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Diesel Exhaust Exposure and N-acetyl-cysteine Supplementation *in-utero* and Atherosclerotic Progression in Hyperlipidemic Mice

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Abstract

Diesel Exhaust Exposure and N-acetyl-cysteine Supplementation *in-utero* and Atherosclerotic Progression in Hyperlipidemic Mice

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Background: Ambient air pollution exposures have consistently been found to be positively associated with cardiovascular morbidity and mortality. Fine particle air pollution ($PM_{2.5}$), largely derived from diesel exhaust (DE) emissions, have been emphasized as having the most significant effect on cardiovascular health. $PM_{2.5}$ exposure during pregnancy can have deleterious effects on fetal development, decrease birth weight, and is associated with an increased risk of later life metabolic diseases. The adverse intrauterine conditions may also promote risk of atherosclerosis in adulthood. The mechanism of $PM_{2.5}$-mediated effects remains unclear, but there is extensive evidence for the generation of reactive oxygen species and ensuing oxidative stress as a central driver of these observations. N-acetylcysteine is an antioxidant and precursor for glutathione (GSH), the major determinant of intracellular reductive potential. In this study, we investigate the effect of DE exposure and NAC supplementation *in-utero* on adult susceptibility to atherosclerosis.

Methods: Female 8- 16 week old ApoE-/- mice (n= 35) were time-mated to ApoE-/- males. Pregnant females were randomly sorted to a treatment group: Diesel exhaust (~300 μm^3 $PM_{2.5}$ for 6 hours/day, 5 days/week) and control water (DC), diesel exhaust and NAC (500 mg/kg/day) (DN), filtered air and NAC (FN), or filtered air and control water (FC). Females were exposed during gestation only and were transferred with their pups to filtered air with control water at birth. All surviving offspring (n=190) were weighed each postnatal week until 16 weeks. At 8, 12 and 16 weeks, plasma was collected for measurement of total cholesterol, total triglyceride, HDL, LDL, and VLDL cholesterol and triglyceride fractions. Mice were sacrificed at 16 weeks. Urine was collected at 16 weeks for determination of 8-isoprostane. DNA from livers was isolated for assessment of 8-hydroxy-deoxyguanosine levels. mRNA from liver, lung, and aortas was extracted to measure expression of heme oxygenase-1 (HO-1) and glutamate cysteine ligase modifying and catalytic subunits (GCLm and GCLc). Aortic sinuses were processed for histology and imaged to assess lesion progression. Differences among treatment groups from the FC group were analyzed using ANOVA. Males and females in each treatment group as well as litter generation aortic lesion areas were separately analyzed.

Results: The DC dams had significantly smaller litters than FC dams and there was a trend toward higher mortality in the DC group. Week 1 mean weights for the FC, FN, DC, and DN treatment groups were 4.59±0.3 g, 5.13±0.4 g, 4.45±0.38 g, and 5.84±0.4 g, respectively. Weight growth rate in weeks 1- 8 were: FC= 0.23±0.05 g, FN= 0.38±0.13 g, DC= 0.45±0.16 g, and DN= 0.12±0.09 g. There were no week 8-16 weight gain or final weight significant differences among treatment groups. No major trends were observed in 8, 12, and 16 week lipid profiles among treatment and sex groups. Statistical significance was not observed from the results of 8-OHdG, 8-isoprostanes (urine and liver), or GCLm, GCLc, and HO-1 mRNA expression analyses. Mean

aortic lesion areas were not different among treatment groups. Combined offspring from dams second litters had significantly decreased aortic lesion area means compared to first litter offspring.

Comment: The results of this research suggest that *in utero* DE exposure and NAC supplementation does not influence later life redox homeostasis or the risk for increased atherosclerosis progression. Further studies should be conducted in order to fully elucidate the effects of DE on the *in utero* environment, the pathology of offspring mortality, and of litter generation and maternal characteristics on atherosclerotic development.

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Introduction

Atherosclerosis is a major health problem globally, driving cardiovascular disease as the leading cause of mortality (WHO 2011). Considerable epidemiological evidence links ambient air pollution with increased cardiovascular morbidity and mortality (Yin et. al. 2013). Air pollution is a complex mixture of gaseous and particle phases. The particulate matter (PM) component is classified by particle size fractions and the aerodynamic diameter, which determines lung deposition location. There is particularly strong evidence for a causal role of fine (PM< 2.5um) and ultrafine (PM< 100nm) particles that can reach deep into the lung in the progression of atherosclerosis (Araujo and Nel 2009). Diesel exhaust (DE) emission, containing a large amount of ultrafine particles, is a major contributor to urban air pollution. These small particles are highly reactive in the cellular environment (Akimoto, 2003) and can enter deep into the lung and into the circulatory system (Oberdörster et al., 2004 and Elder et al., 2006). The pro-atherogenic actions of inhaled DE is tied to DE particles' ability to induce systemic pro-oxidative conditions in adults (Yin et. al. 2013). However, the long-term health implications of *in-utero* DE inhalation and the mechanisms by which DE PM could enhance atherosclerosis are not well understood.

There is a long lag time between onset of atherosclerosis and its clinical manifestation. (Palinski and Napoli 2002). Evidence suggests that the process of atherosclerosis begins in early life. During fetal development, the prodromal stages of atherosclerotic lesions are already formed in offspring of human and mouse hypercholesterolemic mothers (Palinski and Napoli 2002). Barker and colleagues originally described the concept of fetal programming in their work on undernutrition and its subsequent alteration of human fetal growth and induction of permanent changes in physiological structure, function, and metabolism *in-utero* (Hales and

Barker 1992). The rapid fetal and infant growth experienced as a result of maternal undernutrition has been linked to adult obesity and metabolic diseases (Desai et. al. 2005; Osmond et. al. 1993; Fernandez-Twinn et al 2006). These changes are thought to be a critical component in adult vulnerability to cardiovascular disease (Hales and Barker 1992). Similar to the effects described by Barker and colleagues, there is evidence that PM exposure *in-utero* may alter development, leading to reduced birth weights in humans (Weldy et al. 2014). Early life PM exposure has also been associated with risk of obesity and metabolic diseases in children (van den Hooven et. al. 2012). Specifically, mice exposed to DE *in-utero* show increased fetal inflammation and oxidative stress, which have been demonstrated to promote susceptibility to weight gain and subsequently heart failure (Weldy et al. 2014). The current study examines whether DE-induced oxidative stress (an excess level of pro-oxidants without a balanced amount of antioxidants) *in-utero* in hyperlipidemic apolipoprotein E deficient (apoE-/-) mice predisposes offspring to enhanced atherosclerosis.

One consequence of oxidative stress is tissue injury caused by direct damage to macromolecules by reactive oxygen species (ROS) (Ayala et. al. 2014). This direct interaction can result in changes to lipid signaling, gene expression, membrane physiology, and cell survival. ROS can also stimulate the nuclear factor erythroid-2 related factor (Nrf2-Keap1) signaling pathway, important for protection against oxidative toxicity through expression of phase II enzymes (Ning et al. 2012). Paradoxically, evidence suggests that Nrf2 activity may accelerate atherosclerotic lesion development (Howden 2013), which could be because oxidative stress also stimulates other interacting pro-inflammatory pathways such as NF-kB (Kim and Vaziri 2010). The Nrf2-Keap1 pathway leads to expression of heme-oxygenase-1 (HO-1) and glutamate-cysteine ligases (GCL) (Gorrini et al. 2013), the mRNA expression of which are used

as biomarkers of oxidative stress in this study (Callegari et al. 2011). N-acetylcysteine (NAC), added to the drinking water in this experiment, is a thiol containing antioxidant that replenishes intracellular cysteine levels for synthesis of glutathione by GCL (Basyigit et al. 2004). NAC protection against oxidative damage and the severity of atherosclerosis in apoE-/- mice have been documented (Basyigit et al. 2001; Shimada et al. 2011). NAC may also protect against oxidative stress *in-utero* (Moazzen et al. 2014; Covarrubias et al. 2008), and thus be athero-protective during development and in adulthood.

DE exposure-induced oxidative stress may also be deleterious to fetal development through epigenetic modification that programs the fetus for disease development later in life (Crider et al 2012, Matata and Elahi 2011). Many epigenetic changes have been connected to the cardiovascular disease processes (Castro et al. 2003). Epigenetic regulation, through mechanisms such as DNA methylation, changes gene expression and is perpetuated through subsequent cell divisions (Crider et al 2012). Methylation resulting from air pollutants such as DE may be a reason for PM's long term deleterious effects on health (Belinsky et al. 2002, Janssen et. al. 2015). Evidence from animal studies has shown that PM can affect methylation (Belinsky et al. 2002, Janssen et. al. 2015), altering arterial gene expression and the animal's risk for atherosclerosis.

Thus, there are several mechanisms by which DE-induced oxidative stress can lead to enhanced atherosclerosis in adult mice, including: 1) structural alteration of cellular machinery during fetal development (Baccarelli et al. 2010); 2) interference with oxidation-sensitive signaling pathways (Palinski and Napoli 2002); 3) epigenetic modifications. By altering these possible mechanisms, NAC supplementation could mitigate the risk of atherosclerosis and lead to prevention strategies for humans.

The purpose of this study was to investigate the effects of DE exposure in mice *in-utero* and the progression of atherosclerosis in the offspring. Specifically, the study aimed to [1] measure differences in atherosclerotic plaque areas in the aorta of the DE and/or DE plus NAC exposed offspring as compared to *in-utero* filtered air exposure, [2] examine changes in plasma lipids and biomarkers of oxidative stress that may underlie the atherosclerosis progression observed in our primary aim, and [3] to compare among treatment groups the initial weights, final weights, and the rate of weight gain before and after maturity, which may enhance both oxidative stress and cardiovascular disease progression in DE-exposed animals.

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).

Experimental design

ApoE-/- female and male mice of 8-16 weeks of age were obtained housed in a modified specific pathogen free (SPF) University of Washington Brotman vivarium. At 3 different time points, sets of 7-15 mice were transferred to the Northlake Diesel facility at the University of Washington. All adult mice were kept at the University of Washington Northlake Diesel Exhaust Facility housed under SPF conditions in Allentown caging systems (Allentown, NJ, USA) as previously described (Yin et al. 2013). A more detailed description of the facility system has been published elsewhere (Gould et al. 2008). Briefly, the facility provides a controlled inhalation exposure to diluted and mixed diesel exhaust. The concentrations of particulate matter were in the range of 250- 300 $\mu g/m^3$. The mixture of particulate and gaseous pollutants have been characterized and the exposure conditions have been tested to be consistent and replicable.

After one week of adjustment to the facility, each female was placed in one male cage for breeding. Mice were checked each subsequent morning for visible vaginal plugs to ensure that mating took place. Upon confirmation of mating, each female was moved to its randomly assigned exposure or the control group. This study exposed the female mice to four exposure combinations for the full gestation period: 1) DE and NAC drinking water (DN), 2) DE and

control water (DC), 3) Filtered air and NAC drinking water (FN), 4) Filtered air and control water (FC). 2 mg/mL of NAC was added to the water bottles of the DN and FN cages (the same drinking water bottles as the controls) so that dams were consuming approximately 500 mg/kg/day for the duration of their gestation. Diesel exhaust exposures were 6 hours per day for 5 days per week from the determined time of mating until birth (mouse gestation period was approximately 19-21 days). Offspring remained at this facility until weaning at 4 weeks of age. To ensure that the mice were exposed prenatally only, litters and their dam were moved to the filtered air rack with control water on the morning following their birth.

The average water consumption of randomly selected females in each exposure group from 3 matings (n=22) was recorded for the second week until the end of gestation. This comparison eliminated the possibility that different group outcomes were not due to differences in total water consumption.

Weight Measurements

Litters were weighed 7 days after birth and every other day until 4 weeks of age. Each mouse was identified with Permanent marker tail lines until 4 weeks of age when mice were permanently ear-marked with an ear punch. At 4 weeks of age, mouse pups were transferred to the South Lake Union Brotman Vivarium. From 4 weeks of age to 16 weeks, mice were weighed weekly. Final weight was determined the morning of their date of sacrifice.

Plasma Collection

At 8 and 12 weeks blood samples were collected from fasted mice (4 hour fast) using the retro-orbital blood extraction technique. Mice were anesthetized by inhalation of isofluorane, and

2 to 3 capillary tubes (300-microliter tubes) of blood were collected from each mouse. Blood was transferred immediately into a heparin tube. After approximately one hour in the heparin tube, samples were spun in the centrifuge for 10 minutes at 12,000 rpm. Using a clean 200ul pipette, plasma was extracted from the sample into an Eppendorf tube and stored in a -80 degree freezer.

Tissue Sample Collection

At 16 weeks, mice were fasted for 4 hours. Plastic wrap was placed on a clean surface, on which each mouse was placed on for urine collection. Urine was then collected with a 200ul pipette from the plastic wrap. If the mouse did not urinate, urine was collected with a 29-gauge needle from the bladder. Urine samples were transferred to the Eppendorf tube, frozen in liquid nitrogen, and stored in a -80 freezer. Mice were anesthetized by intraperitoneal injection with 35 mg/kg of a 50/50 mix of ketamine hydrochloride and xylazine. After opening the chest cavity, blood was collected by puncture through the left ventricle with a 27-gauge needle. The animal was then perfused through its left ventricle with 3–5 ml of a Phosphate Buffered Saline-EDTA solution (2 mM EDTA in PBS; pH 7.4).

The esophagus and distal colon were cut to remove all intestines. RNase-sprayed and sanitized (wiped with 70% ethanol, placed in glass bead sterilizer for 30 seconds) instruments were placed in a glass bead sterilizer for 30 seconds. Liver sample 1 (left section of left lateral lobe), liver sample 2 (middle section of left lateral lobe), liver sample 3 (right section of left lateral lobe), liver sample 4 (left half of medial lobe), and liver sample 5 (right half of medial lobe) were extracted. Instruments were sanitized (wiped with 70% ethanol, placed in glass bead sterilizer for 30 seconds) between organs. 5 lung samples were cut: sample 1 (bottom half of left lung), sample 2 (top half of left lung), sample 3 (inferior lobe), sample 4 (middle lobe), sample 5

(superior lobe). The abdominal and thoracic aortas were isolated. All tissue samples were placed immediately into their corresponding Eppendorf tube, snap frozen in liquid nitrogen, and stored at -80 Celcius.

The aorta was fixed with 5 ml of formalin. After the initial fixation, the aortic arch and its main branches were dissected. The adventitia was removed as much as possible. The tissue was stored in formalin in a glass vial.

Plasma Lipid Profiles

Total cholesterol was measured in plasma samples from each animal at 8, 12, and 16 weeks using the Sekisui Cholesterol-SL colorimetric kit (Sekisui Diagnostics, MA, USA) according to the manufacturer's instructions. Total Triglyceride was measured with the Wako Diagnostics L-type Triglyceride kit according to the Wako colorimetric kit protocol (Wako Chemicals, VA, USA). Subsequently, an equal amount of plasma from each female or male from each treatment group was pooled and stored at -80° C. Plasma lipoproteins FPLC profile was obtained using mouse plasma with the AKTA purifier FPLC system with a 10/300 column (GE Healthcare, Pittsburgh, PA, USA). The elution was performed in 0.15 M NaCl, 0.01 M Na2HPO4, pH 7.5, 0.02% NaN3 as a running buffer. After loading 100 µl pooled plasma, the system was run with a constant flow rate of 0.5 ml/min and fractions were collected using a fraction collector (Amersham Biosciences, NJ, USA). The first 11 fractions were discarded and fraction numbers 11 to 40 were used for further analysis to determine cholesterol and triglyceride levels of each fraction. The cholesterol and triglyceride content from each fraction was determined using the Sekisui Cholesterol-SL kit (Sekisui Diagnistics, MA, USA) and Wako Diagnostics kit (Wako Chemicals, VA, USA) according to the standard methods.

RNA isolation and Quantitative RT-PCR

The liver sample 1, lung sample 1, and the combined thoracic and abdominal aorta samples were used for DNA and RNA isolation from 3 randomly selected male and 3 selected female animals per exposure group. Frozen tissues were homogenized using a Polytron homogenizer (Thermo Fisher, MA, USA). The DNA and RNA isolation was subsequently carried out using a Qiagen DNA/RNA Mini kit (Qiagen AllPrep DNA/RNA Mini Kit). The Center for Ecogenetics Functional Genomics Laboratory at the University of Washington used a fluorogenic 5' nuclease-based assay to quantitate the mRNA levels of the specific genes. Briefly, reverse transcription was performed according to the manufacturer's protocol using total RNA and the Life Technologies Invitrogen SuperScript III kit (Thermo Fisher Scientific, NY, USA). For gene expression measurements, 500ng of starting total RNA was used to make the cDNAs. 2 µL of cDNA were included in a PCR reaction that also consisted of the TaqMan Gene Expression Master Mix (Thermo Fisher Scientific, NY, USA). The expression levels of GAPDH, HO-1, GCLc, and GCLm genes were assessed using the Life Technologies TaqMan® Gene Expression Assays (Thermo Fisher Scientific, NY, USA) mix according to the manufacturer's protocol. Amplification and detection of PCR amplicons were performed with the ABI PRISM 7900 system (Applied Biosystems Inc., Foster City, CA, USA) with the following PCR reaction profile: 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 30 s, and 62°C for 1 min. The GAPDH amplification plots derived from serial dilutions of an established reference sample were used to create a linear regression formula in order to calculate expression levels, and GAPDH gene expression levels were utilized as an internal control to normalize the data.

8-OH-dG and 8-isoprostane Analysis

Pooled urine from male and female animals of randomly selected litters (3 litters from each exposure group) was analyzed for 8-isoprostane levels. Liver sample 2 from one randomly selected male and one female from each of these litters were also used to measure 8-isoprostane levels. Urinary and liver 8-isoprostane was measured according to the manufacturer's protocol (8-isoprostane ELISA kit, Detroit R&D, MI, USA).

A DNA/RNA Oxidative Damage EIA kit was used to measure the DNA oxidation byproduct 8-hydroxy-2-deoxyguanosine (Cayman Chemical, MI, USA) in the same liver samples used for the mRNA analysis. DNA was isolated from 10-20 mg of liver sample 1 using a DNA/RNA isolation kit (Qiagen AllPrep DNA/RNA Mini Kit). Each sample was then diluted so that 50 ug of DNA was used in the 8-hydroxy-2-deoxyguanosine assay. The competitive immunoassay involves the binding of free 8-hydroxy-2-deoxyguanosine to an antibody coated 96-well plate. The assay and sample concentration of 8-hydroxy-2-deoxyguanosine was carried out as per the manufacturer's instructions.

Histological Analysis

The formalin fixed aortic sinus bisected from the heart was transferred to a pre-labeled biopsy cassette, into a beaker containing 70% ethanol for 25 minutes. Specimens were dehydrated (70% 25 minutes, 95% 2X 25 minutes, 100% 3X 30 minutes). The fixed tissues were processed according to the manufacturer's instructions (GMI Shandon Citadel 1000 Automatic Tissue Processor) and embedded with paraffin wax in steel molds. 5-µm-thick serial sections were prepared using a Spencer Model 820 rotary microtome (American Optical, Buffalo, NY). Negatively-charged slides (Tanner Scientific, Sarasota, FL) with 3 sections per slide were dried

overnight at 20° C. Tissues collection started when the aortic sinus began to form and collection stopped when the aortic sinus was surpassed. Slides were transferred to an oven set to 60° C to bake overnight. Slides were then stored at room temperature in a slide cabinet until they were stained.

Every third slide was stained using the Movat's Pentachrome Stain. Slides were coverslipped using Richard-Allan Scientific Cytoseal XYL (ThermoFisher Scientific, Waltham, MA) and Corning coverglass (Corning, Corning, NY) after staining was completed. Coverslipped slides were dried overnight at 20° C. Stained aortas were imaged using a Nikon Eclipse E400 microscope coupled with a Nikon DS-1 camera (Nikon, Chiyoda, Tokyo). Imaging was performed at 10X magnification using the Nikon Plan Fluor objective. NIS Elements F Package was used to view and save the images for further analysis. Tissue analysis was conducted without knowledge of the tissue treatment group. The cross-sectional area of atherosclerotic lesions was determined by analyzing the Movat-stained tissues using a computer-assisted morphometric analysis application that was calibrated with a 20X ruler/0.7X relay (*Image Pro Plus*, Media Cybernetics, Silver Spring, MD). Only the middle section of stained slides was analyzed for lesion area. The lesion areas were summed on each measured section. The mean of these sums from each animal was used for statistical analysis.

Statistical Analysis

Differences were determined by ANOVA. Boxplots display median values and bars represent the range of values. Aortic lesion areas were analyzed with individual animal means and error bars represent standard error of the means (SEM).

Results

Litter Success and Survival

There was no significant difference in water consumption during gestation among dams with NAC in their water compared to those with control water (p= 0.774). Average water consumption in control water dams and NAC water dams were 7.46 mL/day and 7.58 mL/day. A total of 35 females (FC=9, FN=9, DC=9, DN=8) were bred and 25 females (FC=6, FN=5, DC=8, DN=6) had at least one litter. From all of the timed matings, a total of 190 pups were born, consisting of 104 males, 84 females, and 2 unsexed before their death. Litter sizes ranged from 1 to 9 pups. The number of pups born from each treatment group is: FC= 59 (35 males and 24 females), FN= 39 (21 males, 17 females, 1 unknown), DC= 41 (19 males, 21 females, 1 unknown), DN= 51 (29 males, 22 females). There were 7 dams that had second litters after prior exposure to their same assigned treatment with litter 1. Of these litters, there were 2 FC, 2 FN, 2 DC, and 3 DN treatment groups. The average number of offspring from successful litters in the treatment groups is: FC= 7.3±1.1, FN=6.3±1.9, DC=4.5±2.3, DN= 5.8±4.4. The distribution of litter sizes among treatment groups is displayed in Figure 1. The FC dams had significantly larger litters while DC dams had the smallest litters compared among treatment groups (p= 0.0098). Male to female gender birth ratios for each treatment group were: FC= 1.46, FN= 1.24, DC= 0.9, DN= 1.32. FC had the greatest ratio of male to female offspring while DC had the lowest ratio. Offspring gender ratios among treatment groups are shown in Figure 2.

In total, 165 pups survived to their 16 week date of sacrifice, 94 males and 71 females. Offspring survival per week among treatment groups is displayed in Figure 3. Premature deaths were observed up through week 16 (n=25). There were 3 deaths due to handling at sample collections, but the other mortality was due to unknown causes. There were no significant

differences in mortality among treatment groups, sex, or number of maternal exposures. The mortality rate per 100 mice was: FC= 10.3, FN= 10.5, DC=21.4, and DN= 13.5. There was a trend toward DC animals suffering the most unknown-cause premature deaths, with 19% of their offspring lost before 16 weeks compared to FC group with a 6.9% loss. The DC group had the most mature animal losses among all treatment groups.

Body Weight

The weekly rate of weight gain for weeks 1-8 and weeks 8-16, as well as body weights at week 1 and male and female week 16 were analyzed. The average rate of weight gain for weeks 1-8 was: FC= 2.59±0.06 g, FN= 2.25±0.14 g, DC= 2.4±0.16 g, and DN= 2.61±0.09 g. FN animals had significantly lower week 1-8 rates of growth compared to FC animals ($p= 0.018$). Weekly rates of growth weeks 8-16 were: FC= 0.23±0.05 g, FN= 0.38±0.13 g, DC= 0.45±0.16 g, and DN= 0.12±0.09 g. There were no significant week 8-16 growth rate differences among treatment groups. Body weight growth in combined males and females among all treatment groups during weeks 1- 8 is shown in Figure 4. Male and female week 10- 16 weights among treatment groups are displayed in Figures 5 and 6, respectively.

Week 1 mean weights for the FC, FN, DC, and DN treatment groups were 4.59±0.3 g, 5.13±0.4 g, 4.45±0.38 g, and 5.84±0.4 g, respectively. DN mice had significantly higher week 1 weights compared to the FC treatment mice ($p= 0.001$). Average female FC, FN, DC, and DN 16 week weights were 21.75±3.04 g, 20.73±7.5 g, 22.5±8.85 g, and 23.17±11.44 g, respectively. Average male 16 week weights for FC, FN, DC, and DN treatment groups were 26.7±7.3 g, 26.5±8 g, 27±5.3 g, 26.8±5.5 g, respectively. None of the male ($p = 0.94$) or female ($p = 0.078$)

16 week weights were significantly different among treatment groups. The distribution of every male and female weight each week (weeks 1- 16) are shown in Figure 7.

Lipid Profiles

A summary table of total cholesterol and triglyceride levels with the mean and standard deviation for each treatment group and sex at each time-point are shown in Table 1. Male 8 week total cholesterol (TC) was significantly higher in the DN group than all other treatments and the FC males was lowest (p= 0.001). Female 8 week TC was also highest in the DN group compared among all treatments (p=0.001). However, 12 week male TC was significantly higher in the DC groups compared to all other male groups (p= 0.026), followed by FN, FC, and DN with the lowest cholesterol. Female 12 week TC was not significant, but the trend among treatment groups matched that of 12 week males. There were no significant TC differences among male and female treatment groups at 16 weeks.

There were no significant differences among male or female triglyceride (TG) levels at 8 weeks. Female TG levels were significantly lower in the FC group compared to all other treatment groups at 12 weeks (p= 0.03). There were no significant differences among male 12 week TG levels. Male 16 week TG in the FN group was significantly higher than all other treatment groups and FC group had the lowest level (p= 0.03). 16 week females followed this same pattern in the following order: FN, DC, DN, and FC females (p= 0.055). Insufficient plasma quantities from several mice at each time-point and uneven premature death rates among the groups may partially account for the inconsistent cholesterol and triglyceride results.

A graphical representation of the HDL, LDL, and VLDL cholesterol fractions at 8 weeks for both males and females are displayed in Figures 8 and 9, respectively. None of the

cholesterol or triglyceride fractions were significantly different among treatments. Male combined DC and DN groups tended to have higher LDL cholesterol (LDL-C) at all time-points ($p = 0.14$) than FC and FN males, but also had higher average HDL (HDL-C) levels ($p = 0.13$). Female combined DC and DN group LDL-C averages were significantly higher than combined FC and FN females at collective time points ($p = 0.04$). TC and TG summaries from the pooled group lipid profiling on weeks 8, 12, and 16 in males and in females are displayed in Tables 2-4.

Gene Expression Analysis of the Liver, Lung, and Aorta

To investigate whether *in utero* DE exposure can continue to modulate oxidative stress-activated pathways with age, the expression of 3 Nrf2 pathway target genes were analyzed by qPCR. The adjusted mRNA expression levels for all of the treatments and tissues analyzed did not reach statistical significance ($p < 0.05$) with respect to the expression of HO-1, GCLm, and GCLc due to high variability. In addition, the samples sizes (3 females and 3 males per treatment group) used for this analysis were small. Figures 10- 18 are graphical representations of the expression of these 3 genes in each tissue among treatment groups.

Although there were no significant differences between groups, there were a number of trends observed. Lung ($n=24$) HO-1 mRNA expression in DN animals was almost 5-fold greater than DC animals and 6 times greater than FN animals. There was a nearly 4-fold increase in DN livers ($n=24$) HO-1 expression compared to the DC livers and nearly 5 fold increase from the FC group. HO-1 expression in the aortas ($n=18$) was significantly lower ($p=0.05$) in all groups and did not follow the same pattern as the liver and lung. Instead, the FC aortas had 3.5 times greater expression than DN aortas.

Liver GCLc mRNA was expressed in DN nearly 3-fold more than DC; FN livers also had greater expression than FC livers. Lung GCLc was also 3-fold and 1.5-fold higher in DN and FN compared to their respective controls. This trend did not follow for the aorta analyses. FC aortas had 4-fold greater expression than DN aortas but FC aorta total HO-1 expression remained equivalent to that of FC livers and lungs.

GCLm liver mRNA expression was 2-fold higher in DN than DC animals, while FC and FN expression was intermediate to these groups. Lung DN GCLm expression was over 2-fold higher than DC, FC, and FN ($p = 0.06$). GCLm expression in the aorta again did not follow the trend of corresponding livers and lungs, but had the greatest expression in the FC group that was lower than FC group liver and lung expression.

Although the increase in DN mice liver and lung mRNA expression was not significant, the trend across all 3 genes indicates that exposure to both NAC and diesel exhaust together *in utero* led to the most responsive adult animals with respect to induction of these oxidative stress mRNAs.

<u>8-Isoprostane and 8-Hydroxy-deoxyguanosine ELISA Analysis</u>

To further explore how DE-induced oxidative stress and antioxidants *in utero* influence markers of oxidative stress in adult animals, urinary and liver 8- isoprostanes were measured as well as liver 8-OHdG levels. There were 3 females and 3 males selected from each treatment group (n=24) for analyses. Statistical significance ($p < 0.05$) was not reached for any of the treatment groups with respect to 8-isoprostane or 8-OHdG measurements.

Male and female 8-OHdG levels were higher in DC and FC groups compared to their respective NAC groups. Average FC, FN, DC, and DN levels were 567.3 pg/g, 428.6 pg/g, 572.9

pg/g, and 410.8 pg/g, respectively ($p = 0.13$). A boxplot of 8-OHdG levels among treatment groups is shown in Figure 19.

Liver 8-isoprostanes combined male and female level averages for FC, FN, DC, and DN were 38±1104 pg/g, 251.6±71,224 pg/g, 482.8±786,508 pg/g, and 1,410.8±296,582 pg/g, respectively ($p= 0.24$). DC and DN were 13-fold and 37-fold higher than the FC group, respectively. A boxplot of Liver 8-isoprostane levels among treatment groups is shown in Figure 21.

Urine 8-isoprostanes levels in males were highest in the FC group, which was over 3-fold higher than the DC males. Male FN and DN groups had lower levels than their respective control group ($p= 0.17$). Females did not follow this same trend with their average 8-isoprostane urine levels remaining similar among the treatment groups. A boxplot of urine 8-isoprostane levels among treatment groups is shown in Figure 20.

Histology

There were 130 hearts measured with a range of 10-24 hearts measured per sex per treatment group. Statistical significance ($p< 0.05$) was not reached for any of the treatment groups with respect to average lesion area in the aortic sinus due to high variability among treatment groups. The mean lesion area for all FC, FN, DC, and DN animals were 11,050±9398 μm2 , 15,527±15,527 μm^2 , 13,970± 14,949 μm^2, and 11,830± 7,803 μm^2, respectively ($p= 0.5$). There was another nonsignificant trend toward offspring from larger litters (5 to 9 offspring) to have increased mean aortic lesion areas than offspring from litters with less than 5 total offspring. A comparison of combined male and female mean lesion areas among treatment groups is presented in Figure 22. The distribution of these values in both sexes among treatment

groups is displayed in Figure 24. Figure 25 shows male and female offspring aortic lesion area means by litters size.

There was a significant difference between offspring of first litters (dams with no prior treatment) and offspring of second litters (dams had previously been exposed to their respective treatment). First litter and second litter offspring mean lesion area were 15953.7± 2064 μm^2 and 9660.7± 1274 μm^2, respectively ($p= 0.009$). The distribution of mean aortic lesion areas by maternal exposure (litter 1 and 2) in combined treatment group offspring are presented in Figures 23.

Discussion

Litter Success and Survival

The outcomes from successful matings in this study suggest differences in litter size and survival due to DE and NAC treatment *in utero*. DC dams had the smallest litter sizes and FC dams had the largest litters (Figure 1), indicating that diesel PM exposure may have caused embryo resorption or fetal death. Increased embryo resorption in dams exposed to diesel exhaust during gestation (Weldy et. al. 2014) and elevated intrauterine mortality in epidemiological studies of ambient air pollution (Wang and Pinkerton 2007) have been reported previously. Additionally, evidence from human studies support the role of systemic and placental oxidative stress in the pathology of spontaneous abortion (Gupta et. al. 2007). Oxidant-induced endothelial damage and impaired placental vascularization are thought to be key players in recurrent pregnancy loss (Gupta et. al. 2007; Myatt 2010). Increased litter size in DN dams compared to DC dams may reflect differences in redox homeostasis during pregnancy. In this case, NAC could have supported antioxidant defense systems of DN dams in the setting of DE-induced oxidative stress. NAC supplementation during pregnancy in humans has reduced unexplained recurrent pregnancy loss (Amin et. al. 2008). Similarly, gestational NAC supplementation in mice restored maternal and fetal oxidative balance and resulted in a higher number of living pups born (Buhimschi et. al. 2003). It was also demonstrated that oral NAC did increase maternal liver GSH and suggested that GSH was critical for fetal survival (Buhimschi et. al. 2003).

FC dams tended to have the highest male to female litter ratios and DC dams had the smallest ratios (Figure 2). Low ratios of males to females among live births and high male to female fetal deaths have been observed in human cohorts after acute exposure to environmental stressors (Catalano et. al. 2005; Helle et. al. 2009). Malnutrition during the 1959-1961 Chinese

famine reduced male to female sex ratios in 2 generations (Song 2012). Applied to the setting of this study, DE- induced intra-uterine oxidative stress and depletion of GSH could have changed the offspring sex ratios.

There was premature pup death in all treatment groups throughout the 16 weeks of the study (Figure 3). The high overall rate of death in DC animals, though insignificant, could have been due to the fetal PM-induced oxidative stress. Epigenetic networks that influence DNA methylation and repair and coordinate response to stressors continuously alter the adult phenotype (Boekelheide et. al. 2012). An adverse intra-uterine environment could have induced adaptive fetal genetic, metabolic, and structural responses that were maladaptive in adult DC animals faced with new environmental challenges. It is possible that the NAC supplementation reduced some of the animals long term health risks via a reduction of *in utero* oxidative stress (Boekelheide et. al. 2012). However, measures of uterine oxidative stress were not obtained. Furthermore, to determine a definite cause of death of the offspring, a pathological examination of the mice would have been necessary.

Body Weight

Human studies have associated *in utero* DE, air pollution, and malnutrition with intra-uterine growth restriction (IUGR) and low birth weight (Boekelheide et. al. 2012; Edwards et. al. 2015; van den Hooven et. al. 2012). IUGR predicts a plethora of later life morbidities, including obesity, insulin resistance, and cardiovascular disease (Matata and Elahi 2011). The small average size of DC litters could signify IUGR due to oxidative stress *in utero* (Myatt 2010). Increased maternal oxidative stress in otherwise normal pregnancies has been negatively associated with birth weight (Matata and Elahi 2011). Both FN and DN animals had higher week

1 weights compared to their respective control groups (Figure 4). This result is consistent with prior human gestational antioxidant supplementation trials. For example, Lee and colleagues found that offspring birth weight and length were higher after pregnancies during which the mother consumed high concentrations of antioxidant vitamins, including tocopherols and ascorbic acid, throughout the second trimester (2004). Another epidemiological study showed that infants with low retinol and alpha-tocopherol levels weighed less (Masters et. al. 2007).

It was predicted that the group with the smallest week 1 weights would have rapid growth before maturity. Instead, the offspring of dams supplemented with NAC and exposed to DE were largest at week 1 and had the highest growth rates in the first 2 months. In contrast, the DC animals experienced the lowest rate of growth until 8 weeks of age, and more rapid growth after maturity, possibly indicating a later life “catch-up” growth similar to that seen in the first years of IUGR infants given nutrient-rich diets (Boekelheide et. al. 2012). The slow early DC group growth agrees with the association that Jedrychowski and colleagues found between low body growth rates in children and residence in a highly polluted area (2002). NAC supplementation also could have increased DN growth as dietary cysteine or a cysteine-rich protein supplementation has been reported to elevate weight gain in rats, monkeys, and in humans (Elshorbagy et. al. 2012).

Maternal oxidative stress has been correlated with reduced fetal and early growth (Elahi and Matata 2011). Oxidative stress biomarkers were not measured in the dams or developing fetuses in this study, so it is possible that the concentration, composition, and duration of DE maternal exposure was insufficient to affect redox homeostasis *in utero*. Nevertheless, exposure to concentrated fine and ultrafine particles in mice after a minimum of 40 hours has been reported to increase oxidative stress as indicated by upregulation of Nrf2-regulated genes,

oxidative modification of HDL, lipid peroxidation, and increased ROS production in endothelial cells and macrophages, among other effects (Araujo 2011). Acute DE exposures in humans can also increase inflammatory mediators and alter endothelial function (Casarett and Doull 2013). During pregnancy, air pollution PM exposure led to elevated maternal and fetal inflammatory responses in women that were linked to small for gestational age of infants (van den Hooven et. al. 2012). Hence, it is likely that DE-exposed dams experienced some amount of oxidative stress and inflammation after conception that may have influenced intra-uterine growth.

Although the DC animals were small and had slow growth in early life, their final weights were comparable to that of DN. It was expected that week 16 body weight and overall weight gain would be significantly greater in both DE exposed groups, as *in utero* DE exposure has previously been reported to increase weight gain over time (Weldy et. al. 2014). However, it is also possible that overall DC weights were higher by 16 weeks because of higher mortality of smaller animals prior to 16 weeks (Figure 3). We cannot rule out that despite similar final weights, adiposity may have been different in DN and FN animals since maternal antioxidant supply and redox state is a determinant of offspring adiposity (Sen and Simmons 2010). Body composition can influence adult oxidative stress, lipid profiles, and metabolic disease risks (Sen and Simmons 2010; Shah et. al . 2014; Gostynski et. al. 2004).

Lipid Profiles

Previous concentrated ambient particles (CAPs) exposure studies in ApoE-/- and LDL-R-/- mice reported small increases in TC and TG and decreases in HDL (Sun et. al. 2005; Araujo et. al. 2008; Li et. al. 2013). In addition, data from experimental animal models indicates that maternal sterol metabolism dictates fetal cholesterol levels (Woollett 2005). So, although

maternal lipid profiles were not analyzed in this study, it was expected that the DC group would have the most pro-atherogenic lipid profiles at each time-point. It was also predicted that DN, FC, and FN animals would have healthier lipid profiles compared to the DC group. This would support the hypothesis that DE creates an adverse *in utero* environment that elevates cardiovascular disease risk with age. This expected pattern for TC was not observed at 8 weeks and instead the DC and FC groups TC levels were lower than the DN group. But by 12 and 16 weeks, the DC group had the highest TC levels (Table 1). The inconsistent TC and TG values across time points may reflect the difficulty collecting plasma from smaller mice (Table 2- 4).

Similarly, the exceptionally low HDL-C in some groups may have resulted from the large variation in group sizes used in plasma analysis. Nonetheless, the lack of effects on plasma lipids agrees with other CAPs and DE exposure studies in ApoE-/- mice on chow diets, including research conducted with the same mouse colony and exposure facility (Bai et. al. 2011; Quan et. al. 2010). DE effects on plasma lipids is apparently very dependent on diet (i. e. cholesterol enriched chow), age of the animals, genetic background, and exposure differences (Araujo and Rosenfeld 2015).

Both TC and TG tend to rise with body mass index of adults and children (Gostynski et. al. 2004). Thus, it would be expected that DC mice have high TG since they tended to have higher body weights and TC after week 8. However, their TG levels were not significantly elevated relative to other treatment groups. Use of a cholesterol rich diet in place of the standard chow diet could have magnified any lipid effects of *in utero* DE exposure. However, there was concern that since high cholesterol diet- induced hyperlipidemia independently promotes atherosclerotic plaque development, this would hide the effects of DE itself on atherosclerosis (Araujo and Rosenfeld 2015).

As predicted, there was a trend of higher average LDL-C in DC animals than FC animals at all time-points, but the pattern among all groups was inconsistent. FN males had the highest 16 week LDL-C and TG, which correlated with their large average lesion area. Also unexpected was that groups with high LDL-C tended to have high HDL-C as well. HDL function was not measured and loss of its athero-protective functions may have explained this result. Normally HDL is protective against atherosclerosis, and cardiovascular disease is associated with HDL that has lost its anti-oxidant and anti-inflammatory protective activities (Smith 2010). Furthermore, induction of HDL reverse cholesterol transport dysfunction is thought to be a good biomarker of enhanced susceptibility to atherosclerosis development (Khera et. al. 2011). Yin et al. (2013) have shown, in the same exposure facility and mouse colony as our study, that DE exposure of Apo E-/- mice leads to loss of the athero-protective properties of HDL. This might be a reason why despite high HDL-C in FN animals, their mean lesion areas tended to be the largest.

Other qualitative effects on plasma lipids not measured in this study may be better indicators of atherosclerosis progression. Motor vehicle emissions and ambient PM in animal models resulted in enhanced susceptibility of LDL and VLDL to oxidation or increased oxidized LDL levels (Araujo and Rosenfeld 2015). Also not measured in our study, 12-HETEs and 13-HODEs are products of lipid oxidation that were increased in mice after DE and ambient PM inhalation (Yin et. al. 2013; Soares et. al. 2009). These plasma biomarkers may be more sensitive and informative on the acute and chronic pro-atherosclerotic effects of air pollutants than plasma lipid quantification alone (Araujo and Rosenfeld 2015).

Gene Expression Analysis of the Aorta, Liver, and Lung

Although statistical significance was not reached, there was a trend of elevated expression in all 3 mRNAs measured in lungs and livers of DN animals. It was expected that DE-exposed animals would have the highest expression of these Nrf2 dependent genes since this pathway is responsive to oxidative stress (Lin et. al. 2010). It is possible that DC animals were not able to upregulate the GCLc and GCLm genes as strongly as DN because both DE and NAC exposures have previously been shown to independently induce the Nrf2 pathway (Weldy et. al. 2011; Lin et. al. 2010). This expression pattern may have reached significance with a higher NAC concentration or extended NAC treatment throughout lactation. The reduced DN aortic lesion areas from the DC group are consistent with the observations of Callegari et. al. who reported an elevated rate of atherosclerotic lesion development in ApoE-/- mice with GCLm deficiency and a mild protective effect of GCLc transgene in macrophages against atherogenesis (2011).

Cumulative evidence supports the protective role of the Nrf2 pathway on lung and liver oxidative injury and protection from atherosclerosis (Mimura and Itoh 2015). Our results are in agreement with this. However, other studies found conflicting results in which the absence of Nrf2 expression has also been shown to reduce atherosclerotic lesion progression in mouse models (Barajas et. al. 2011). In these experiments, Nrf2 activation adversely altered expression of genes involved in lipid metabolism, plasma lipoproteins and hepatic lipids, and macrophage deposition in aortic lesions (Barajas et. al. 2011). A possible reason for the different result is that Nrf2 activation stimulates several interacting genes and complex pathways. Also, Nrf2 expression in ApoE-null mice accelerates the late but not the early stages of atherosclerotic plaque progression (Mimura and Itoh 2015). In the current studies, advanced stages of

atherosclerosis were not observed in DN animals at 16 weeks and may not have been significantly influenced by a change in Nrf2 mediated gene expression.

Also downstream of Nrf2, there was a trend toward higher liver and lung HO-1 expression in DN animals. This in part aligns with recent reviews on HO-1 expression that suggest that HO-1 in the vasculature plays a protective role against inflammation and atherogenesis (Araujo et. al. 2012; Durante 2011). However, in the current study aortic expression of HO-1 was lowest in the DN group. Overall, the elevated HO-1 expression in the liver and lung of the DN group and lower aortic lesion areas compared to the DC group agrees with these reviews describing HO-1 as a protective agent (Araujo et. al. 2011).

8-Isoprostane and 8-Hydroxy-deoxyguanosine ELISA Analysis

There was a tendency toward both NAC supplemented groups to have low liver 8-OHdG (Figure 19). This was the expected result because by replenishing liver GSH, gestational NAC might reduce oxidative stress and DNA damage. NAC supplementation has previously been reported to attenuate DE- and ischemia reperfusion- induced increases in plasma and liver 8-OHdG levels in humans (Yamamoto et. al. 2013; Keles et. al. 2008). The 8-OHdG result and high DN week 1 weights also parallels that of human cohorts of IUGR neonates who experienced small increases in DNA damage (Negi et. al. 2012).

Liver 8-isoprostane levels did not follow the same pattern as 8-OHdG and were unexpectedly higher in groups treated with NAC compared to their respective controls. Also unanticipated, urine 8-isoprostanes were somewhat lower in both DE exposed groups. Our results disagree with previous findings that 8-isoprostanes increase foam cell formation by mechanisms such as inhibition of foam cell apoptosis and elevated MSR-1 expression (Scholz et.

al. 2004). Because human IUGR neonates have elevated lipid peroxidation in epidemiological studies, it was anticipated that DC animals would similarly have high lipid peroxidation (Hracsko et. al. 2008). Also in human trials, urine 8-isoprostane was used as a marker of oxidative stress and correlated with the severity of coronary artery disease in patients with established atherosclerotic disorders (Basarici et. al. 2008). It is possible that given a longer time frame for atherogenesis to occur in this study, DC animals' would have had higher urine and liver 8-isoprostanes.

Histology

There were no statistically significant effect of DE exposure *in utero*, on atherosclerosis in adulthood. The lack of aortic lesion area results among treatment groups may be a result of sufficient upregulation of the maternal oxidant defense systems in response to DE that protected the developing fetus from oxidative stress mediated adverse changes without NAC supplementation.

Though not significant, the observed survival differences among the treatment groups may have confounded the mean lesion area results if only the healthiest animals were used for lesion analysis. There were fewer total DC and DN hearts to analyze at 16 weeks and larger equal male and female group sizes may have reduced the variability within groups. Median lesion area values (data not shown) did reflect the predicted outcome that DC males and females would have the largest lesion areas. A possible explanation for the insignificant results was the 16 week time frame since many lesions at this time point were small and in some animals lesions were entirely absent. Additional time may have made the differences among groups more distinguishable and reduced within group variability. It is possible that the higher antioxidant gene expression helped reduce the aortic lesion areas in DN animals compared to DE-exposure

alone animals since these enzymes are part of the athero-protective Nrf2 pathway in PM exposure research (Ji et. al. 2013). Another factor that may have contributed to the lack of differences was the relatively short exposure period ending at birth instead of at weaning. Previous *in utero* DE studies reported cardiovascular effects after a longer maternal exposure that included lactation (Weldy et. al. 2014). Early life exposures in addition to *in-utero* may be necessary or may have a greater impact on cardiovascular health.

Also statistically non-significant is that mean aortic lesion areas tended to be proportional to litter size (Figure 25). Our result was unexpected since it was thought that offspring born in smaller litters would have elevated atherosclerosis progression because the small litter size may indicate poorer maternal health and an adverse intra-uterine environment. There is evidence for rodent offspring reared in small litters to have increased retroperitoneal fat, enhanced capacity to store glucose, and to accumulate fat (Young 2006). It is suggested that these tendencies are an adaptive response to over-nutrition during early life of these offspring (Young 2006). Previous studies report that epigenetic modifications of major insulin signaling pathway genes could result in the development of insulin resistance in small litter rats (Liu et. al. 2013). Offspring that tend to develop this metabolic syndrome phenotype would also be at risk of elevated atherogenesis. In contrast, our results may imply that small litter offspring received intrauterine and lactation nutrient restriction. Previous work in primates characterized the fetuses of triplet pregnancies, compared to single or twin, as experiencing intrauterine nutritional restriction (Rutherford et. al. 2014). Thus, according to the fetal origin of diseases hypothesis that fetal malnutrition leads to elevated risk of cardiovascular disease later in life (Elahi and Matata 2011), it follows that larger litter pups would have (non-significantly) enhanced lesion areas.

It was also predicted that offspring from first exposure dams would have smaller lesion areas than offspring from dams with multiple exposures. Such results would have indicated that consecutive exposures had an additive effect on dams with the combined DE exposure or NAC further increasing maternal oxidative stress. However, second litter animals had significantly smaller lesion areas (Figure 23). Our data is consistent with there not being oxidative stress differences in the treatment groups. Although not measured in placentas and uteri, the lack of differences in oxidative stress among the offspring may indicate other important changes among dams between their first and second litters. While age itself is associated with higher oxidative stress, age between litters was not very different (1- 2 months difference). One explanation for our results could be that maternal behavior changed between first and second litters due to maternal experience. It has previously been reported that maternal behavior can alter offspring epigenetic regulation and is linked to altered phenotypes (Weaver et. al. 2005). For example, in a rat model, decreased maternal behaviors such as pup licking epigenetically altered the hippocampal glucocorticoid receptor expression and enhanced glucocorticoid feedback sensitivity (Weaver et. al. 2004). A human cohort analysis also suggested a link between prenatal exposure to glucocorticoids or stress and CVD up to 40 years later (Plana-Ripoll et. al. 2015). Therefore, it could be theorized that dams were more stressed (elevated endogenous glucocorticoids) and were poorer mothers with their first litters, and this 'programmed' first litters for increased atherosclerotic progression.

Another reason for smaller lesions in second litters could be that during the second pregnancy, dams upregulated antioxidant defense systems after being 'primed' during their original exposure. Several antioxidant genes have been reported to be upregulated after DE exposure, PM, or NAC treatment (Araujo 2009; Izigov et. al. 2011), which could be

advantageous to the developing fetuses. However, it appears that FC first litters also had higher lesion areas than second litters despite dams having no prior treatments. Moreover, differences in fetal or later life oxidative stress markers between litter generations were not outcomes looked at in the current study. Future studies could determine the reproducibility of these outcomes and potential mechanisms mediating these effects.

Limitations

Despite the substantial number of total animals born, male and female treatment groups had uneven post-natal mortality and fairly small sample sizes at 16 weeks. In addition, there were no dams inspected during gestation and no necropsies performed so the reasons for the loss of these animals can only be speculated. In order to minimize offspring mortality, mice pups were not weighed until postnatal day 7. Placenta to fetal measurements as well as birth weight would have helped establish if IUGR had occurred.

Maternal biomarkers of oxidative stress and antioxidant defense genes expressed in the uterus and placenta would have provided insight about the environment of the developing fetuses. This information would add to the associations being made between *in utero* conditions and adult health outcomes. In addition to mRNA analysis, products of HO-1 could inform us if expression is translated to function and how this might play a protective role in offspring from each treatment. Maternal and placental GSH levels could indicate whether the insignificant differences in GCLc and GCLm gene expression or the NAC supplementation actually changed these measurements as predicted.

Innominate arteries were collected from the subjects of this study but have not yet been analyzed. Lesions in this artery may be less variable and provide positive results. Future studies

should also measure plaque composition in order to provide more information about the plaque stability and cardiovascular related mortality risks among treatment groups.

Lipid analysis was limited due to missing samples from animals at each time-point and large samples size ranges. This may have skewed the results because of differences in animal size and fragility that dictated which animals provided sufficient plasma. Analysis of LDL oxidation and lipid peroxidation products would have been further indication of oxidative stress and atherosclerotic progression among treatment groups. Also, HDL function was also not measured in this study, but could have confirmed whether the high HDL in DE-exposed groups was potentially protective or was instead contributing to atherogenesis.

Many of the outcomes had under-powered small sample sizes with randomly selected animals. In order to explore the mechanisms behind lesion area differences among treatment groups and within group differences, it will also be important to measure oxidative stress and possibly inflammation biomarkers in dams and placentas. This could also help elucidate how consecutive multiple maternal exposures could program offspring differently.

Body weights were not measured at birth, which meant that any rapid “catch up” growth in the first week is missing from our analyses. Many fetal programming studies look at birth weight and infant accelerated growth as strong indicators of metabolic diseases in adulthood. In addition, epidemiological studies have found ponderal index (birthweight to crown- heel- length ratio) to be more indicative of later life outcomes than weight alone. Insignificant final weight results could also be due to the limited time frame of the study and significance may have been observed in final weights of older mice (i.e. 32 weeks of age).

It was a limitation of this study that only 3 genes were looked at in the mRNA analysis. There are numerous known genes influencing atherosclerotic progression and many more that

are potentially altered by DE and NAC (Lusis 2012). The simultaneous expression of these genes together likely influenced the results. In addition, translation in proteins and protein function needs to be taken into consideration in the evaluation of DE and NAC health effects. Since the mitochondria is particularly susceptible to oxidative damage and is known to be altered by DE, mitochondrial gene expression and DNA content would help elucidate the molecular mechanisms of DE health consequences (Janssen et. al. 2012). Also important for the determination of developmental programming, are epigenetic analyses. Epigenetic alterations *in utero* have been shown to influence cardiovascular risk in adults (Matata and Elahi 2011), but was not directly looked at in this study.

In order to further investigate differences between litter generations, additional studies should conduct a study with larger and equal sized groups. It would be important to look into possible mechanisms involving oxidative and glucocorticoid stress *in utero* and in early life.

Figures and Tables

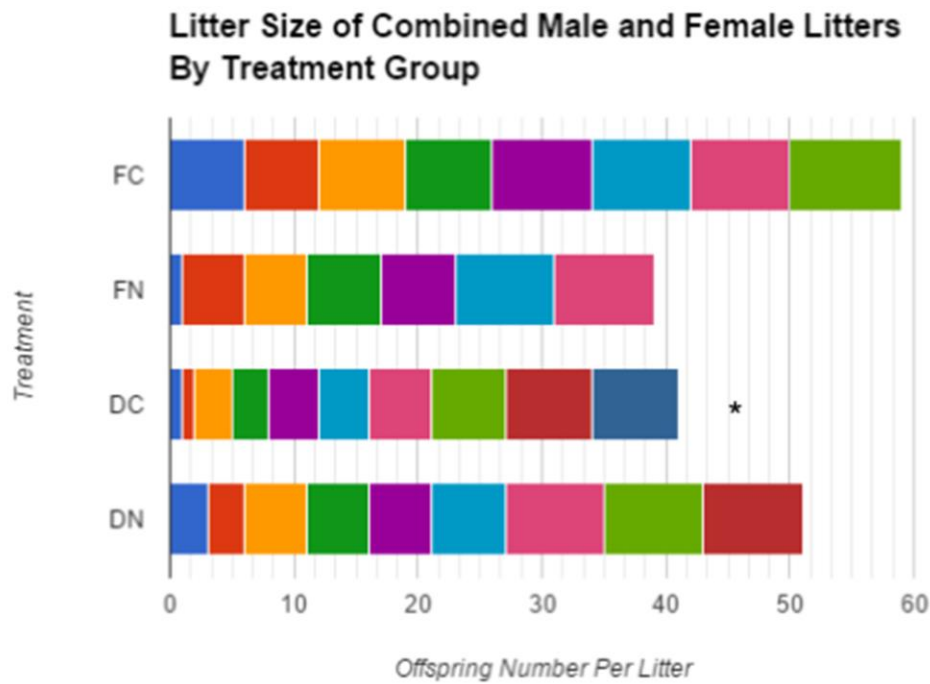

Figure 1. *Litter size distribution among combined male and female treatment groups. Each color represent each recorded litter and the relative litter size from all dams in treatment groups. Litters in which the number of offspring could not be counted due to cannibalism are not shown. * = statistically significant difference in litter sizes from the FC treatment group (p< 0.05).*

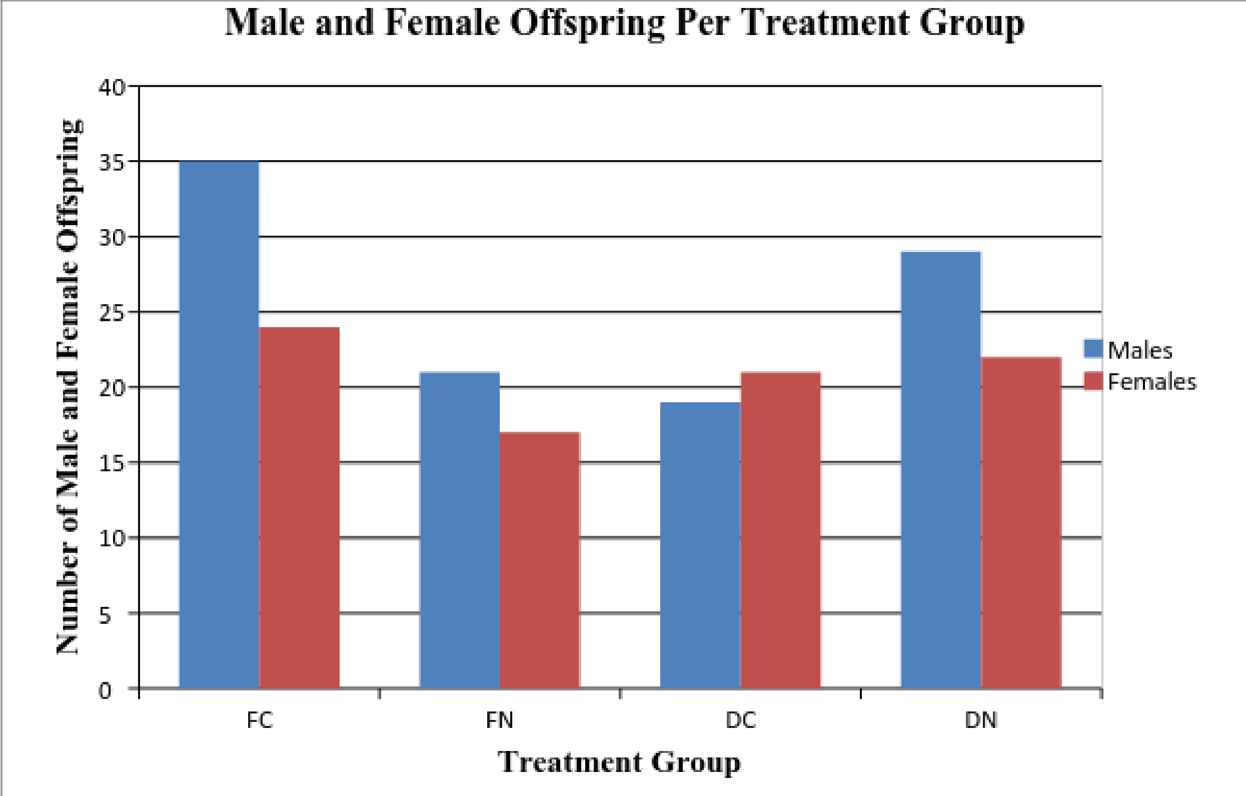

Figure 2. *Distribution of male and female offspring born from combined litters in each treatment group. Offspring totals represent animals that survived to 4 months of age when sex was determined.*

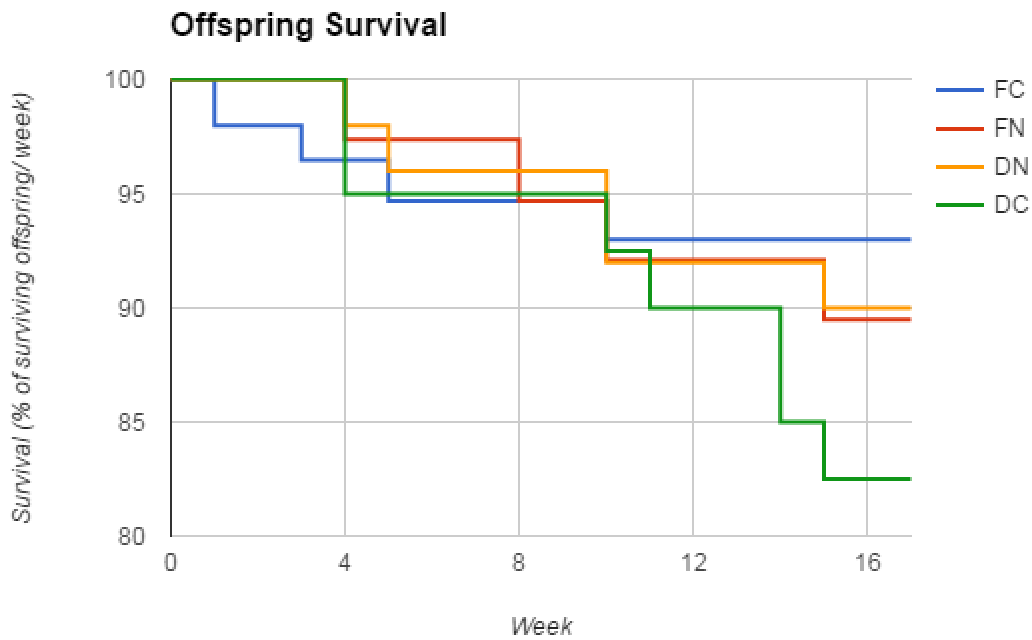

Figure 3. *Weekly survival of combined male and female offspring among treatment groups. The mortality rate per 100 mice was: FC= 10.3, FN= 10.5, DC=21.4, and DN= 13.5.*

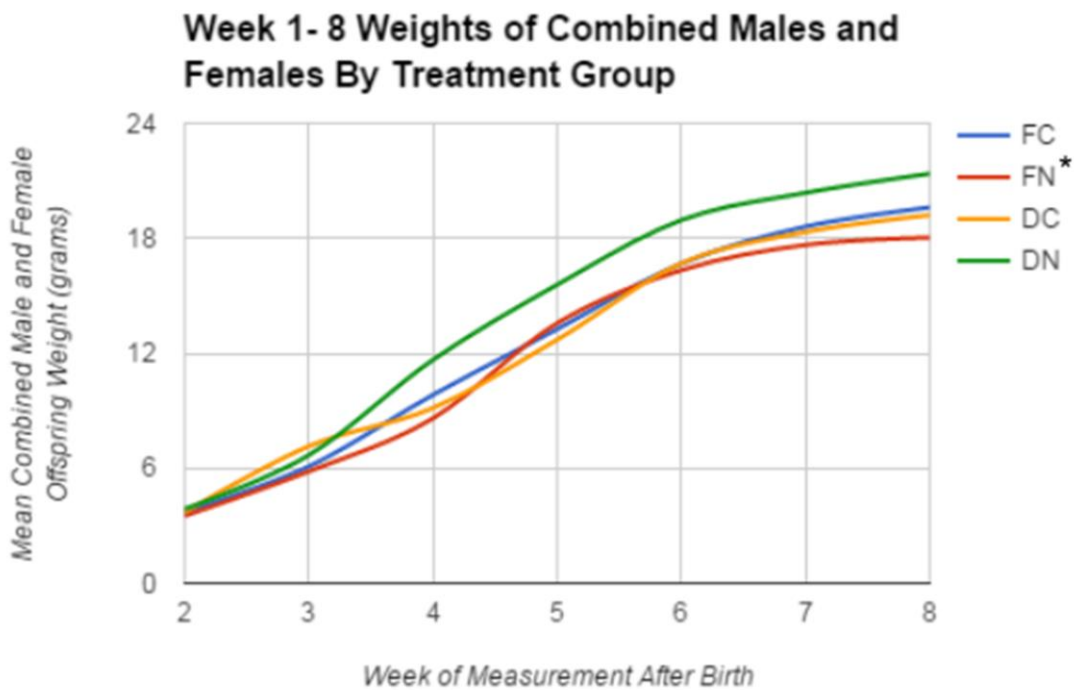

Figure 4. *Weekly growth rates of combined male and female animals among treatment groups. Initial weights were recorded postnatal day 7. The average rate of weight gain for weeks 1-8 in each treatment group was: FC= 2.59±0.06 g, FN= 2.25±0.14 g, DC= 2.4±0.16 g, and DN=*

*2.61±0.09 g. * = statistically significant difference in weight gain per week compared to the FC group (p< 0.05).*

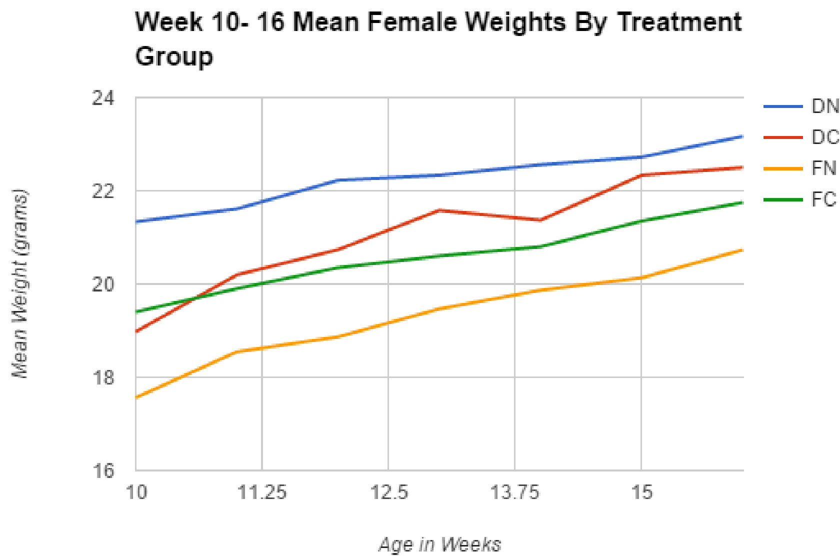

Figure 5. *Weekly growth rates of female surviving animals among treatment groups from week 10 through week 16. Final weights were recorded at 16 weeks of age. Mean female 16 week weights were: FC= 21.75±3.04 g, FN= 20.73±7.5 g, DC= 22.5±8.85 g, and DN= 23.17±11.44 g*

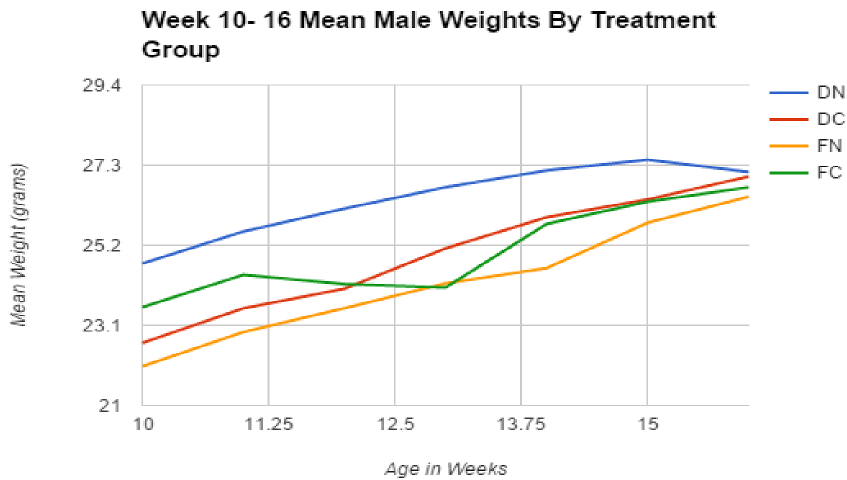

Figure 6. *Weekly growth rates of male surviving animals among treatment groups from week 10 through week 16. Final weights were recorded at 16 weeks of age. Mean male 16 week weights were FC= 26.7±7.3 g, FN= 26.5±8 g, DC= 27±5.3 g, DN= 26.8±5.5 g.*

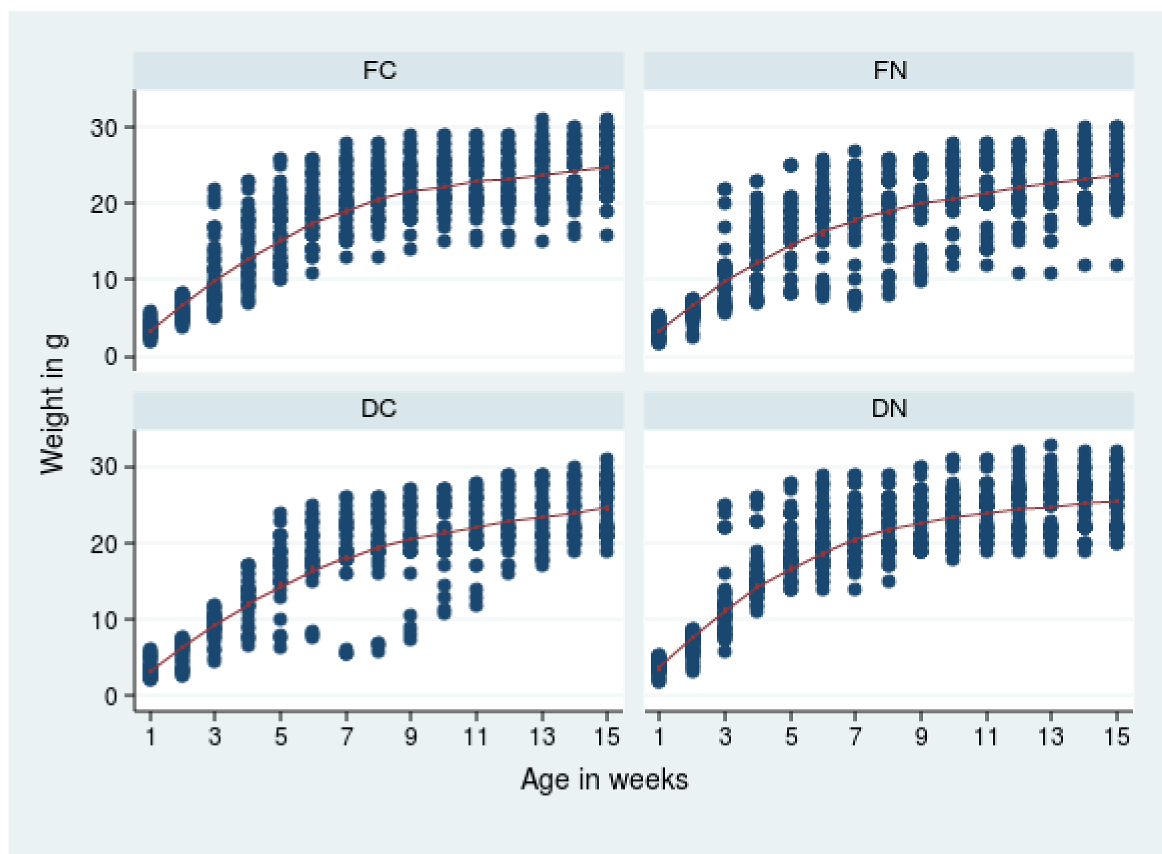

Figure 7. *Distribution of weekly weights and mean growth rates of combined male and female animals among treatment groups. Initial weights were recorded postnatal day 7 and final weights were at 16 weeks of age.*

Total Triglyceride (mg/dL)	**FC**	**FN**	**DC**	**DN**
8 Weeks *	76.7 (42.7) n= 36	88.2 (51.6) n= 18	65.6 (25.2) n= 18	64.6 (18.9) n= 16
12 Weeks	68.5 (35.8) n= 26 (females*)	65.2 (27.8) n= 29	65 (24.8) n= 18	71.9 (38.3) n= 21
16 Weeks	333.4 (233.6) n= 18	108.7 (158.1) n= 14 (males*)	168.4 (124.1) n= 15	312.6 (253.2) n= 15
Total Cholesterol (mg/dL)	**FC**	**FN**	**DC**	**DN**
8 Weeks *	253.9 (46.7) n= 35	310 (84.3) n= 19	259.5 (54.6) n= 18	378.4 (93.5) n= 16 (males*, females *)
12 Weeks *	322.9 (93.5) n= 29	326.9 (107.6) n= 29	330.5 (134.1) n= 23 (males*)	252.6 (84.3) n= 21
16 Weeks	259.4 (131.2)	363.5 (76.9)	367.6 (116.3)	296.3 (123.6)

	n= 27	n= 15	n= 16	n= 15

Table 1. *Summary Statistics of 8, 12, and 16 week Total Triglyceride and Total Cholesterol among combined male and female treatment groups. Values represent mean, standard deviations is in parentheses, n= sample size used for each measurement. *= significantly different value from the FC combined male and female group. Sex specific significant differences in male or females only group analyses noted with sex in parentheses.*

8 Week Treatment group, gender	**VLDL Cholesterol (mg/dL)**	**LDL Cholesterol (mg/dL)**	**HDL Cholesterol (mg/dL)**	**VLDL Triglyceride (mg/dL)**	**LDL Triglyceride (mg/dL)**	**HDL Triglyceride (mg/dL)**
FC Females (n=14)	63.3	92.1	27.6	64	0	0
FC Males (n=22)	59	89.1	37.9	118	0	0
DC Females (n=11)	62.3	84.6	30.1	64	0	0
DC Males (n=7)	66.4	110.8	52.0	106	0	0
FN Females (n=7)	52.1	87.0	32.6	53	0	0
FN Males (n=12)	45.0	84.8	39.9	105	0	0
DN Females (n=11)	64.9	101.9	35.9	3.1	0	0
DN Males (n=5)	50.8	79.7	39.5	43.5	0	0

Table 2. *Total Cholesterol and Triglyceride VLDL, LDL, and HDL by sex among treatment groups at 8 weeks. The sum of pooled male and pooled female fractions 13-17 represent VLDL, 18-27 represent LDL, and 28-33 represent HDL. No significant differences ($p<0.05$) in treatment groups from the FC group were observed.*

12 Week Treatment group, gender	**VLDL Cholesterol (mg/dL)**	**LDL Cholesterol (mg/dL)**	**HDL Cholesterol (mg/dL)**	**VLDL Triglyceride (mg/dL)**	**LDL Triglyceride (mg/dL)**	**HDL Triglyceride (mg/dL)**
FC Females (n=8)	51.2	51	19.3	53.9	53.6	0
FC Males (n=19)	43.1	51.4	51	52	56.2	0
DC Females (n=9)	17.3	188.8	109.4	27.7	3.4	0

DC Males (n=9)	9.8	155.3	100	15.2	20.8	0
FN Females (n=13)	10	11.4	0.5	52.5	12.6	0
FN Males (n=18)	19.4	28.1	12.1	105	0	0
DN Females (n=9)	17.5	158	130.6	13.4	44.2	0
DN Males (n=17)	35.3	258.2	65.66	6.4	29.6	0

Table 3. *Total Cholesterol and Triglyceride VLDL, LDL, and HDL by sex among treatment groups at 12 weeks. The sum of pooled male and pooled female fractions 13-17 represent VLDL, 18-27 represent LDL, and 28-33 represent HDL. No significant differences (p<0.05) in treatment groups from the FC group were observed.*

16 Week Treatment group, gender	**VLDL Cholesterol (mg/dL)**	**LDL Cholesterol (mg/dL)**	**HDL Cholesterol (mg/dL)**	**VLDL Triglyceride (mg/dL)**	**LDL Triglyceride (mg/dL)**	**HDL Triglyceride (mg/dL)**
FC Females (n=9)	16.5	26.4	4.4	49.2	44.4	0
FC Males (n=14)	30.8	56.9	47.9	34.5	32.4	0
DC Females (n=7)	50.7	75.8	5.2	53.9	51.6	42.1
DC Males (n=5)	62.8	75.15	70.1	43.8	28.2	0
FN Females (n=9)	86.8	11	21.1	17.4	30	0
FN Males (n=5)	41.4	109	0	63.9	57.6	0
DN Females (n=8)	65.3	57.3	0	26	6.2	0

DN Males (n=15)	42.4	50.5	19.3	53	40.2	41.4

Table 4. *Total Cholesterol and Triglyceride VLDL, LDL, and HDL by sex among treatment groups at 16 weeks. The sum of pooled male and pooled female fractions 13-17 represent VLDL, 18-27 represent LDL, and 28-33 represent HDL. No significant differences (p<0.05) in treatment groups from the FC group were observed.*

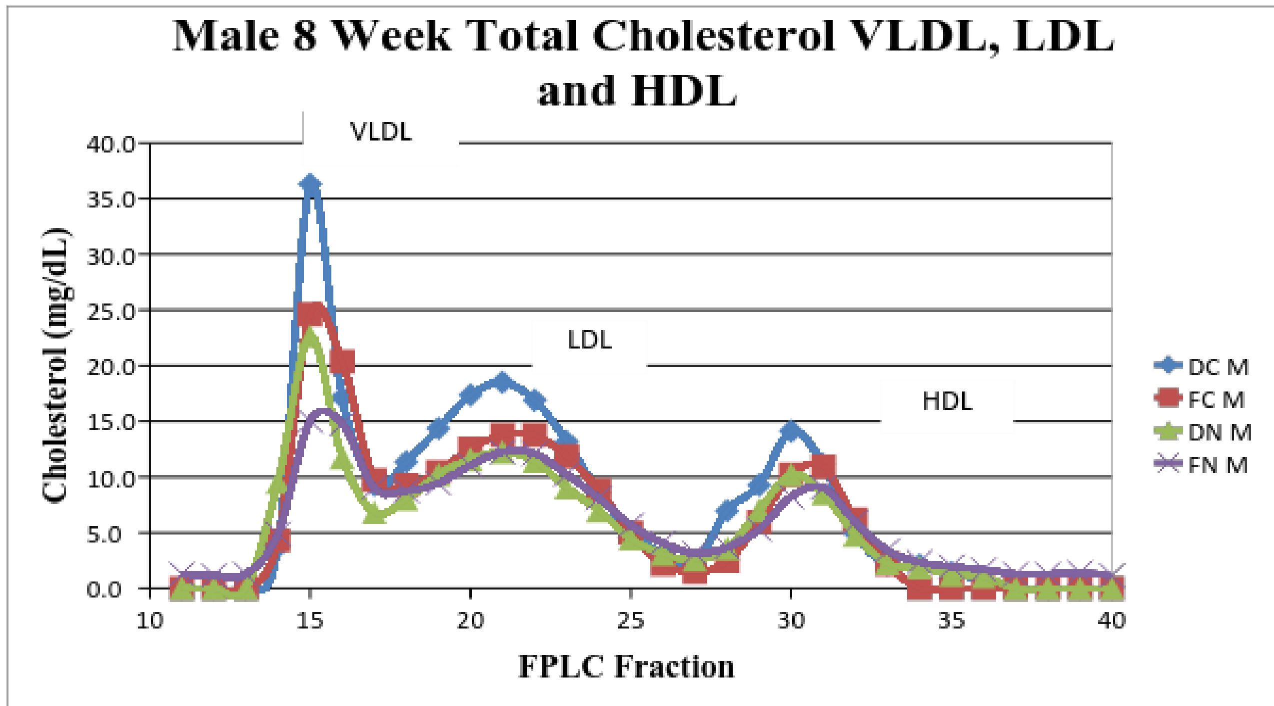

Figure 8. *Total 8 week cholesterol VLDL, LDL, and HDL fractions among male treatment groups. Male samples used for TC and TG analysis were pooled and separated into fractions using FPLC. The sum of fractions 13-17, 18- 27, and 18-27 were used to represent VLDL, LDL, and HDL, respectively.*

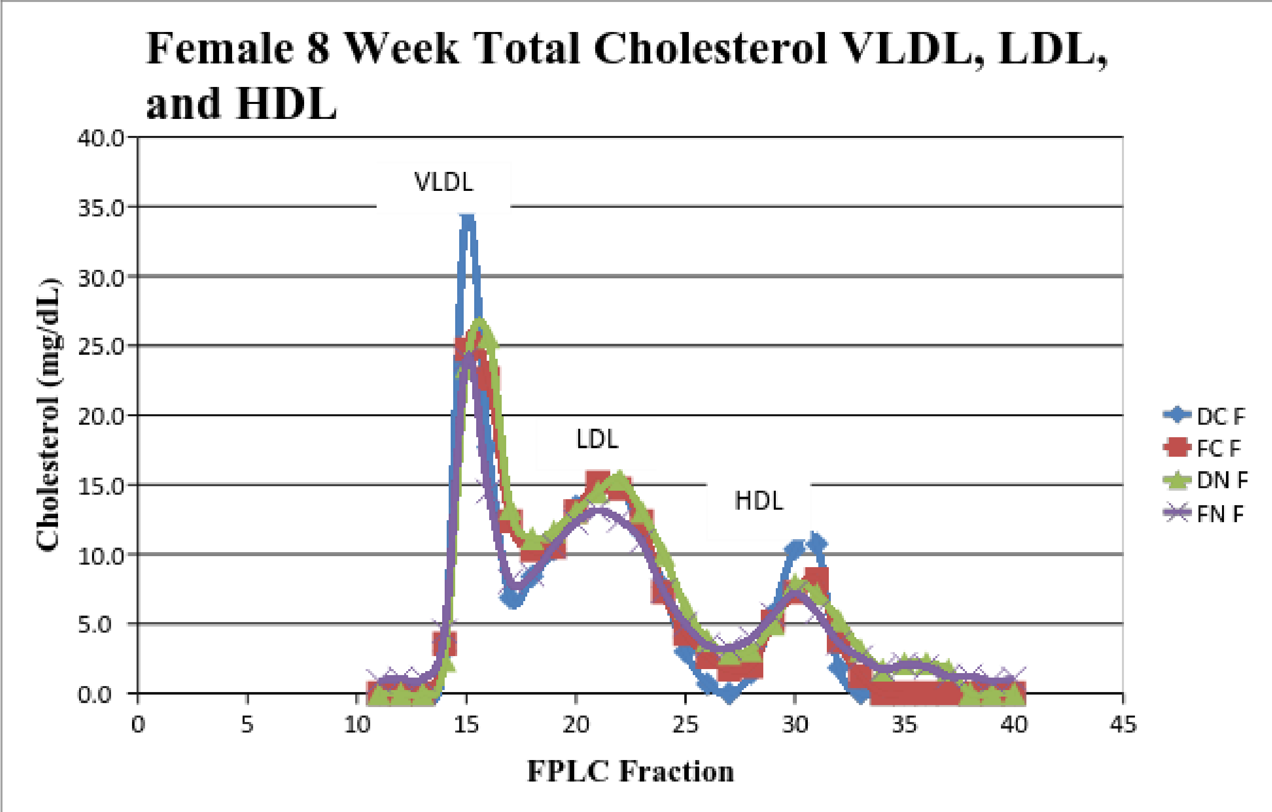

Figure 9. *Total 8 week cholesterol VLDL, LDL, and HDL fractions among female treatment groups. Female samples used for TC and TG analysis were pooled and separated into fractions using FPLC. The sum of fractions 13-17, 18- 27, and 18-27 were used to represent VLDL, LDL, and HDL, respectively.*

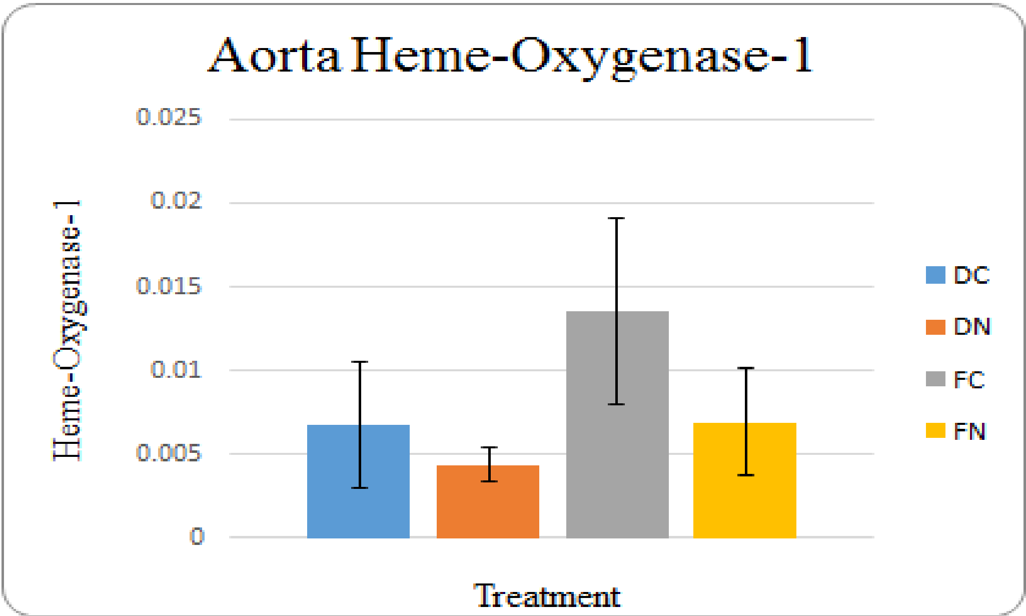

Figure 10. *Real-time PCR assessment of mRNA levels for aorta HO-1 at 16 weeks, and normalized to GAPDH. Error bars represent SEM. Tissue samples from randomly selected male and female mice within each treatment group were analyzed together (n=18; FCn=5, FNn=4, DCn=4, DNn=5). No significant differences (p<0.05) in treatment groups from the FC group were observed.*

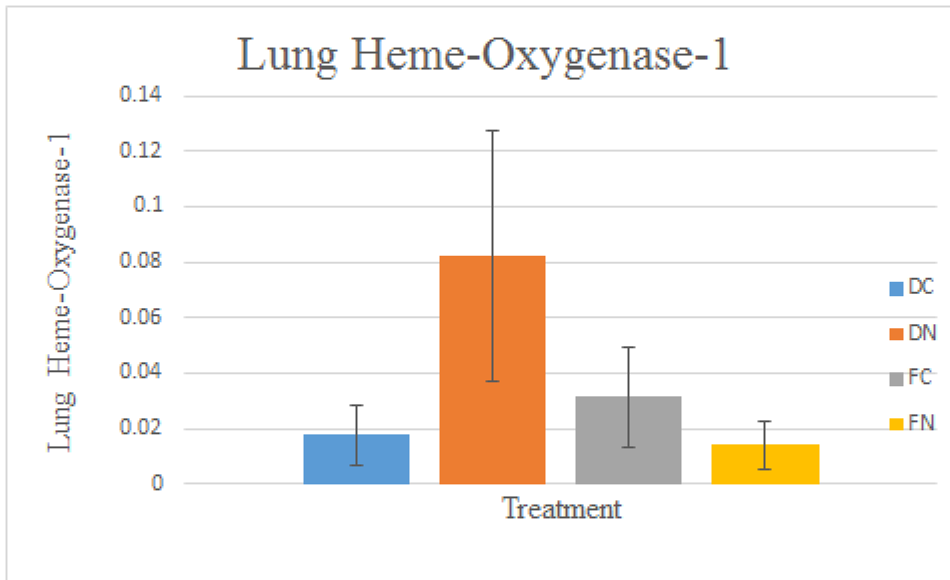

Figure 11. *Real-time PCR assessment of mRNA levels for lung HO-1 at 16 weeks, and normalized to GAPDH. Error bars represent SEM. Tissue samples from randomly selected male and female mice within each treatment group were analyzed together (n=24; FCn=6, FNn=6, DCn=6, DNn=6). No significant differences (p<0.05) in treatment groups from the FC group were observed.*

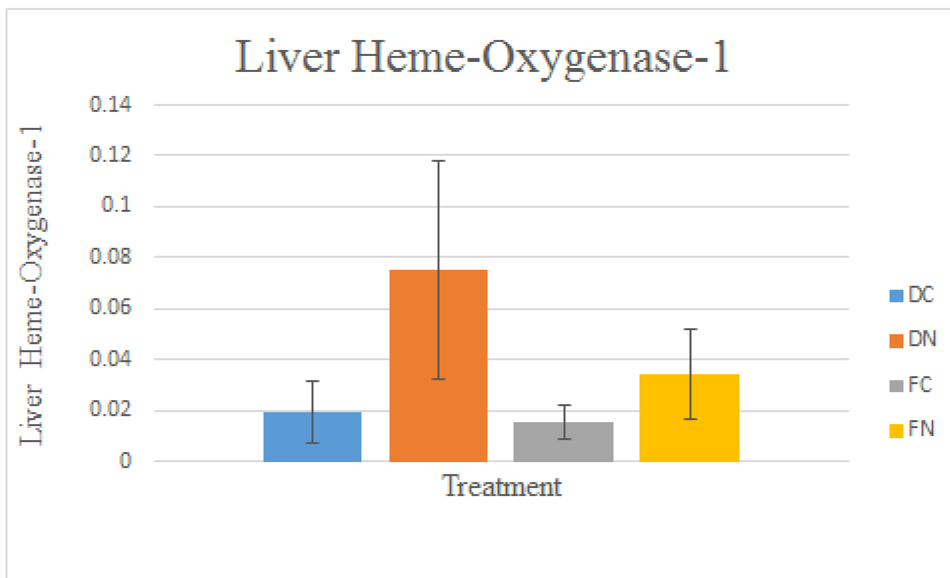

Figure 12. *Real-time PCR assessment of mRNA levels for liver HO-1 collected at 16 weeks, and normalized to GAPDH. Error bars represent SEM. Tissue samples from randomly selected combined male and female mice within each treatment group were analyzed together (n=24;*

FCn=6, FNn=6, DCn=6, DNn=6). No significant differences (p<0.05) in treatment groups from the FC group were observed.

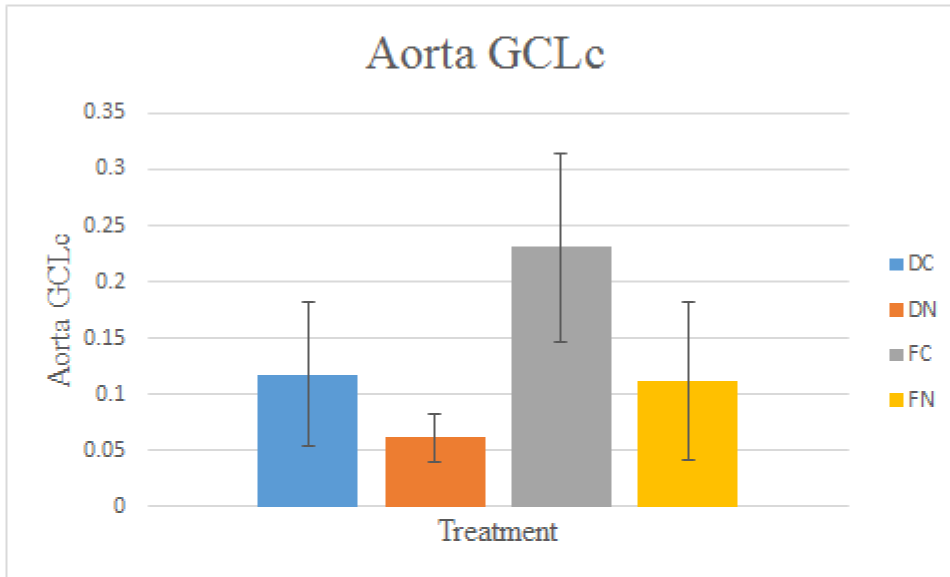

Figure 13*. Real-time PCR assessment of mRNA levels for Aorta GCLc collected at 16 weeks, and normalized to GAPDH. Error bars represent SEM. Tissue samples from randomly selected male and female mice within each treatment group were analyzed together (n=18; FCn=5, FNn=4, DCn=4, DNn=5). No significant differences (p<0.05) in treatment groups from the FC group were observed.*

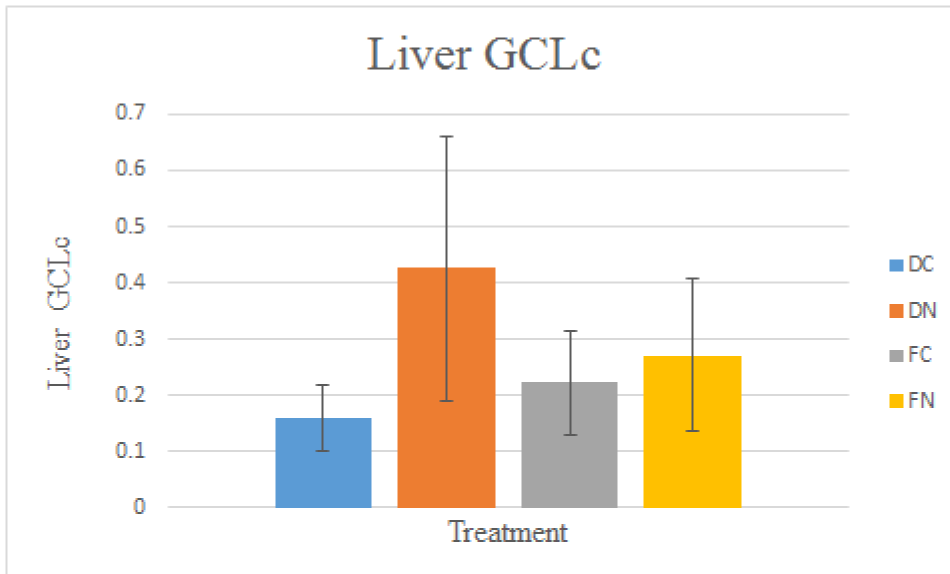

Figure 14*. Real-time PCR assessment of mRNA levels for liver GCLc at 16 weeks, and normalized to GAPDH. Error bars represent SEM. Tissue samples from randomly selected male and female mice within each treatment group were analyzed together (n=24; FCn=6, FNn=6, DCn=6, DNn=6). No significant differences (p<0.05) in treatment groups from the FC group were observed.*

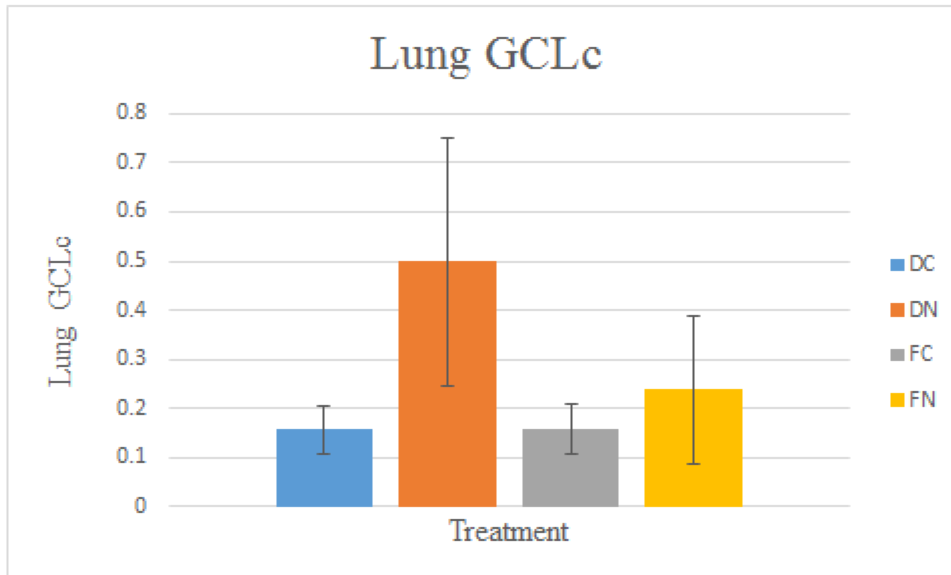

Figure 15. *Real-time PCR assessment of mRNA levels for lung GCLc collected at 16 weeks, and normalized to GAPDH. Error bars represent SEM. Tissue samples from randomly selected male and female mice within each treatment group were analyzed together (n=24; FCn=6, FNn=6, DCn=6, DNn=6). No significant differences (p<0.05) in treatment groups from the FC group were observed.*

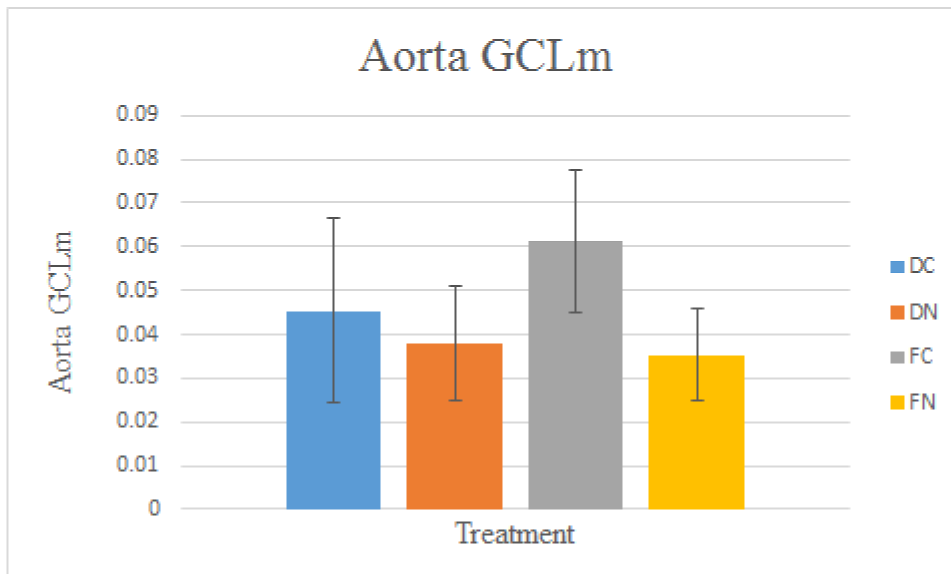

Figure 16. *Real-time PCR assessment of mRNA levels for aorta GCLm collected at 16 weeks, and normalized to GAPDH. Error bars represent SEM. Tissue samples from randomly selected male and female mice within each treatment group were analyzed together (n=18; FCn=5, FNn=4, DCn=4, DNn=5). No significant differences (p<0.05) in treatment groups from the FC group were observed.*

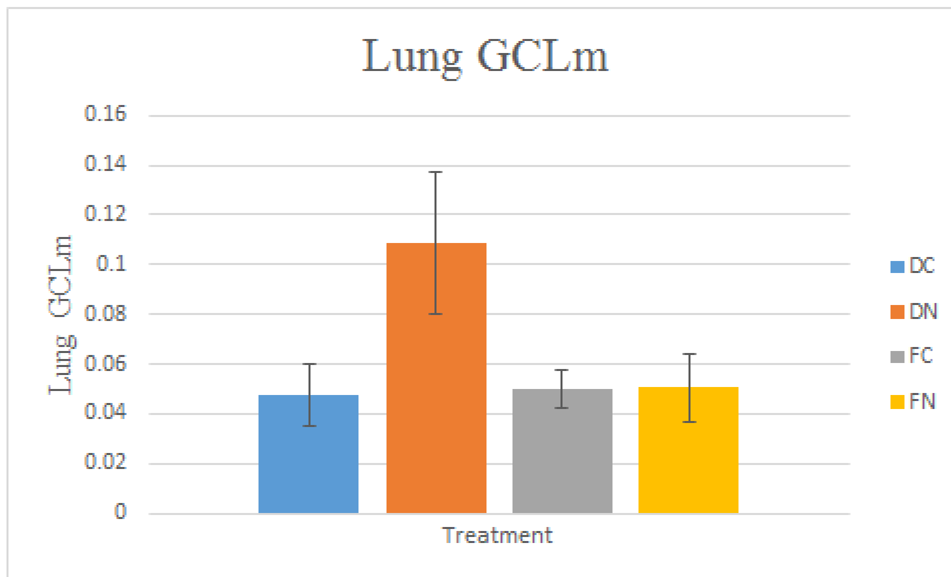

Figure 17. *Real-time PCR assessment of mRNA levels for lung GCLm collected at 16 weeks, and normalized to GAPDH. Error bars represent SEM. Tissue samples from randomly selected male and female mice within each treatment group were analyzed together (n=24; FCn=6, FNn=6, DCn=6, DNn=6). No significant differences (p<0.05) in treatment groups from the FC group were observed.*

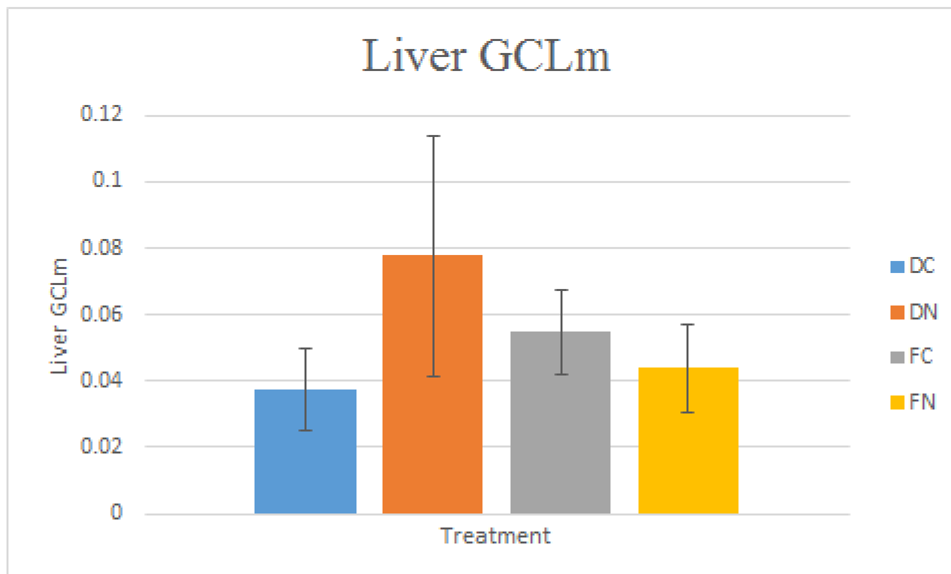

Figure 18. *Real-time PCR assessment of mRNA levels for liver GCLm collectedat 16 weeks, and normalized to GAPDH. Error bars represent SEM. Tissue samples from randomly selected male and female mice within each treatment group were analyzed together (n=24; FCn=6, FNn=6, DCn=6, DNn=6). No significant differences (p<0.05) in treatment groups from the FC group were observed.*

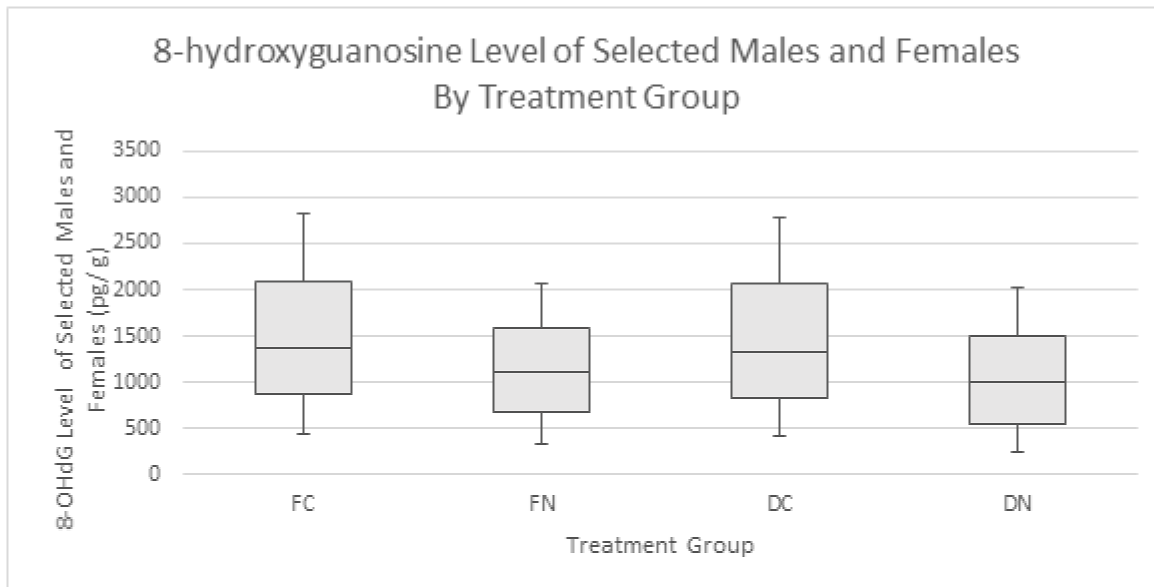

Figure 19. *ELISA assessment of liver 8-OHdG in randomly selected mice from each treatment and sex. Boxes representing median and interquartile range. Bars represent the range of values. Liver samples from randomly selected male and female mice (3 males and 3 females from each treatment group) were analyzed together (n=24). No significant difference (p<0.05) in treatment groups from the FC group were observed.*

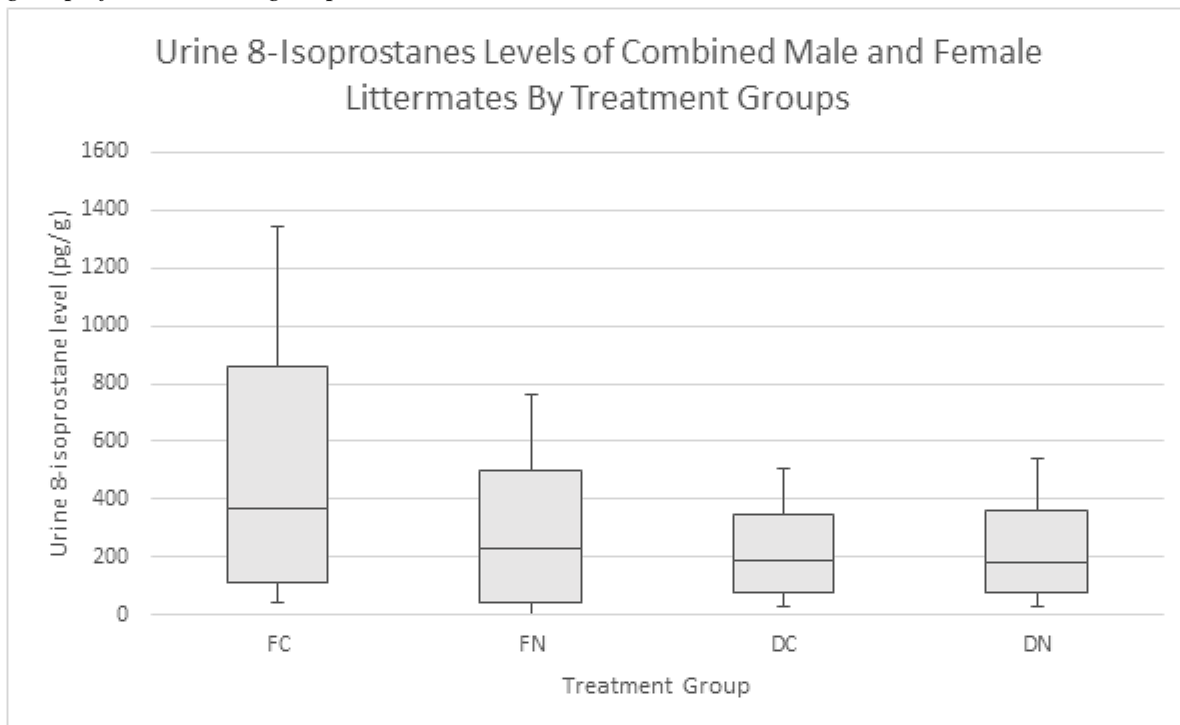

Figure 20. *ELISA assessment of pooled urine 8-isoprostane levels from randomly selected litters from each treatment and sex. Boxes representing median and interquartile range. Bars represent the range of values. Pooled urine samples from females and pooled urine from males of*

randomly selected litters of each treatment group was used for analysis (n=24). No significant difference (p<0.05) in treatment groups from the FC group were observed.

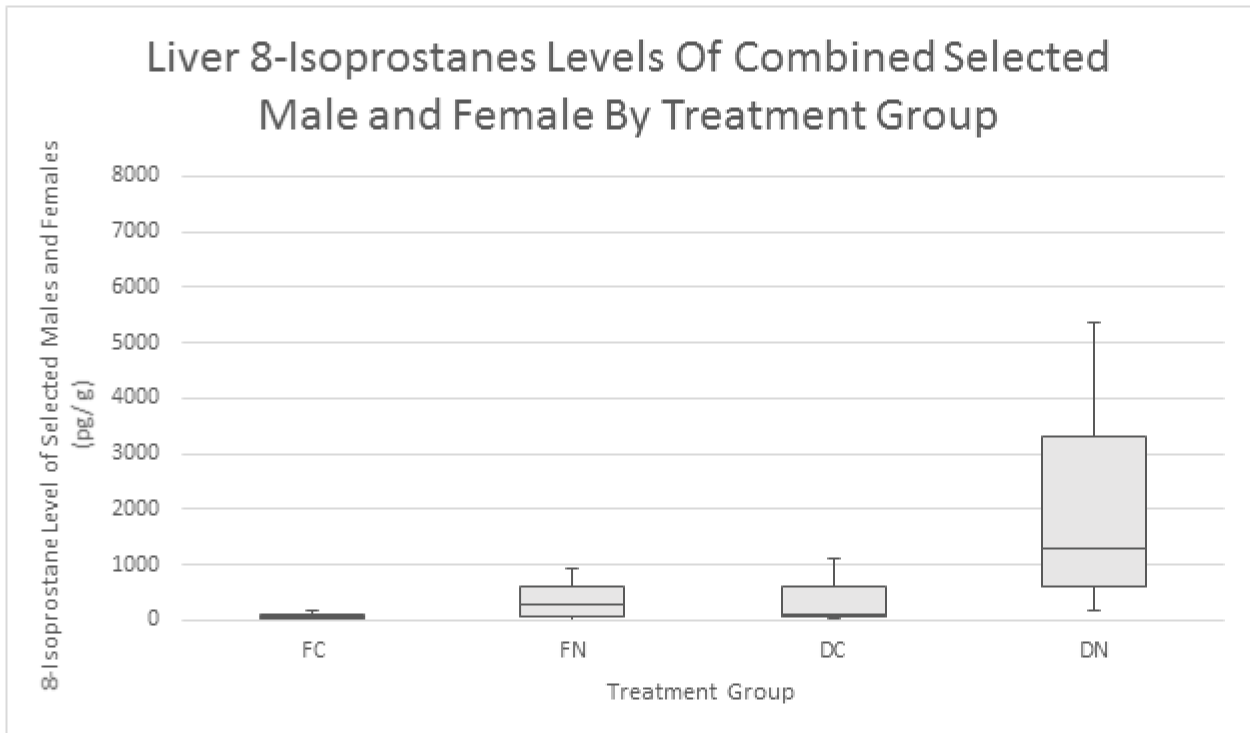

***Figure 21**. ELISA assessment of liver 8-isoprostane levels in randomly selected mice from each treatment and sex. Boxes representing median and interquartile range. Bars represent the range of values. Liver samples from randomly selected male and female mice combined for analysis in each treatment group (n=12). No significant difference (p<0.05) in treatment groups from the FC group were observed.*

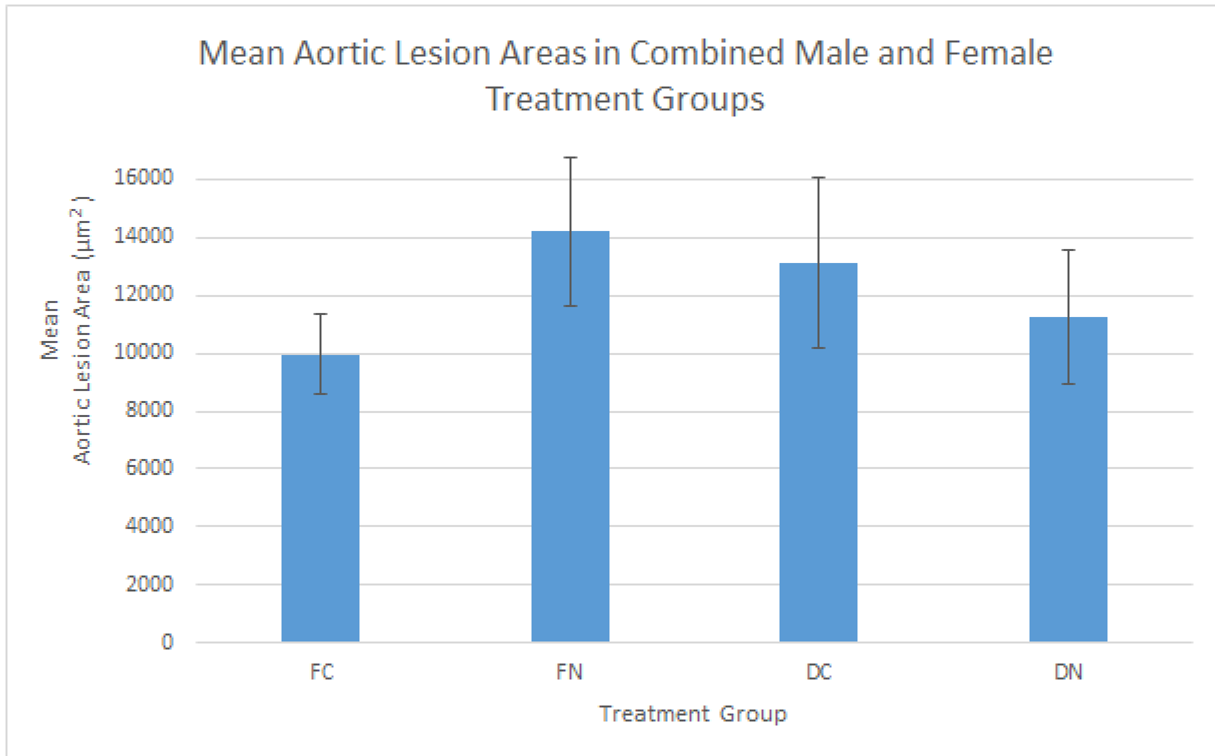

Figure 22. *Average aortic lesion area of combined male and female mice in each treatment group. Mean lesion size from each animal used for analysis. Bars represent Mean ± SE. (n= 130; FCn= 45, FNn= 33, DCn= 25, DNn= 26). No significant difference (p<0.05) in treatment groups from the FC group were observed.*

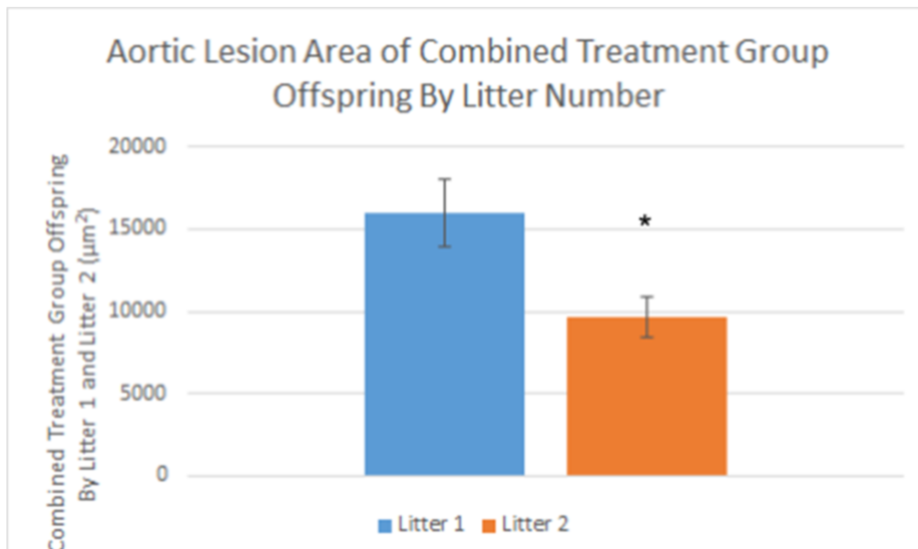

Figure 23. *Distribution of average lesion areas in first maternal exposure and second maternal exposure (second litter offspring) combined treatment group offspring. Mean lesion size from each animal used for analysis (Generation 1 n= 56; Generation 2 n= 60). *= Statistically*

significant difference between combined generation 1 offspring lesion areas and combined generation 2 offspring lesion areas (p< 0.05).

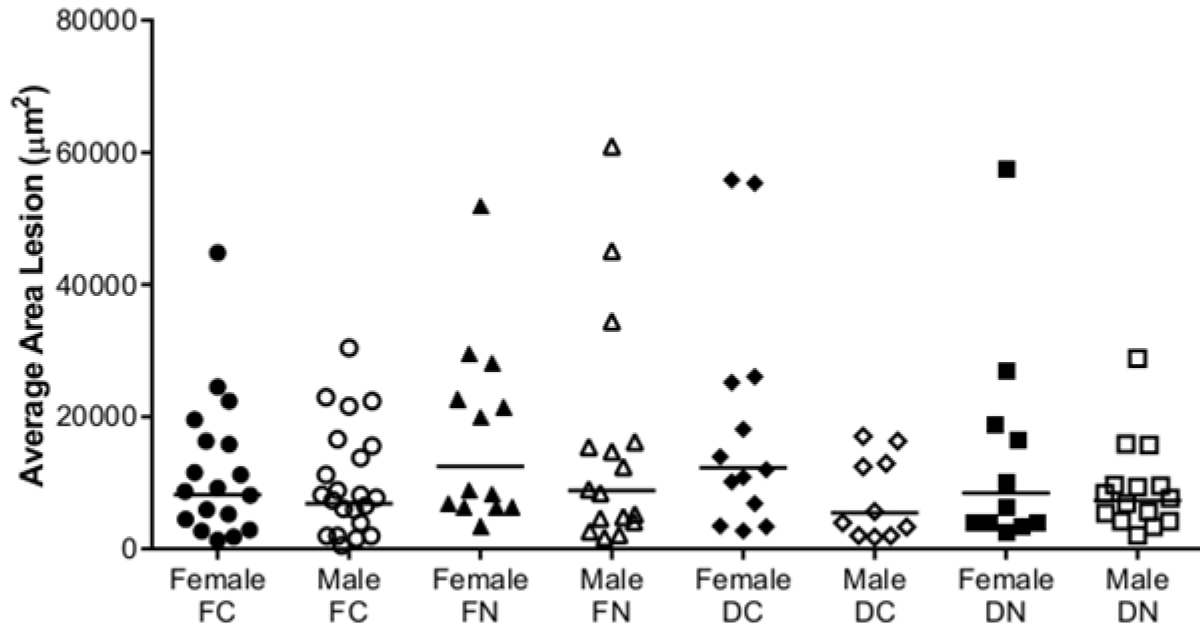

Figure 24. *Range of average aortic lesion area values from each male and female offspring analyzed in each treatment group. Horizontal bars represent treatment and sex group means. No significant difference (p<0.05) in treatment groups from the FC group were observed.*

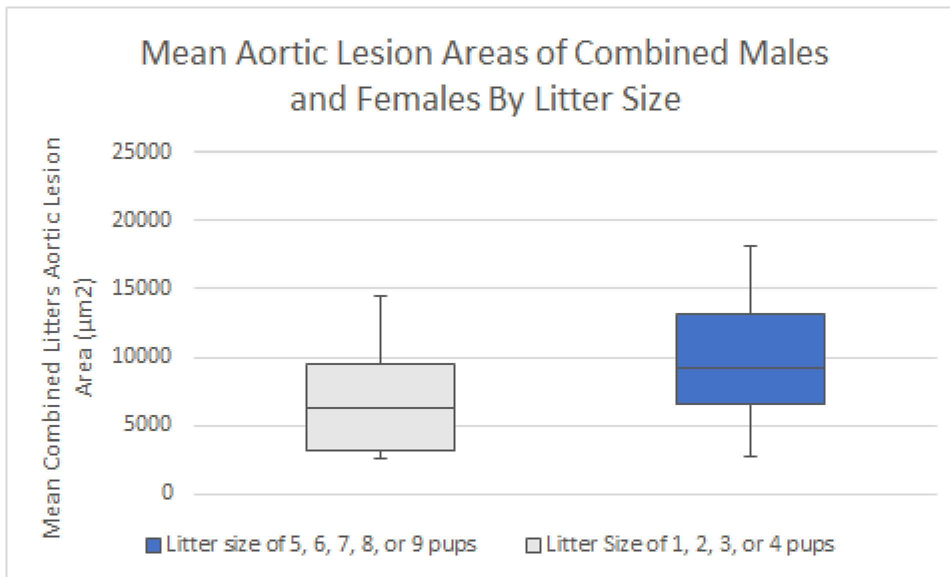

Figure 25*. Boxplots of mean lesion areas in combined litters of less than 5 pups and combined litters of 5 or more pups. Mean lesion size from each animal used for analysis.(Litter size <5*

pups= 6 litters; Litter size 5- 9 pups= 16 litters). There was no statistically significant difference between combined <5 pups litters and 5+ pups litters lesion areas (p< 0.05).

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