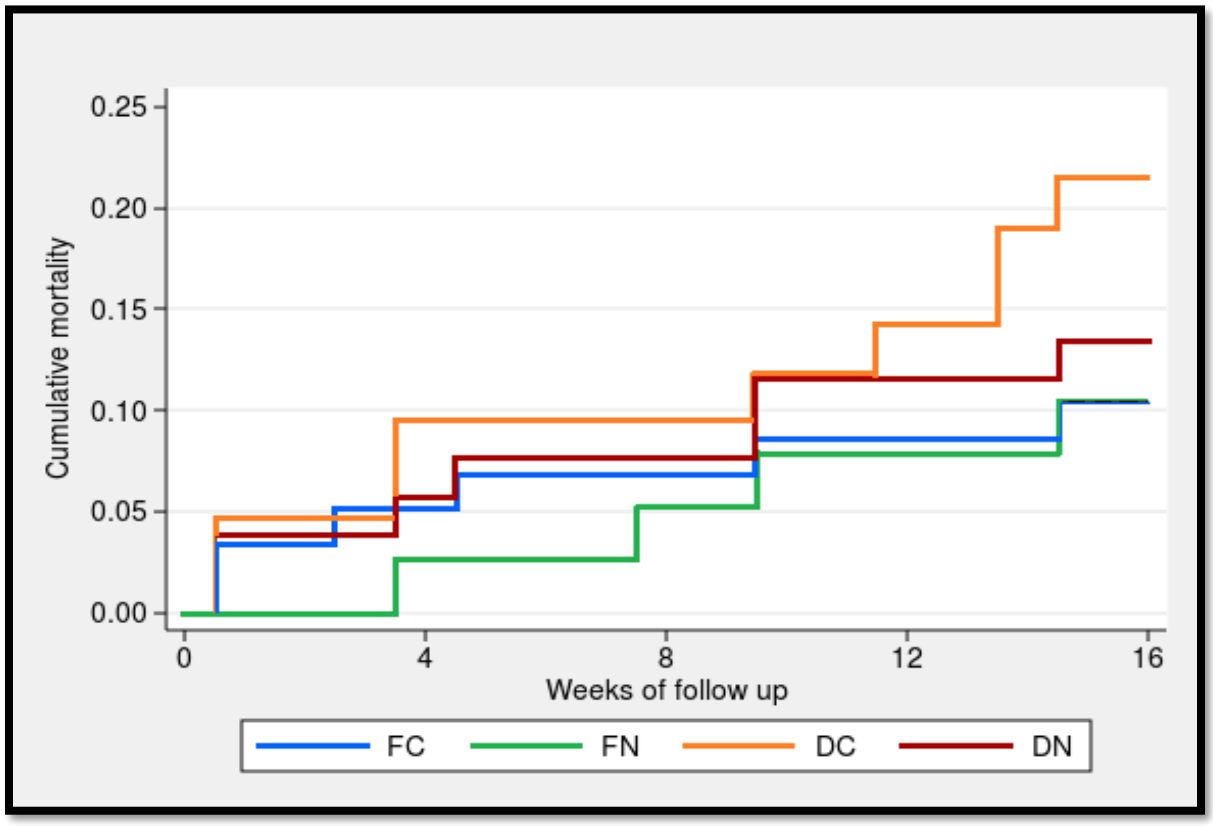


***Figure 9**: Cumulative Mortality from birth to 16 weeks, stratified according to treatment group*



Appendix I

Cardiac Tissue Collection and Processing:

This was done at 16 weeks of age. The mice were fasted for 4 hours prior to the sacrifice. Mice were anesthetized by intraperitoneal injection with 35 mg/kg of a 50/50 mix of ketamine hydrochloride and xylazine. Chest cavity was opened by sectioning through the sternum and ribs. Isolation of mouse aorta was done based on the method described by Palinski and colleagues in 2002 (Palinski & Napoli, 2002). The aorta was then perfused with 3-5 ml of PBS-EDTA solution (2mM EDTA in PBS; pH 7.4)

RNAse-sprayed instruments which had been placed in a glass bead sterilizer for about half a minute were used for the dissection. The heart specimens with the aorta *in-situ* were isolated from the mediastinum. Each specimen was fixed with 5 ml of formalin and stored in a glass vial. The IAs were isolated from the formalin fixed hearts and transferred into pre-labelled tissue cassettes.

Appendix II

Histological Analysis:

The cassettes with IAs were immersed in a solution of 70% ethanol for 25 minutes. Tissue specimens were dehydrated by sequentially immersing the cassettes in the following concentrations of ethanol: 70% for 25 minutes, then in 95% (2X) for 25 minutes each time and 100% 3X 30 minutes each time. The fixed tissues were processed with Shandon Citadel Automatic Tissue Processor and embedded with paraffin in steel molds. Once these have cool down and the molds have set, 5µm thick tissue sections were obtained using a Cryotome

(Reichert Scientific Instruments, USA). Serial transections of the IAs were saved for differential staining. Every slide was mounted with 5 serial sections and labelled appropriately. These were dried overnight at 20 degrees Celsius and baked at 45-50 degree Celsius for a period of 30 minutes. Every fifth slide from the rack was stained with Movat Pentachrome stain; as described by Russell Jr. (1972); and cover-slipped.

The histological sections were viewed using the Nikon Eclipse e400 microscope and examined electronically using Image Pro Plus software (*Media Cybernetics,* Rockville MD) and Nikon DS-1 Camera. The magnification was calibrated with a 20X ruler/0.7X relay.

References:

- Barker, S. G., L. C. Tilling , and G. C. Miller . et al. The adventitia and atherogenesis: removal initiates intimal proliferation in the rabbit which regresses on generation of a 'neoadventitia'. Atherosclerosis 1994

- Benbrahim-Tallaa, L., Baan, R. A., Grosse, Y., Lauby-Secretan, B., El Ghissassi, F., Bouvard, V., & International Agency for Research on Cancer Monograph Working Group. (2012). Carcinogenicity of diesel-engine and gasoline-engine exhausts and some nitroarenes. *The lancet oncology*, *13*(7), 663-664.

- Bolton, J. L., Auten, R. L., & Bilbo, S. D. (2014). Prenatal air pollution exposure induces sexually dimorphic fetal programming of metabolic and neuroinflammatory outcomes in adult offspring. *Brain, behavior, and immunity*, *37*, 30-44.

- Brook, R. D., Xu, X., Bard, R. L., Dvonch, J. T., Morishita, M., Kaciroti, N., ... & Rajagopalan, S. (2013). Reduced metabolic insulin sensitivity following sub-acute exposures to low levels of ambient fine particulate matter air pollution. *Science of the Total Environment*, *448*, 66-71.

- Budinger, G. S., McKell, J. L., Urich, D., Foiles, N., Weiss, I., Chiarella, S. E., ... & Mutlu, E. A. (2011). Particulate matter-induced lung inflammation increases systemic levels of PAI-1 and activates coagulation through distinct mechanisms. *PLoS One*, *6*(4), e18525.

- Caldji, C., Diorio, J., & Meaney, M. J. (2000). Variations in maternal care in infancy regulate the development of stress reactivity. *Biological psychiatry*,*48*(12), 1164-1174.

- Chan, S. H., Van Hee, V. C., Bergen, S., Szpiro, A. A., DeRoo, L. A., S. London, Julian D. Marshall, Joel D. Kaufman,& Sandler, D. P. (2015). Long-Term Air Pollution Exposure and Blood Pressure in the Sister Study. *Environ Health Perspect*.

- Chen, H., Burnett, R. T., Kwong, J. C., Villeneuve, P. J., Goldberg, M. S., Brook, R. D., & Copes, R. (2013). Spatial association between ambient fine particulate matter and incident hypertension. *Circulation*, CIRCULATIONAHA-113.

- Chen, S. Y., Su, T. C., Lin, Y. L., & Chan, C. C. (2012). Short-term effects of air pollution on pulse pressure among nonsmoking adults. *Epidemiology*, *23*(2), 341-348.

- Diesel Exhaust/Diesel Particulate Matter Hazard Alert Manual https://www.osha.gov/SLTC/dieselexhaust/

- Dover, G. J. (2009). The Barker Hypothesis: How Pediatricans Will Diagnose and Prevent Common Adult-Onset Diseases. *Transactions of the American Clinical and Climatological Association*, *120*, 199–207.

- Duan, Z., Du, F. Y., Yuan, Y. D., Zhang, Y. P., Yang, H. S., & Pan, W. S. (2013). [Effects of PM2. 5 exposure on Klebsiella pneumoniae clearance in the lungs of rats]. *Zhonghua jie he he hu xi za zhi= Zhonghua jiehe he huxi zazhi= Chinese journal of tuberculosis and respiratory diseases*, *36*(11), 836-840.

- Feng, S., Gao, D., Liao, F., Zhou, F., & Wang, X. (2016). The health effects of ambient PM 2.5 and potential mechanisms. Ecotoxicology and environmental safety, 128, 67-74.
- Finegold JA, Asaria P, Francis DP. Mortality from ischaemic heart disease by country, region, and age: Statistics from World Health Organisation and United Nations. *International Journal of Cardiology*. 2013;168(2):934-945. doi:10.1016/j.ijcard.2012.10.046.
- Gualtieri, M., Øvrevik, J., Mollerup, S., Asare, N., Longhin, E., Dahlman, H. J., ... & Holme, J. A. (2011). Airborne urban particles (Milan winter-PM2. 5) cause mitotic arrest and cell death: effects on DNA, mitochondria, AhR binding and spindle organization. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *713*(1), 18-31.
- Gurgueira, S. A., Lawrence, J., Coull, B., Murthy, G. K., & González-Flecha, B. (2002). Rapid increases in the steady-state concentration of reactive oxygen species in the lungs and heart after particulate air pollution inhalation. *Environmental health perspectives*, *110*(8), 749.
- H.E. Wichmann, H. E. (2007). Diesel exhaust particles. *Inhalation toxicology*, *19*(S1), 241-244.
- Hellings, W. E., Peeters, W., Moll, F. L., Piers, S. R., van Setten, J., Van der Spek, P. J., ... & Pasterkamp, G. (2010). Composition of carotid atherosclerotic plaque is associated with cardiovascular outcome a prognostic study. *Circulation*, *121*(17), 1941-1950.
- Hesterberg, T. W., Long, C. M., Bunn, W. B., Sax, S. N., Lapin, C. A., & Valberg, P. A. (2009). Non-cancer health effects of diesel exhaust: a critical assessment of recent human and animal toxicological literature. *Critical reviews in toxicology*, *39*(3), 195-227.
- Hirano, S., Furuyama, A., Koike, E., & Kobayashi, T. (2003). Oxidative-stress potency of organic extracts of diesel exhaust and urban fine particles in rat heart microvessel endothelial cells. *Toxicology*, *187*(2), 161-170.
- Iacobellis, G., & Leonetti, F. (2005). Epicardial adipose tissue and insulin resistance in obese subjects. *The Journal of Clinical Endocrinology & Metabolism*, *90*(11), 6300-6302.
- Jiang, R., Jones, M. J., Sava, F., Kobor, M. S., & Carlsten, C. (2014). Short-term diesel exhaust inhalation in a controlled human crossover study is associated with changes in DNA methylation of circulating mononuclear cells in asthmatics. *Particle and fibre toxicology*, *11*(1), 1.
- Kockx, M. M., De Meyer, G. R., Buyssens, N., Knaapen, M. W., Bult, H., & Herman, A. G. (1998). Cell composition, replication, and apoptosis in atherosclerotic plaques after 6 months of cholesterol withdrawal. *Circulation Research*, *83*(4), 378-387.
- Koike, E., Hirano, S., Shimojo, N., & Kobayashi, T. (2002). cDNA microarray analysis of gene expression in rat alveolar macrophages in response to organic extract of diesel exhaust particles. *Toxicological Sciences*, *67*(2), 241-246.
- Kolodgie, F. D., Burke, A. P., Nakazawa, G., & Virmani, R. (2007). Is pathologic intimal thickening the key to understanding early plaque progression in human atherosclerotic disease? Arteriosclerosis, Thrombosis, and Vascular Biology, 27(5), 986-989.
- Li, R., Ning, Z., Cui, J., Khalsa, B., Ai, L., Takabe & Hsiai, T. (2009). Ultrafine particles from diesel engines induce vascular oxidative stress via JNK activation. *Free radical biology and medicine*, *46*(6), 775-782.

- Lehman, Sam J. et al. “Peri-Aortic Fat, Cardiovascular Disease Risk Factors, and Aortic Calcification: The Framingham Heart Study.” *Atherosclerosis* 210.2 (2010): 656–661. *PMC*. Web. 1 May 2016.
- Liguori, A., D’Armiento, F. P., Palagiano, A., Palinski, W., & Napoli, C. (2008). Maternal C-reactive protein and developmental programming of atherosclerosis. *American journal of obstetrics and gynecology*, *198*(3), 281-e1.

- Lucking, A. J., Lundback, M., Mills, N. L., Faratian, D., Barath, S. L., Pourazar, J., ... & Sandstrom, T. (2008). Diesel exhaust inhalation increases thrombus formation in man†. European heart journal.

- Lund, G., Andersson, L., Lauria, M., Lindholm, M., Fraga, M. F., Villar-Garea, A., ... & Zaina, S. (2004). DNA methylation polymorphisms precede any histological sign of atherosclerosis in mice lacking apolipoprotein E. *Journal of Biological Chemistry*, *279*(28), 29147-29154.

- Maiellaro, K., & Taylor, W. R. (2007). The Role of the Adventitia in Vascular Inflammation. *Cardiovascular Research*, *75*(4), 640–648. http://doi.org/10.1016/j.cardiores.2007.06.023

- Manka, D., Chatterjee, T. K., Stoll, L. L., Basford, J. E., Konaniah, E. S., Srinivasan, R., … Weintraub, N. L. (2014). Transplanted perivascular adipose tissue accelerates injury-induced neointimal hyperplasia: role of MCP-1.*Arteriosclerosis, Thrombosis, and Vascular Biology*, *34*(8), 1723–1730. http://doi.org/10.1161/ATVBAHA.114.303983

- Mendis, S., Davis, S., & Norrving, B. (2015). Organizational Update The World Health Organization Global Status Report on Noncommunicable Diseases 2014; One More Landmark Step in the Combat Against Stroke and Vascular Disease. *Stroke*, *46*(5), e121-e122.

- Miller MR, McLean SG, Duffin R, et al. Diesel exhaust particulate increases the size and complexity of lesions in atherosclerotic mice. *Particle and Fibre Toxicology*. 2013;10:61. doi:10.1186/1743-8977-10-61.

- Napoli, C., Infante, T., & Casamassimi, A. (2011). Maternal–foetal epigenetic interactions in the beginning of cardiovascular damage. *Cardiovascular research*, cvr201.

- Nelin, T. D., Joseph, A. M., Gorr, M. W., & Wold, L. E. (2012). Direct and Indirect Effects of PM on the Cardiovascular System. *Toxicology Letters*,*208*(3), 293–299. http://doi.org/10.1016/j.toxlet.2011.11.008

- Omar, A., Chatterjee, T. K., Tang, Y., Hui, D. Y., & Weintraub, N. L. (2014). Proinflammatory phenotype of perivascular adipocytes. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *34*(8), 1631–1636. http://doi.org/10.1161/ATVBAHA.114.303030

- Palinski, W. U. L. F., & NAPOLI, C. (2002). The fetal origins of atherosclerosis: maternal hypercholesterolemia, and cholesterol-lowering or antioxidant treatment during pregnancy influence in utero programming and postnatal susceptibility to atherogenesis. *The FASEB Journal*, *16*(11), 1348-1360.

- Raanan, R., Harley, K. G., Balmes, J. R., Bradman, A., Lipsett, M., & Eskenazi, B. (2015). Early-life exposure to organophosphate pesticides and pediatric respiratory symptoms in the

CHAMACOS cohort. Environmental health perspectives, 123(2), 179.

- Russell Jr, H. K. (1972). A modification of Movat's pentachrome stain. *Archives of pathology*, *94*(2), 187.

- Salim Yusuf, Srinath Reddy, Stephanie Ôunpuu, and Sonia Anand, Global Burden of Cardiovascular Diseases: Part I: General Considerations, the Epidemiologic Transition, Risk Factors, and Impact of Urbanization, *Circulation. 2001;104:2746-2753, doi:10.1161/hc4601.099487*

- Simon, A., Megnien, J. L., & Chironi, G. (2010). The value of carotid intima-media thickness for predicting cardiovascular risk. Arteriosclerosis, thrombosis, and vascular biology, 30(2), 182-185.

- Shimada, A., Kawamura, N., Okajima, M., Kaewamatawong, T., Inoue, H., & Morita, T. (2006). Translocation pathway of the intratracheally instilled ultrafine particles from the lung into the blood circulation in the mouse.*Toxicologic Pathology*, *34*(7), 949-957.

- Srivastava, S., D'Souza, S. E., Sen, U., & States, J. C. (2007). In utero arsenic exposure induces early onset of atherosclerosis in ApoE−/− mice.*Reproductive Toxicology*, *23*(3), 449-456.

- Thacher, J. D., Gruzieva, O., Pershagen, G., Neuman, Å., Wickman, M., Kull, I., ... & Bergström, A. (2014). Pre-and postnatal exposure to parental smoking and allergic disease through adolescence. Pediatrics, 134(3), 428-434.

- Villeneuve, P. J., Weichenthal, S. A., Crouse, D., Miller, A. B., To, T., Martin, R. V., ... & Burnett, R. T. (2015). Long-term Exposure to Fine Particulate Matter Air Pollution and Mortality Among Canadian Women. *Epidemiology*,*26*(4), 536-545.

- Wadhwa, P. D., Buss, C., Entringer, S., & Swanson, J. M. (2009). Developmental Origins of Health and Disease: Brief History of the Approach and Current Focus on Epigenetic Mechanisms. *Seminars in Reproductive Medicine*, *27*(5), 358–368. http://doi.org/10.1055/s-0029-1237424

- Wang, G., Zhen, L., Lü, P., Jiang, R., & Song, W. (2013). Effects of ozone and fine particulate matter (PM2. 5) on rat cardiac autonomic nervous system and systemic inflammation. Wei sheng yan jiu= Journal of hygiene research,42(4), 554.

- Weaver, I. C., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., ... & Meaney, M. J. (2004). Epigenetic programming by maternal behavior. *Nature neuroscience*, *7*(8), 847-854.

- Weintraub N. L., Manka, D., Chatterjee, T. K., Stoll, L. L., Basford, J. E., Konaniah, E. S., Srinivasan, R., …. (2014). Transplanted perivascular adipose tissue accelerates injury-induced neointimal hyperplasia: role of MCP-1.*Arteriosclerosis, Thrombosis, and Vascular Biology*, *34*(8), 1723–1730.

- Weintraub, N. L.,Rajsheker, S., Manka, D., Blomkalns, A. L., Chatterjee & T. K., Stoll, L. .,(2010). Crosstalk between perivascular adipose tissue and blood vessels. *Current opinion in pharmacology*, *10*(2), 191-196.

- Weldy, C. S., Liu, Y., Liggitt, H. D., & Chin, M. T. (2014). 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*, *9*(2), e88582.

- Whitaker, R. C., Wright, J. A., Pepe, M. S., Seidel, K. D., & Dietz, W. H. (1997). Predicting obesity in young adulthood from childhood and parental obesity. *New England Journal of Medicine*, *337*(13), 869-873.

- Wold, L. E., Simkhovich, B. Z., Kleinman, M. T., Nordlie, M. A., Dow, J. S., Sioutas, C., & Kloner, R. A. (2006). In vivo and in vitro models to test the hypothesis of particle-induced effects on cardiac function and arrhythmias.*Cardiovascular toxicology*, *6*(1), 69-78.

- WHO factsheet 2014. Global status report on non-communicable diseases 2014 http://www.who.int/mediacentre/factsheets/fs317/en/