EFFECTS OF THE USE OF ASSISTED REPRODUCTION AND HIGH CALORIC DIET CONSUMPTION ON BODY WEIGHT AND CARDIOVASCULAR HEALTH OF JUVENILE MOUSE OFFSPRING

A Thesis

Presented to the Faculty of the Graduate School

At the University of Missouri

In Partial Fulfillment

Of the Requirement for the Degree

Master of Science

By

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DECEMBER 2013

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EFFECTS OF THE USE OF ASSISTED REPRODUCTION AND HIGH CALORIC DIET CONSUMPTION ON BODY WEIGHT AND CARDIOVASCULAR HEALTH OF JUVENILE MOUSE OFFSPRING

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ACKNOWLEDGEMENTS

There are many people I would like to thank for helping me out in one way or another during my past two years here. First and foremost, I would like to thank my two major professors Dr. Rocío Rivera and Dr. Luis Martínez-Lemus for their patience with me, as I came here with very little laboratory experience. I could not have made it through this thesis work without their constant support and belief in me. I would also like to thank to thank the other two memebers of my committee, Dr. Laura Schulz and Dr. Duane Keisler for their valuable input for this new project. I would further like to thank the Miller Fellowhsip Committee for giving me the support to learn new, valuable research skills. Also, thank you to my undergraduate professors for helping me have the courage to try the new experience of a research lab.

Next, I would like to thank the current and previous members of the lab who added so much positivity to my experience here. I would like to thank Chen for always being there, whether it was for homework help or simply for telling jokes; Matt for his patience with me when initially trying to train me with the pipette; and Verónica for offering her support to me at any and ALL times. Further, I need to thank our undergraduates Laura Moone and Jasmine Randle. While I did not get to know them for long, they added a fresh, fun feeling in the lab. Thank you to Francisco and Christopher in the Martínez-Lemus lab for always lending me a helping hand when I needed one. I would also like to thank the other graduate students, especially Holly, Dane, Liz and Katy for always making me feel at home here.

I would also like to thank my family, and my soon-to-be family for being there for me in any way possible during my studies. Thank you to my fiancé Matt for always

sending me his love and support, even from two hours away. A big thank you to my brother for giving me somewhere to go when I needed to be out of my apartment, and for allowing me to play with his kitties when I was missing mine from home. Last, but certainly not least, I want to thank my parents for their constant love, encouragement, and support no matter what the situation. I would truly not be where I am today without the help of all of the aforementioned people.

PREFACE

Obesity and Cardiovascular Disease (CVD) are two current epidemics faced globally. While the onset of these diseases can be contributed partly to a person's lifestyle, the mechanism behind them is still largely unknown. The Developmental Origins of Health and Disease Hypothesis (DOHaD) asserts that the environment in which a fetus develops can affect the subsequent adult disease state. This was discovered from the initial association between maternal undernutrition and the increased risk for CVD in the offspring.

Two suboptimal maternal environments, maternal obesity and the environment formed with the use of assisted reproductive technologies (ART), have also been associated with increased risk of CVD in children. In this thesis, I will mainly describe how the DOHaD hypothesis has emerged to include maternal obesity and ART due to their increasing prevalence. Obesity in women is a risk factor for infertility; therefore, many obese women are now using ART to conceive. Both of these suboptimal environments for fetal development have been associated with adverse metabolic and cardiovascular outcomes in the offspring, including high blood pressure, also known as hypertension.

One mechanism for the development of hypertension associated with CVD is inward remodeling of the vasculature. This is where the smooth muscle cells reorganize, forming a smaller lumen and creating more resistance to blood flow. Based on previous research in our lab, Matrix metalloproteinases (MMPs) involved in the degradation of the extracellular matrix (ECM) are suggested to be involved in this inward remodeling. The

activity of the MMPs can be increased by reactive oxygen species (ROS) derived from enzymes such as NADPH oxidase (NOX), and decreased by the endogenous tissue inhibitors of matrix metalloproteinases (TIMPs).

Studies have examined the effects of maternal obesity and ART separately on the subsequent disease state of the offspring, but data are lacking for the effect that the two environments have when they occur simultaneously. We hypothesize that obesity and ART independently and synergistically adversely affect the cardiovascular health and body weight of the offspring. We further hypothesize that these two suboptimal maternal environments will be associated with higher blood pressure in the offspring, and that this is mediated by the MMPs, TIMPs and NOXs.

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NOMENCLATURE

AA	aortic aneurysm
AAA	abdominal aortic aneurysm
ACE	angiotensin converting enzyme
ADA	American Diabetes Association
ANGII	angiotensin II
ART	Assisted Reproductive Technologies
BMI	body mass index
BSA	bovine serum albumin
CDC	Centers for Disease Control
CHR	Center for Human Reproduction
CVD	Cardiovascular disease
DOHaD	Developmental Origins of Health and Disease
eCG	equine chorionic gonadotropin
ECM	extracellular matrix
FOAD	Fetal Origins of Adult Disease
FSH	follicle stimulating hormone

GnRH	gonadotropin releasing hormone
H_2O_2	hydrogen peroxide
HF	high fat (includes high fructose in Ch III)
hMG	human menopausal gonadotropin
ICSI	intracytoplasmic sperm injection
IDF	International Diabetes Foundation
IL-6	interleukin-6
IVF	*in vitro* fertilization
IU	international units
LGA	large for gestational age
LH	luteinizing hormone
LOI	loss-of-imprinting
MAP	mean arterial pressure
MII	metaphase II
MMP1/2/7/8/9	matrix metalloproteinase 1/2/7/8/9
MS	Metabolic Syndrome
NCBI	National Center for Biotechnology Information

NCI	National Cancer Institute
NE	norepinephrine
NEFA	nonesterified fatty acid
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NOX	NADPH oxidase
NOX2	NADPH oxidase 2
O_2^-	superoxide
PPARα	peroxisome proliferator-responsive element α
PUFA	polyunsaturated fatty acid
RAS	renin-angiotensin system
ROS	reactive oxygen species
SART	Society for Assisted Reproductive Technologies
SBP	systolic blood pressure
SO	superovulation
SOD	superoxide dismutase
T2DM	Type II Diabetes Mellitus
TIMPs 1-4	tissue inhibitor of matrix metalloproteinase 1-4

WHF	World Heart Federation
WHO	World Health Organization

CHAPTER 1

Introduction of Cardiovascular, Metabolic and Reproduction Terms

This section aims to orient the reader to some of the cardiovascular, metabolic and reproduction terms that are helpful in fully understanding the variety of topics presented in this thesis. Definitions are listed *verbatim* or nearly *verbatim* and the proper source is accredited.

1.1 Cardiovascular Terms

Cardiovascular Disease- Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels and they include:

- coronary heart disease – disease of the blood vessels supplying the heart muscle
- cerebrovascular disease - disease of the blood vessels supplying the brain
- peripheral arterial disease – disease of blood vessels supplying the arms and legs
- rheumatic heart disease – damage to the heart muscle and heart valves from rheumatic fever, caused by streptococcal bacteria
- congenital heart disease - malformations of heart structure existing at birth
- deep vein thrombosis and pulmonary embolism – blood clots in the leg veins, which can dislodge and move to the heart and lungs.

(WHO 2013b)

Atherosclerosis- the process in which deposits of fatty substances, cholesterol, cellular waste products, calcium and other substances build up in the inner lining of an artery (AHA 2013).

Aortic abdominal aneurysm- An aortic aneurysm (AA) is a ballooning or dilatation of the aorta, the large artery that carries blood from the heart through the chest and abdomen. AAs are classified according to their location; in the chest, it is called a thoracic AA, in the abdomen an abdominal AA (AAA), and across both areas a

thoracoabdominal AA [see **Fig. 1.1** for thoracic aorta and abdominal aorta; (CDC 2011)].

Mean Arterial Pressure (MAP)- MAP, the product of cardiac output (CO) and total peripheral resistance (TPR), is the time-averaged blood pressure within the arterial circuit, as well as the average pressure in the arteries throughout the cardiac cycle (Henry *et al.* 2002).

Systolic Blood Pressure (SBP)- In a blood pressure measurement (*i.e.* 120/80), the upper number ("120") is the systolic blood pressure, which is the highest pressure in blood vessels and happens when the heart contracts, or beats. Normal blood pressure is 120/80 (WHO 2013d).

Diastolic Blood Pressure (DBP)- The lower number ("80") is the diastolic blood pressure, which is the lowest pressure in blood vessels in between heartbeats when the heart muscle relaxes (WHO 2013d).

Hypertension- a systolic blood pressure equal to or above 140 mm Hg and/or diastolic blood pressure equal to or above 90 mm Hg (WHO 2013d).

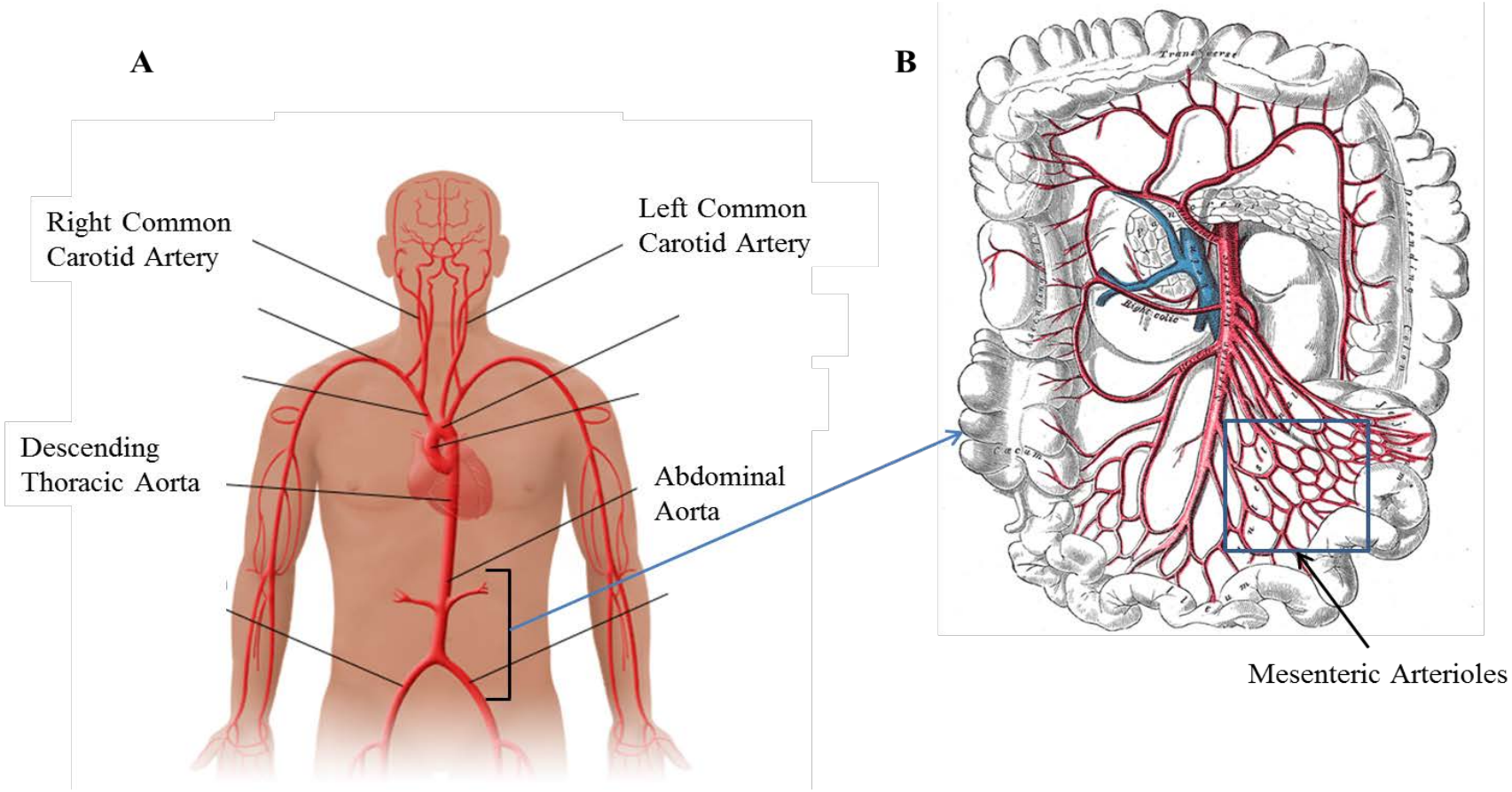


Figure 1.1 Diagram of vasculature commonly mentioned in the thesis. The image of the human (A) was modified from (Mayer 2013). It shows the left and right common carotid arteries, the abdominal aorta and the thoracic aorta. The image of the large intestine (B) was modified from (Reminetskyy) and is used to display the mesenteric arterioles.

Renin-Angiotensin System (RAS)- a peptidergic system with endocrine characteristics. The substrate of the system, angiotensinogen, an α-glycoprotein, is released from the liver and is cleaved in the circulation by the enzyme renin that is secreted from the juxtaglomerular apparatus of the kidney to form the decapeptide angiotensin (ANG) I. **ANG I** is then activated to the octapeptide **ANG II** by angiotensin converting enzyme (**ACE**), a membrane-bound metalloproteinase, which is predominantly expressed in high concentrations on the surface of endothelial cells in the pulmonary circulation. ANG II, considered the main effector peptide of the RAS, then acts on specific receptors (Paul *et al.* 2006).

Inward eutrophic remodeling- inward, eutrophic remodeling of arterioles does not involve cellular proliferation and thus it is due to rearrangement of the same vascular smooth muscle cells (VSMCs) around a smaller lumen (Prewitt *et al.* 2002)

Endothelial Dysfunction- The term endothelial dysfunction has been used to refer to several pathological conditions, including altered anticoagulant and anti-inflammatory properties of the endothelium, impaired modulation of vascular growth, and dysregulation of vascular remodeling. However, in much of the literature this term has been used to refer to an impairment of endothelium-dependent vasorelaxation caused by a loss of nitric oxide (NO) bioactivity in the vessel wall (Cai & Harrison 2000).

Coronary Heart Disease/Coronary Artery Disease/Ischemic Heart Disease- Disease of the blood vessels supplying the heart muscle (WHO 2013a).

Extracellular Matrix- The extracellular matrix (ECM) is the noncellular component present within all tissues and organs, and provides not only essential physical scaffolding

for the cellular constituents but also initiates crucial biochemical and biomechanical cues that are required for tissue morphogenesis, differentiation and homeostasis (Frantz *et al.* 2010).

1.2 Metabolic Terms

Macrosomia- Macrosomia is a term mostly used for newborns with a birthweight in weight units above a certain limit. However, there is no general agreement what this limit should be. Human birth weights above 4,000, 4,200 and 4,500 g are being used as definitions of newborn macrosomia (Henriksen 2008).

Body mass index (BMI)- Body Mass Index (BMI) is a simple index in humans of weight-for-height that is commonly used to classify underweight, overweight and obesity in adults. It is defined as the weight in kilograms divided by the square of the height in metres [kg/m^2; (WHO 2006)].

Overweight and Obesity- Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health. The WHO definition is:

- a BMI greater than or equal to 25 is overweight
- a BMI greater than or equal to 30 is obesity.

(WHO 2013e)

Morbidly obese is a BMI greater than or equal to 40 (Dokras *et al.* 2006).

Type II Diabetes Mellitus- This form of diabetes, which accounts for ~90-95% of those with diabetes, previously referred to as non-insulin-dependent diabetes, type 2 diabetes, or adult-onset diabetes, encompasses individuals who have insulin resistance and usually have relative (rather than absolute) insulin deficiency (ADA 2013).

Glucose Intolerance (impaired glucose tolerance)- Impaired glucose tolerance (IGT), along with impaired fasting glucose (IFG), is recognised as being a stage before diabetes when blood glucose levels are higher than normal (IDF 2013).

Hyperinsulinemia- Hyperinsulinemia is defined in the presence of fasting insulin levels greater than 30 micro units (µU)/ml or when the sum of insulinemic values at the second and third hours of the glycemic curve is greater than 60 µU/ml (Lavinsky *et al.* 2004).

Hyperleptinemia- elevated plasma leptin levels (Ren 2004).

Metabolic Syndrome- There is no internationally agreed definition for the Metabolic Syndrome. The following, which does not imply causal relationships, is suggested as a working definition: glucose intolerance, or diabetes mellitus and/or insulin resistance together with two or more of the other components listed below.

1. Impaired glucose regulation or diabetes

2. Insulin resistance (under hyperinsulinaemic euglycaemic conditions, glucose uptake below lowest quartile for background population under investigation)

3. Raised arterial pressure ≥160/90 mmHg

4. Raised plasma triglycerides (≥1.7 mmol l^{-1}; 150 mg dl^{-1}) and/or low HDL-cholesterol (<0.9 mmol l^{-1}, 35 mg dl^{-1} men; <1.0 mmol l^{-1}, 39mg dl^{-1} women)

5. Central obesity (males: waist to hip ratio >0.90; females: waist to hip ratio >0.85) and/or BMI >30 kg m^{-2}

6. Microalbuminuria (urinary albumin excretion rate ≥20 mg min^{-1} or albumin:creatinine ratio ≥20 mg g^{-1})

(Alberti & Zimmet 1998)

Gestational Diabetes Mellitus- any degree of glucose intolerance with onset or first recognition during pregnancy (ADA 2013).

1.3 Reproduction Terms

Follicle Stimulating Hormone (FSH)- FSH (follicle stimulating hormone) is a hormone released by the pituitary gland which stimulates the growth of follicles and has a role in the maturation of oocytes (Gleicher 2013).

Luteinizing Hormone (LH)- LH is produced by gonadotropic cells in the anterior pituitary gland. In females, a sharp rise in LH triggers ovulation and development of the corpus luteum (Choi *et al.* 2013).

Anovulation- Disorders of anovulation account for about 30% of infertility and often present with irregular periods (oligomenorrhea) or an absence of periods [amenorrhea; (Katsikis *et al.* 2006)].

Preeclampsia- Preeclampsia refers to a syndrome characterized by the new onset of hypertension and proteinuria after 20 weeks of gestation in a previously normotensive woman (Ghulmiyyah & Sibai 2012).

Syncytiotrophoblast- The syncytiotrophoblasts are a continuous, specialized layer of uterine epithelial cells. They cover the entire surface of villous trees and are in direct contact with maternal blood (Wang 2010).

Assisted Reproductive Technologies (ART)- ART includes all fertility treatments in which both eggs and sperm are handled. In general, ART procedures involve surgically removing eggs from a woman's ovaries, combining them with sperm in the laboratory, and returning them to the woman's body or donating them to another woman (CDC 2012).

Female Infertility- In general, infertility is defined as not being able to get pregnant (conceive) after one year of unprotected sex (CDC 2013).

***In vitro* fertilization (IVF)**- IVF involves extracting a woman's eggs, fertilizing the eggs in the laboratory, and then transferring the resulting embryos into the woman's uterus through the cervix (CDC 2012).

Intracytoplasmic sperm injection (ICSI)- A procedure in which a single sperm is injected directly into an egg; this procedure is commonly used to overcome male infertility problems (CDC 2012).

Embryo transfer- Placement of embryos into a woman's uterus through the cervix after IVF (CDC 2012).

Ovarian Stimulation-The use of drugs (oral or injected) to stimulate the ovaries to develop follicles and eggs (CDC 2012).

CHAPTER 2

Literature Review

2.1 General Introduction

CVD is the leading cause of death both in the United States and world-wide. According to World Health Organization (WHO) data, it is estimated that CVD will account for as many as 23.3 million deaths annually by 2030. Despite the increasing prevalence of the disease, the mechanism behind the development of CVD is largely unknown.

Low birth weight can be attributed to poor maternal nutrition and has been associated with an increased risk for CVD. Therefore, both human and animal models of maternal undernutrition have been used to examine the effect of maternal nutrition on offspring CVD. The hypothesis that the environment an individual is exposed to while *in utero* can affect the individual's subsequent disease state in adulthood became known as the Fetal Origins of Adult Disease (FOAD), and more recently renamed the Developmental Origins of Health and Disease (DOHaD) hypothesis.

While originally the DOHaD hypothesis was used to describe the effects of maternal undernutrition on offspring, it now encompasses other suboptimal maternal environments including maternal obesity and overweight. Obesity is an epidemic both in the United States and world-wide. Over 300 million women and 200 million men in 2008 were considered obese by the World Health Organization. Further, over 64% of women of reproductive age in the United States are overweight. Assisted reproductive technologies (ART) also provide an altered environment for fetal development. ART is

used to overcome infertility which affects approximately 10 percent of women ages 15-44 in the United States. While most children conceived with the use of ART are healthy, both maternal obesity and the use of ART have been associated with adverse outcomes in the children such as increased peripheral body fat, lowered glucose tolerance and increased systolic blood pressure associated with hypertension.

A proposed mechanism of hypertension involves inward remodeling of the vasculature, resulting in increased resistance to blood flow. Previous work in our laboratory has suggested that this inward remodeling is a result of increased activity of matrix metalloproteinases (MMPs). Both obesity and the use of ART can result in increased oxidative stress. Oxidative stress has been associated with up regulated production of reactive oxygen species (ROS) by NADPH Oxidase (NOX) which can result in increased activity of the MMPs. MMP activity can be decreased by the tissue inhibitors of matrix metalloproteinases (TIMPs).

Women who are obese have an increased risk for infertility, leading many of these women to use ART in order to conceive. There are many studies that examine the effects of these two environments alone, but knowledge is lacking on what effect these two maternal environments have on the disease state of the offspring when used in combination. For my thesis research, I studied the effects of these two suboptimal maternal environments on body weight and blood pressure in mouse offspring and sought to determine whether a mechanism involving the MMPs, TIMPs and NOX-derived ROS is involved in increased offspring blood pressure.

This literature review will provide background information that is helpful for fully understanding the information presented in the research presented in Chapter 3. The topics covered include CVD, the DOHaD hypothesis, and current knowledge about the effects of maternal overweight/obesity and ART on offspring development and subsequent disease state.

Note: The standards about the use of capital and lower case letters when writing about a gene, RNA or protein can vary between species and between journals (*i.e. MMP2* vs. *Mmp2* for RNA in humans and mice, respectively). The research conducted as part of this thesis was prepared for submission to the journal *Reproduction*. For simplicity, the gene and protein nomenclature will be presented in the format required by *Reproduction* throughout the entire thesis. *Reproduction* requires that all genes/DNA/RNA be italicized and in all capital letters for humans, non-human primates and domestic species (*i.e. MMP2*) and with only the first letter capitalized in mice and rodents (*i.e. Mmp2*). Protein nomenclature for all of the above-mentioned species is the same, with all letters capitalized and no italics (*i.e.* MMP2). If I am referring to the group as a whole (*i.e.* the matrix metalloproteinases), I will list it in all capital letters (*i.e.* the MMPs).

2.2 Cardiovascular Disease

a) Introduction to Cardiovascular Disease

The number one cause of death world-wide is Cardiovascular Disease (CVD), which accounted for nearly 30% of deaths in 2008 (WHO 2013b). Most of the deaths from CVD occur in low- and middle- income countries. The incidences of CVD are

becoming more common and it is estimated that CVD will globally account for 23.3 million deaths per year by 2030 (WHO 2013b). Obesity and high blood pressure are two common risk factors for CVDs. Some common forms of CVDs include coronary, peripheral, and cerebrovascular diseases of the vessels supplying the heart, arms and legs, and brain; respectively (WHO 2013b). Heart attack and stroke are two other acute forms of CVD which occur most commonly due to lipid buildup in the arteries (WHO 2013b). CVD in children also includes congenital heart disease, which is present from birth (WHF 2012). The global epidemic of obesity, which will be discussed later, has also led to an increase in the prevalence of cardiovascular risk factors in children with the potential to lead to premature CVD (WHF 2012).

b) Introduction to the Cardiovascular System

To understand CVD and its pathologies, one must first understand how the cardiovascular system functions physiologically. While the main focus of this section will be on the arteries, which carry blood away from the heart to supply nutrients to the tissues, I will first briefly mention the cardiovascular system as a whole. The cardiovascular system is also known as the circulatory system due to its role of circulating blood throughout the body. The system consists of the heart and the vasculature, including the arteries, veins, capillaries and lymphatics. Blood is transported by the arteries to the tissues of the body to supply oxygen and other nutrients, and the veins transport deoxygenated blood back to the heart and then the lungs to become reoxygenated (Barrett 2012). The capillaries are where the nutrient exchange occurs in the tissues and the lungs (NCI 2012). The amount of nutrients that reach the tissues depends both on the functionality of the heart and of the vasculature. Blood flow

is directed with each heart beat by a pressure gradient from the large blood vessels near the heart to the smaller peripheral vessels (London and Pannier, 2010).

The arterial system consists of both macrovasculature including the aorta and the large arteries, and the microvasculature which includes the small arteries, arterioles and capillaries (Barrett 2012). The aorta and larger arteries near the heart contain large amounts of elastin and are distensible to accommodate large amounts of blood with each pump of the heart (Hendry *et al.* 2012). The macrovasculature can hypertrophy and become stiffer and more resistant to flow (Briet & Schiffrin 2013). This has been associated with increased risk for death due to cardiovascular events (Vlachopoulos *et al.* 2010). However, the main focus of this review will be on the microvasculature which is the primary source of resistance to blood flow (Barrett 2012).

The small arteries and arterioles provide most of the resistance in the cardiovascular system, and they are where the main structural changes attributed to increased peripheral resistance occur (Mulvany & Aalkjaer 1990). Microvasculature includes the blood vessels with diameters smaller than 150µM, and its main function is to transport nutrients and oxygen in accordance to the demand of the tissues (Clough and Norman, 2011). From the outermost to the innermost layers, the arteries include the adventitia made of connective tissue, the media composed of smooth muscle and the intima which includes the endothelium and connective tissue (Ross & Glomset 1976). The microvasculature has more smooth muscle in its walls, and is capable of a larger change in diameter than the macrovasculature (Hendry *et al.* 2012).

One of the contributing factors to the amount of blood and nutrients that reach the tissues is total peripheral resistance. Peripheral resistance is determined largely by the diameter of the blood vessels (Hendry *et al.* 2012). The diameter of the vasculature can change through a narrowing (vasoconstriction) or an increase in the diameter of the blood vessel [vasodilation; (Barrett 2012)].

Vasoconstriction and vasodilation can occur due to various stimuli including mechanical stretch, sympathetic nerve activities, neurohormonal agents and endothelial-derived products. Stretch due to increased blood pressure can be sensed by baroreceptors which respond by decreasing sympathetic nerve activity, resulting in vasodilation (Green & Heffron 1968). Norepinephrine (NE) and Angiotensin II (ANGII) are two neurohormonal agents that are potent vasoconstrictors. An endothelial product that can increase the diameter of the blood vessels is nitric oxide (NO), which is a potent vasodilator (Barrett 2012). In physiological conditions, vasoconstriction and vasodilation create a blood supply to the tissues that is nearly equivalent to that tissue's oxygen demand (Saltin *et al.* 1998). However, vasoconstriction and vasodilation can also become pathophysiological as will be discussed later.

c) Hypertension and Cardiovascular Disease

Systolic blood pressure (SBP; the maximum pressure in the arteries during contraction of the heart) above 140 mmHg and diastolic blood pressure (the maximum pressure in the arteries during relaxation of the heart) above 90 mmHg is considered hypertension (WHO 1999). Over 16% of deaths each year are attributed to hypertension (WHO 2013b). Cardiovascular complications due to the effects of hypertension are vast

and include aneurysms, hypertrophy of the left ventricle, hemorrhage and renal failure (Hollander 1976). Lowering blood pressure in hypertensive patients has been associated with decreased morbid cardiovascular outcomes (Taguchi & Freis 1975, Czernichow *et al.* 2011).

Hypertension has been shown to increase the rate of atherosclerosis (thickening of vessel walls due to fatty deposits) development, especially in the macrovasculature (Robertson & Strong 1968). This has been demonstrated in a model of non-human primates where hypertension accelerated the development of atherosclerosis when compared to primates without hypertension (Hollander *et al.* 1993). Hypertension can also result in hypertensive vascular disease, which creates a narrowing of the lumen in the arteries (Hollander 1976). The lesions formed in hypertensive vascular disease are different than those found in atherosclerosis because these lesions are formed without the presence of excess lipids (Hollander *et al.* 1968). Instead, they are associated with increased mucopolysaccharides, electrolytes and water in the media of the vessels.

Increase of vascular resistance, or the resistance that the vasculature provides against the flow of blood, in all major organ systems is one of the defining characteristics in hypertension (Heagerty *et al.* 1993). Many mechanisms have been proposed to be involved in the development of hypertension. One such mechanism involves the renin-angiotensin system (RAS) where a study revealed that blocking the conversion of Angiotensin I to the potent vasoconstrictor ANGII by Angiotensin Converting Enzyme (ACE) lowered blood pressure in spontaneously hypertensive rats (Antonaccio *et al.* 1979). Blood pressure reduction by inhibition of the RAS has also been observed in humans (Herrera-Acosta *et al.* 1985, Ajayi *et al.* 1986).

Mean arterial pressure (MAP) is determined by cardiac output and total peripheral resistance, so increasing total peripheral resistance can cause an increase in MAP (London & Pannier 2010). As mentioned above, the arterioles are the main resistance vessels, so they are a main contributor to the total peripheral resistance. Increased peripheral resistance and a diminished vasodilation response to flow in the microcirculation has been associated with early hypertension (Schwartzkopff *et al.* 1993). Too much impedance to blood flow due to vascular resistance can result in pressure build-up towards the heart, forcing the heart to work harder with each pump (London and Pannier, 2010). Hypertension has been associated with a decreased vasodilatory response to acetylcholine, showing a decrease in endothelial function (Rizzoni *et al.* 1996). The impairment of vasodilation in resistance vessels can also result in inward eutrophic remodeling (Pistea *et al.* 2005).

Inward eutrophic remodeling occurs when the already existing smooth muscle cells rearrange creating a smaller lumen (Heagerty *et al.* 1993, Rizzoni *et al.* 2003a). Models of hypertension have shown that the inward remodeling that occurs in the arterioles is eutrophic (Korsgaard *et al.* 1993, Rizzoni *et al.* 1996). That is, there is a reduction in the passive luminal diameter of the vessels without significant changes in the cross-sectional area, represented by the amount of material in the vascular wall [reviewed in (Mulvany 1999)]. The inward eutrophic remodeling of the arterioles has been associated with increased risk of life-threatening cardiovascular events such as stroke or myocardial infarction (Rizzoni *et al.* 2003b, Mathiassen *et al.* 2007). This inward remodeling could also be a result of increased RAS activity and sympathetic nerve

activation, as prolonged exposure to NE+ANGII has been shown to result in inward remodeling in arterioles (Martinez-Lemus *et al.* 2011).

d) Vascular Development

The cardiovascular system is the first physiological system to develop in the fetus, so it is prone to damage from early insults in the maternal environment (Clough and Norman, 2011). The placenta is responsible for delivering nutrients to and removing wastes from the fetus [reviewed in (Maltepe *et al.* 2010)]. Angiogenesis, or the formation of new blood vessels, is an important process during placentation allowing for rapid growth of the developing fetus (Maltepe *et al.* 2010). Once implantation occurs, blood flow to the placenta is established by remodeling of the maternal spiral arteries [reviewed in (Red-Horse *et al.* 2004)]. The spiral arteries provide blood to the placental intervillous space starting at about 12 weeks of human pregnancy (Burton *et al.* 1999). Due to the delayed maternal blood flow, the fetus and placenta develop in a low oxygen environment during the first trimester of pregnancy (Watson *et al.* 1998).

The placental vasculature is not innervated, therefore its tone depends on the release of vasodilators and vasoconstrictors (Fox & Khong 1990). The tone of the vasculature in the placenta can be controlled by stimuli such as the RAS and the potent vasodilator NO (Wang 2010). The fetal vascular bed in the placenta has low vascular resistance, allowing for high blood flow to the developing fetus (Trudinger *et al.* 1985). After birth the infant's vascular resistance rises to regulate the blood flow as in adults (Barrett 2012). In the placenta, the arteries stem from the fetus carrying deoxygenated blood to a mass of capillary loops associated with the syncytiotrophoblast which connects

the fetus to the mother. This is where the blood becomes oxygenated before returning to the fetus through a vein within the umbilicus (Wang 2010). The blood flow provides the source of nutrients for the developing fetus, so an impairment in the blood flow in the placenta (*i.e.* decreased vasodilation due to decreased NO availability) can adversely affect fetal development (Wang 2010). The RAS is also present in the placenta, and higher placental ANGII receptor AT1R levels have been found in pregnancies complicated by preeclampsia (Mistry *et al.* 2013).

2.3 Possible Genes Involved in Poor Cardiovascular Health and Hypertension Development

a) Introduction to the Proposed Mechanism of Hypertension

It has previously been shown that inward eutrophic remodeling occurs in hypertension (Korsgaard *et al.* 1993, Rizzoni *et al.* 2003b). Also, it has recently been proposed that the development of hypertension can be mediated by vascular remodeling induced by oxidative stress and the increased activity of matrix metalloproteinases [MMPs; (Martinez-Lemus *et al.* 2011)]. This section will summarize how reactive oxygen species (ROS) and MMPs have been associated with poor cardiovascular health and how they might be involved in the vascular remodeling associated with hypertension.

b) Matrix Metalloproteinases

The MMPs are part of a family of zinc-dependent endopeptidases, and one source of their production is cells in the walls of blood vessels (Raffetto & Khalil 2008). MMPs

were first discovered by Gross and Lapiere in 1962 for their involvement in the dissolution of structures such as the tail and gills in tadpole morphogenesis (Gross & Lapiere 1962). The 23 MMPs found in humans are subdivided into five groups, namely the collagenases, gelatinases, matrilysins, stromelysins, and membrane-type MMPs. These groups are classified by their substrates and sequence homologies [reviewed in (Lindsey & Zamilpa 2012)]. MMP expression is usually low in most tissues, but expression can be upregulated in several ways, such as by inflammatory cytokines and growth factors (Nagase & Woessner 1999).

The MMPs are secreted as inactive pro-MMPs, and the inactive state is maintained by a cysteine residue found in the propeptide domain that ligates the catalytic zinc [*i.e.* the "cysteine switch"; (Van Wart & Birkedal-Hansen 1990)]. The cysteine switch refers to the ability of the MMP to be activated when the propeptide domain is removed and the cysteine residue no longer blocks the activity of the catalytic domain (Van Wart & Birkedal-Hansen 1990). The catalytic domain has a zinc binding motif and the structure is maintained by a structural zinc ion and calcium ions (Bode *et al.* 1993). Most MMPs also contain a *C*-terminal hemopexin-like domain which is required for the cleavage of triple helical collagen (Bode 1995). However, this hemopexin-like domain is absent in the matrilysin MMP7 (Gaire *et al.* 1994). Activation of MMPs occurs when the cysteine switch is disrupted and the pro-domain is removed [reviewed in (Nagase 1997)]. MMPs can be activated by proteinases (including other MMPs) as well as by sulfhydryl reactive agents like oxidized glutathione and by denaturants such as urea (Nagase 1997).

MMPs are best known for their role in the degradation of the extracellular matrix (ECM), but they also participate in the cleavage of extracellular receptors (*e.g.* the β2

adrenergic receptor) resulting in reduced vasodilation (Rodrigues *et al.* 2010), disruption of cellular tight junctions (Yang *et al.* 2007), and shedding of vasoactive factors associated with the ECM (*e.g.* heparin-binding epidermal growth factor) which is then able to interact with the epidermal growth factor receptor to promote vasoconstriction (Hao *et al.* 2004). They have physiological roles such as involvement in placentation (Onogi *et al.* 2011) and wound healing (Salo *et al.* 1994), but they have also been implicated in pathological conditions like cancer (Friedberg *et al.* 1998) and stroke (Cojocarui *et al.* 2012).

In the vasculature, MMPs are involved in vasoconstriction as well as reduction in vasodilation. MMPs can degrade the ECM allowing for movement of the smooth muscle cells during vascular remodeling (Martinez-Lemus & Galinanes 2011). They can also cleave β2 adrenergic receptors, resulting in decreased vasodilation (Rodrigues *et al.* 2010). This can induce hypertension which is associated with inward remodeling, so it has been proposed that the MMPs might be involved in inward eutrophic remodeling through their vasomotor activities (Martinez-Lemus & Galinanes 2011).

In support of this idea, it has been shown that the activity of MMPs in the plasma is elevated in hypertension (Friese *et al.* 2009). It has also been shown that inward remodeling induced by NE and ANGII (potent vasoconstrictors) was prevented when MMPs were inhibited (Martinez-Lemus *et al.* 2011). The MMPs that are of particular interest in the studies of hypertension are MMP2, MMP9, and MMP7, which will be discussed below.

Matrix Metalloproteinase 2

MMP2 is a type IV collagenase/gelatinase A that was first described in the 1970's (Sellers *et al.* 1978). The gene encoding MMP2 is found on chromosome 16 in humans (Visse & Nagase 2003) and chromosome 8 in mice (Becker-Follmann *et al.* 1997). MMP2 can break down both collagen and elastin in the vessel walls (Keeling *et al.* 2005), and is able to bind to collagen and gelatin due to an insert of three repeats of a type III fibronectin domain into its catalytic domain (Allan *et al.* 1995).

It has been suggested that MMP2 is involved in the NE and ANGII induced inward remodeling in rat cremaster arterioles (Martinez-Lemus *et al.* 2011). Further, Chesler *et al.* (1999) suggest that MMP2 and MMP9 (discussed below) are both involved in the early remodeling associated with hypertension in porcine arteries (Chesler *et al.* 1999). Interestingly, MMP2 can induce vasoconstriction by cleaving big endothelin-1 which is only able to produce vasoconstriction in its cleaved form (Fernandez-Patron *et al.* 1999), and has been shown to induce inward remodeling in isolated arterioles (Bakker et al., 2004). Higher levels of serum MMP2 and MMP9 in humans results in an increased risk for heart failure (Collier *et al.* 2011).

In children, increased circulating levels of MMP2 were associated with an increase in SBP (Sesso & Franco 2010). MMP2 is also elevated in individuals with hypertension and end-stage renal disease (Friese *et al.* 2009). Bergman *et al.* (2007) demonstrated a pathophysiological role of *Mmp2* in the heart of mice (Bergman *et al.* 2007). By creating a cardiac-specific active *Mmp2* with a mutation in the pro-domain, they showed that *Mmp2* can lead to systolic dysfunction and left ventricular remodeling.

Matrix Metalloproteinase 9

MMP9 is a 92kDa gelatinase, also known as gelatinase B. Activation of MMP9 occurs when its secreted zymogen form is cleaved to the 82kDa active form by peptidases, including other MMPs (Wilhelm *et al.* 1989). The gene encoding MMP9 is located on chromosome 20 in humans (Visse & Nagase 2003) and 2 in mice (Leco *et al.* 1997). MMP9 has a similar structure and function to MMP2, but it cannot digest type I , II or III collagens (Visse & Nagase 2003).

MMP9 is associated with outward remodeling in response to high intraluminal pressure (Lehoux *et al.* 2004) which is proposed to be a compensatory mechanism in order to increase vascular compliance (Martinez-Lemus & Galinanes 2011). However, aneurysms can result from distension of the blood vessel if the upregulation of *MMP9* is chronic (Keeling *et al.* 2005). This is demonstrated in a mouse model where knocking out *Mmp9* made the mice resistant to the formation of aneurysms (Longo *et al.* 2002). Also, it has been shown in humans that the active concentration of MMP9 in abdominal aortic aneurysm is positively associated with the thickness of the aneurysm (Khan *et al.* 2012).

Increased plasma MMP9 in children has been shown to be a predictor of vascular dysfunction (Sesso & Franco 2010). It has also been shown that higher plasma levels of MMP9 are associated with increased SBP (Friese *et al.* 2009). Increased blood pressure with MMP9 could be due to decreased vasodilation, as it has been demonstrated by Rodrigues *et al.* (2010) that injections of MMP9 and/or MMP7 (discussed below) into mesenteric venules in rats resulted in decreased arteriolar diameter (Rodrigues *et al.*

2010). A reduced arteriolar diameter in response to MMP9 seems contradictory because MMP9 has been associated with outward remodeling (Lehoux *et al.* 2004), but Rodrigues *et al.* (2010) propose that cleavage of the β2 adrenergic receptor by MMP9 and MMP7 in their study is responsible for the reduced vasodilation and arteriolar diameter (Rodrigues *et al.* 2010).

Matrix Metalloproteinase 7

MMP7 is 28kDa in its pro-enzyme form, and is cleaved to a 19kDa active form, and it is also known as matrilysin (Crabbe *et al.* 1992). MMP7 is the smallest MMP because it lacks a hinge region and *C*-terminal hemopexin-like domain (Gaire *et al.* 1994). This enzyme was first discovered in the postpartum rat uterus in 1980 (Sellers & Woessner 1980). The gene encoding MMP7 is located on chromosome 11 in humans (Visse & Nagase 2003) and on chromosome 9 in mice (Yamazaki *et al.* 1998).

In humans, elevated serum MMP7 has been associated with an increased risk of CVD (Tuomainen *et al.* 2012). Knockdown of *Mmp7* in a model of vasoconstrictor-induced hypertension in spontaneously hypertensive rats stops the development of cardiac hypertrophy and reduces the severity of hypertension (Wang *et al.* 2009). It was later shown that *Mmp7* is involved in upregulation of MMP2 in ANGII-induced hypertension in mice (Odenbach *et al.* 2011). In that study, MMP2 had a reduced ability to increase blood pressure when MMP7 was knocked down using a small interfering RNA. MMP7 has also been implicated in the development of atherosclerotic plaques by increasing vascular smooth muscle cell apoptosis (Williams *et al.* 2010).

c) Tissue Inhibitors of Matrix Metalloproteinases

The tissue inhibitors of MMPs (TIMPs) regulate the activities of the MMPs endogenously. Four TIMPs have been described in humans [*i.e.* TIMP 1-4; reviewed in (Nagase & Woessner 1999)]. In the TIMPs, disulfide bonds between the first cysteine and the 70th cysteine residues are critical for the inhibition of the MMPs by allowing binding of the TIMPs to the active site in the MMPs (Gomis-Ruth *et al.* 1997). TIMPs have low selectivity for the inhibition of the MMPs, and they inhibit the MMPs in a 1:1 ratio (Willenbrock & Murphy 1994). All TIMPs contain 12 cysteine residues that form six stable loops held together by disulphide bonds. The first three loops and the last three loops are found in the N- and C- terminal subdomains, respectively. The N- terminus is primarily responsible for the interaction between the TIMPs and the MMPs (Willenbrock & Murphy 1994). The size of the TIMPs ranges from 21 to 29 kDa (Murphy *et al.* 1991). While there are four TIMPs, I will focus on TIMP1 in this review because it is the one we used in our research.

Tissue Inhibitor of Matrix Metalloproteinase 1

TIMP1 can endogenously inhibit all of the MMPs aside from MMP14 (Will *et al.* 1996). The gene for TIMP1 is found on the X chromosome in humans (Anderson & Brown 2005) and in mice (Isensee *et al.* 2008). It has been shown that TIMP1 can form a complex with pro-MMP9 and control the rate of MMP9 activation (Itoh & Nagase 1995).

TIMP1 is elevated in the serum of people with an increased risk of CVD (Tuomainen *et al.* 2012). Infusion of *Timp1* in rat aortas has been shown to block the development of aortic aneurysms (Allaire *et al.* 1998). *Timp1* knockout mice also

develop larger aneurysms than control mice after aortic elastase infusion (Eskandari *et al.* 2005).

TIMP1 also has increased expression in Takayasu's arteritis (TA), an inflammatory disease found primarily in the aorta (Mahajan *et al.* 2012). Mice fed a high fat, high sugar "Western" diet had increased liver inflammation and expression of *Timp1* (Ishimoto *et al.* 2013). The balance between TIMPs and MMPs is controlled in physiological conditions, but the balance can be disrupted in pathophysiological conditions like inflammation and tumor progression [Reviewed in (Gomez *et al.* 1997)].

d) Reactive Oxygen Species and NADPH Oxidase

One way that MMP activity can be increased is by increased levels of ROS (Martinez-Lemus *et al.* 2011). Oxidative stress associated with increased levels of ROS can occur during pathophysiological conditions like CVD [reviewed extensively by (Montezano & Touyz 2013)]. As discussed below, NADPH Oxidase (NOX) derived ROS could provide a mechanism for the association between oxidative stress and CVD.

Reactive Oxygen Species

ROS are small molecules derived from oxygen that act as oxidizing agents such as superoxide (O_2^-), peroxyl and hydroxyl radicals, and hydrogen peroxide [H_2O_2; (Bedard & Krause 2007)]. O_2^- generation, where oxygen accepts one electron, is usually the first step in the production of ROS [**Fig. 2.1**; (Klebanoff 1980)]. O_2^- can then be converted to other radicals such as H_2O_2 by superoxide dismutase [**Fig. 2.1**; (Li *et al.* 2001)]. While ROS are important for physiological processes like cell migration and

proliferation, an over production of ROS can result in adverse outcomes like cell death, cancer, diabetes and CVD (Igosheva *et al.* 2010, Garrido-Urbani *et al.* 2011). NOX, which is the main enzyme responsible for ROS production in the vasculature will be discussed below, as well as how ROS produced by NOX have been associated with CVD.

NADPH Oxidase

NOX was first discovered as part of a respiratory burst in cells that had been described early in the 20th century (Baldridge 1932, MacLeod 1943), and it was then discovered that NOX was responsible for the production of O_2^- and H_2O_2 during this event (Rossi & Zatti 1964, Babior *et al.* 1973). NOX family members on the membrane of the mitochondria reduce oxygen into O_2^- by helping transport electrons across the membrane (Garrido-Urbani *et al.* 2011). All NOX enzymes have an NADPH binding site, a FAD-binding region, six transmembrane domains and four heme-binding histidines (Bedard & Krause 2007). FAD is involved in the regulation of electron transfer by NOX (Hashida *et al.* 2004).

NOX is composed of several subunits that act as an enzyme complex to produce O_2^-, which can then be converted to other forms of ROS (Bedard & Krause 2007). The first NOX discovered was gp91^{phox} [NOX2; (Royer-Pokora *et al.* 1986)], and several isoforms have been discovered since. The NOX family is now known to include seven isoforms with two subunits that contribute to organization of the complex (p47^{phox} and NOXO1), two subunits that help activate the complex (p67^{phox} and NOXA1) and

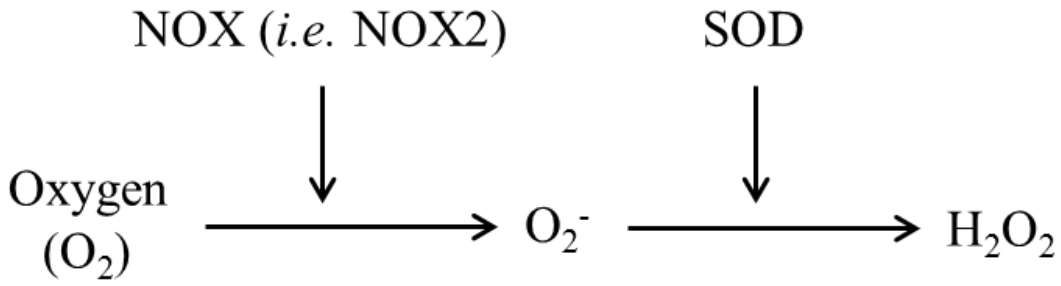


Figure 2.1 Production of superoxide and hydrogen peroxide from oxygen. The donation of an electron by NOX to turn O_2 into O_2^- is usually the first step in ROS production. O_2^- can then be converted to another ROS H_2O_2 by SOD. NOX= NADPH Oxidase. O_2^-= superoxide. SOD= superoxide dismutase. H_2O_2= hydrogen peroxide.

maturation factors for large members of the NOX family [DUOXA1 and DUOXA2; (Bedard & Krause 2007)]. NOX is the main producer of ROS in the vascular wall (Cai *et al.* 2003).

NADPH Oxidase 2

NOX2, also known as CYBB and $gp91^{phox}$, is a membrane-bound component of NADPH oxidase and is encoded by a gene located on the X-chromosome in humans (Bedard & Krause 2007) and mice (Chandra *et al.* 2011). NOX2 is heavily glycosylated (Harper *et al.* 1985). Glycosylation of NOX2 has been shown to increase the production of ROS (Cai *et al.* 2010).

The mechanism for NOX2 activation has been described by Bedard and Krause (Bedard & Krause 2007). NOX2 forms a complex with cytochrome b-245 light chain ($p22^{phox}$), which is translocated to the membrane by phosphorylated $p47^{phox}$. This brings $p40^{phox}$ (a small subunit) into the complex allows the "activator subunit" $p67^{phox}$ to interact with NOX2. The GTPase Rac then interacts with NOX2 followed by interaction with $p67^{phox}$, and completes the assembly and activation of the complex (Bedard & Krause 2007).

Nox2 gene expression can be regulated by the promoter region (Skalnik *et al.* 1991), and also by upstream repressing factors and activating factors such as nuclear factor kappa-light-chain-enhancer of activated B cells [NFκB; (Anrather *et al.* 2006)]. NOX2 *de novo* protein synthesis can also be induced in resistance arteries by ANG-II (Touyz *et al.* 2002). H_2O_2, which is a downstream product from O_2^- degradation, can act

in a feed-forward mechanism by further increasing the production of O_2^- by NOX2 (Li *et al.* 2001).

A role for NOX2 in several CVD states has been demonstrated in humans and in rodents. *Nox2* is elevated in myxomatous mitral valve disease, a disease associated with valve thickening and matrix remodeling in mice (Hagler *et al.* 2013). *NOX2* expression is also higher in aortic abdominal aneurysms in humans (Guzik *et al.* 2013). Women who are deficient in NOX2 display less vascular damage and atherosclerosis (Violi *et al.* 2013).

Sukumar *et al.* (2013) demonstrated in a mouse model that knocking down *Nox2* reduced O_2^- levels and improved vascular function in insulin-resistant endothelial cells (Sukumar *et al.* 2013). Another study revealed that mice without *Nox2* had less vascular dysfunction and reduced O_2^- levels after induced high pressure in cerebral arterioles when compared to wild-type mice (Chan & Baumbach 2013). Also, mice lacking *Nox2* develop ANGII-induced hypertension more slowly than those that have wild-type *Nox2* (Haque & Majid 2011).

e) Relationship between ROS and MMPs

Increased oxidation due to higher levels of ROS can activate the MMPs by the inhibition of the nuclear hormone receptor peroxisome proliferator-responsive element α [PPARα; (Garrido-Urbani *et al.* 2011)]. PPARα normally acts in an anti-angiogenic manner by inhibiting the NFκB pathway, involved in producing more MMPs. Therefore, when PPARα is inhibited by ROS more MMPs are able to be produced through the NFκB pathway, allowing for more remodeling (Garrido-Urbani *et al.* 2011). NFκB is also an

activating factor that induces greater production of ROS by NOX (Anrather *et al.* 2006). PPARα has also been shown to be downregulated with ANGII stimulation, resulting in increased expression of inflammatory cytokines in rat vascular smooth muscle cells (Ji *et al.* 2009).

Oxidative stress can increase the activity of several MMPs including MMP2 and MMP9 in both neonatal and adult cardiac fibroblasts (Siwik *et al.* 2001). Increased ROS can also increase the expression of MMPs in vascular smooth muscle cells (Zhang *et al.* 2013). In rat embryonic heart cells, increased NOX-derived ROS results in increased expression of *Mmp9* (Yang *et al.* 2013). In a model of NE and ANGII stimulation in rat cremaster arteries, O_2^- was the ROS responsible for activating MMPs in the inward remodeling process (Martinez-Lemus *et al.* 2011). It was also shown that when ROS were inhibited during NE and ANGII stimulation, the inward remodeling did not occur. This shows that ROS are required for the inward remodeling induced by NE and ANGII (Martinez-Lemus *et al.* 2011). Mechanical stretch in vasculature which is associated with hypertension also increases *Mmp2* expression due to an increase in NOX-derived ROS (Grote *et al.* 2003).

When increased ROS interact with NO, they form a molecule called peroxynitrite which has been shown to inactivate TIMP1 (Frears *et al.* 1996) and activate MMPs in smooth muscle cells (Rajagopalan *et al.* 1996). Peroxynitrite can also activate MMP1, 8 and 9 at a posttranslational level; however, this action does not involve removing the MMP pro-domain (Okamoto *et al.* 2001).

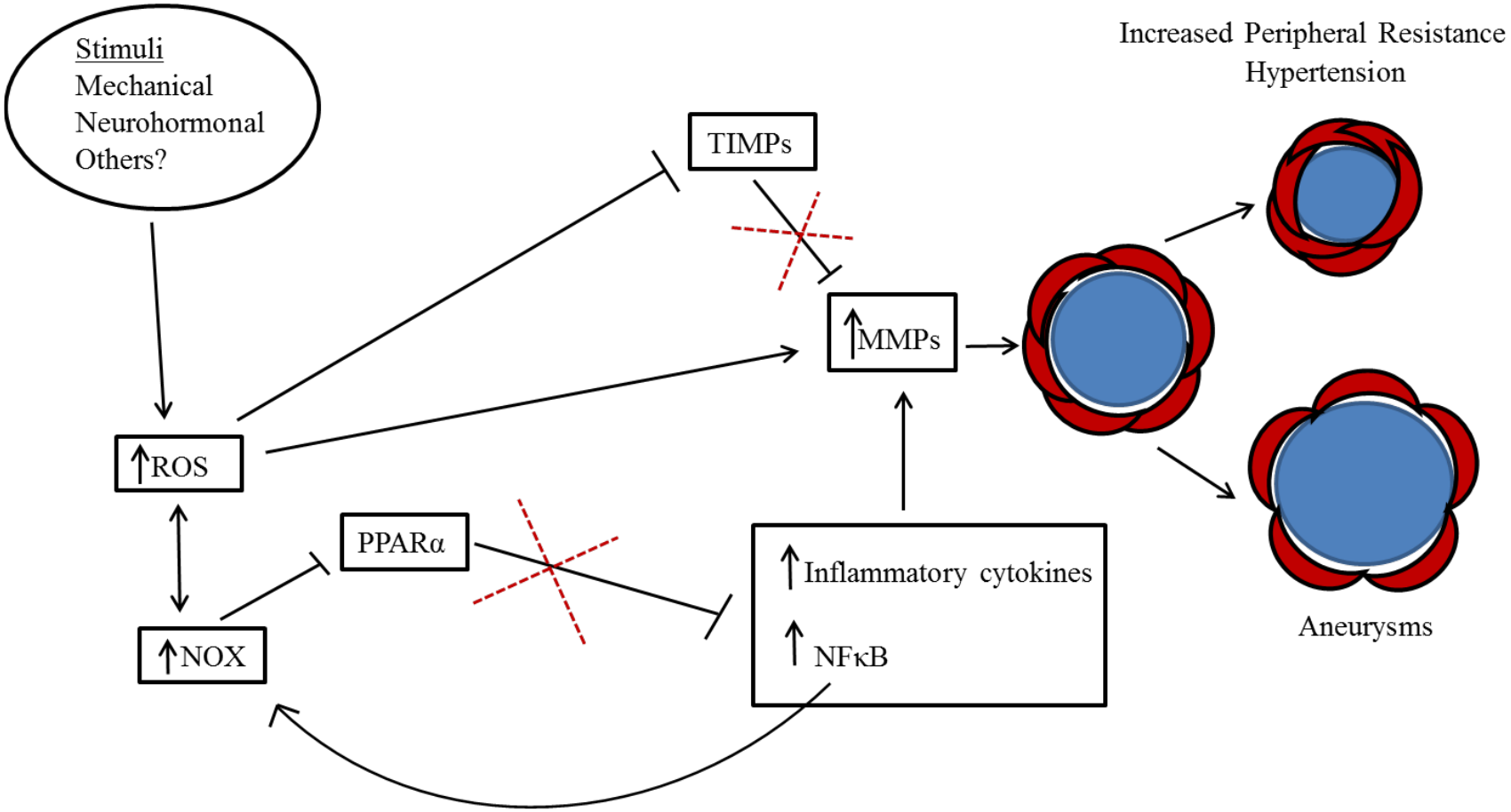

Stimuli
Mechanical
Neurohormonal
Others?
TIMPs
Increased Peripheral Resistance
Hypertension
MMPs
ROS
PPARα
Inflammatory cytokines
NFκB
NOX
Aneurysms

Figure 2.2

Figure 2.2 Involvement of MMPs, TIMPs, NOX and ROS in vascular remodeling. Stimuli such as increased angiotensin II (ANGII, neurohormonal) or increased vascular stretch (mechanical) can result in increased production of reactive oxygen species (ROS) through NADPH oxidase (NOX). Increased ROS can increase the expression and activity of matrix metalloproteinases (MMPs) and decrease the activity of their endogenous inhibitors tissue inhibitors of matrix metalloproteinases (TIMPs). Increased NOX results in inhibition of the nuclear receptor PPAR-α, thereby increasing inflammatory cytokines and NFκB due to decreased inhibitory activities of PPAR-α. The NFκB pathway and inflammatory cytokines can both increase the activity of MMPs. NFκB can also cause an elevated increase in ROS, further perpetuating the pathway. Increased activity and expression of MMPs results in degradation of the ECM allowing repositioning of vascular smooth muscle cells in inward and outward remodeling associated with adverse outcomes such as hypertension and aneurysms, respectively. Other stimuli that can result in increased ROS will be discussed further in this review. Red x's represent a decrease in inhibition by PPARα and TIMPs.

f) Conclusion

This section has shown how the MMPs can be involved in the development of CVD. Along with adverse consequences on cardiovascular health, MMPs and ROS have also been proposed to be involved in the inward eutrophic remodeling associated with the development of hypertension (Martinez-Lemus & Galinanes 2011). A summary of some ways that the NOX-produced ROS, MMPs and TIMPs can interact to affect vascular remodeling and cardiovascular health is represented in **Fig. 2.2**.

2.4 Developmental Origins of Health and Disease Hypothesis

a) Introduction to the DOHaD Hypothesis

The idea that the *in utero* environment can affect the development of disease in adulthood became aptly known as the Fetal Origins of Adult Disease Hypothesis [FOAD; Reviewed in (Calkins & Devaskar 2011, Poston 2011)]. Dr. David Barker discovered this phenomenon in retrospective human studies, and the hypothesis is now commonly being tested in animals and humans to shed light on what suboptimal maternal environments could be contributing to the widespread occurrences of obesity and CVD.

Barker's studies followed the initial observation by Forsdahl that poor nutrition in childhood followed by overnutrition as an adult in a Norwegian population resulted in an increased risk of coronary heart disease (Forsdahl 1977). The FOAD hypothesis has now been termed the Developmental Origins of Health and Disease hypothesis (DOHaD) to encompass the effects that can happen prior to the fetal stage [*i.e.* the oocyte and early

embryo; (Gillman *et al.* 2007)]. This section will discuss observations found in studies regarding maternal undernutrition and its effects on the offspring. Maternal undernutrition is a topic that has been extensively studied since the initial observations that famine exposure during pregnancy resulted in adverse outcomes in the children (Gilbert *et al.* 2005, Watkins *et al.* 2008, Lan *et al.* 2013, Tchoukalova *et al.* 2013).

b) Barker's Cardiovascular Studies

Dr. David Barker and colleagues have performed numerous retrospective studies regarding maternal exposure to famine and its effects on the future disease state of their children as adults. These studies stemmed from the initial observation that sharp increases in the occurrence of CVD correlated not with areas that were known for consuming an unhealthy diet, but rather with areas that had experienced high levels of past infant mortality associated with low birth weight (Barker & Osmond 1986). Low birth weight has since been associated with adverse outcomes including increased risks for coronary heart disease, hypertension and stroke (Barker *et al.* 1989, Eriksson *et al.* 2000, Painter *et al.* 2006a). Further studies have also revealed associations between experiencing famine *in utero* and the subsequent development of CVD, Type II Diabetes Mellitus (T2DM), obesity, stroke and psychological disorders (Barker *et al.* 1989, Hales *et al.* 1991, Brown 2000, Lussana *et al.* 2008). A sample of findings from three of the cohort studies commonly used to retrospectively study the effects of low birth weight, early development and maternal undernutrition in humans are discussed below.

Hertfordshire Cohort Study

Midwives in the Hertfordshire area began recording birth weights at the births they attended in 1911, and other health officials visited the homes throughout infancy to perform health records and to measure the weight at one year of age (Barker *et al.* 2009). It was discovered that men and women who had lower birth weights had an increased risk of dying from coronary heart disease (Barker *et al.* 1989, Osmond 1993). A follow-up study in males who had low birth weights showed that they acquired higher cholesterol levels with the consumption of high fat foods, which can result in an increased risk for coronary heart disease (Robinson 2006). Males from the Hertfordshire Cohort also had greater risk for impaired glucose tolerance and T2DM if they had low birth weight (Hales *et al.* 1991).

Helsinki Cohort Studies

Birth records were also well-recorded in Helsinki from 1934-1944 and followed 13,345 men and women (Eriksson *et al.* 2001, Osmond *et al.* 2007). The results from this birth cohort have been used in many diverse studies that have examined topics ranging from cardiovascular development to obesity and psychological outcomes. An association between low birth weight and increased adiposity and serum triglycerides was made from this cohort (Kajantie *et al.* 2008). Another study found that increased childhood body mass index (BMI) resulted in less cardio respiratory fitness in adulthood (Salonen *et al.* 2011).

Yet another study found that increased CVD death in females was associated with low length at birth and low birth weight in females and males, respectively (Kajantie *et al.* 2005). Interestingly, fetal growth restriction; specifically, a lower birth weight with

a higher head circumference, increased the risk for coronary heart disease (Eriksson *et al.* 2001). Similarly, results were obtained in an earlier Helsinki birth cohort study (from 1924 to 1933) where men who had low birth weight were shown to have an increased risk for stroke as adults (Eriksson *et al.* 2000). The placenta is involved in nutrient transfer to the growing fetus, and a thin placenta—associated with shallow invasion of the spiral arteries and reduced nutrient transfer to the fetus—has been shown to increase risk for sudden cardiac death in men and women in the Helsinki Cohort Studies (Barker *et al.* 2012).

Dutch Famine

Low birth weight can result from decreased nutrient availability or decreased nutrient transfer from the mother (Harding 2001). With the observations that low birth weight could affect the development of the offspring, studies of maternal exposure to famine became of interest. The Dutch famine in the 1940's is an example of a time when maternal nutrition was shown to affect the development of offspring [(Tobi *et al.* 2009), reviewed in (Calkins & Devaskar 2011)]. The famine occurred over a short period of time in 1944 and 1945, but detailed records of people born in Amsterdam during this time period were kept, allowing for studies to be performed in this population (Carroll *et al.* 2012). The short length of the famine in a previously well-nourished population allows for determination of undernutrition effects at specific time points in pregnancy (Roseboom *et al.* 2006). One of the first observations from the children born during the famine was that they had lower birth weights (Smith 1947).

Exposure to famine *in utero* can lead to adverse metabolic outcomes in the offspring. For example, in the Dutch famine 50-year-old women who had experienced

famine *in utero* during the first 16 weeks of gestation tended to have a higher BMI (Ravelli *et al.* 1999), and children of women who experienced the famine during the last 32 weeks of pregnancy tended to have a lower glucose tolerance (Ravelli *et al.* 1998) than those that did not experience famine. Lipid profiles of children exposed to famine *in utero* have been shown to be increased (Lussana *et al.* 2008), and an effect on increased adiposity persisted into the next generation (Painter *et al.* 2008).

Other studies have found links between *in utero* exposure to the Dutch famine and adverse psychological outcomes including Major Affective Disorder and Schizophrenia (Brown 2000, Brown & Susser 2008). Women exposed to famine *in utero* also had increased risk for breast cancer; especially when they were exposed during early gestation (Painter *et al.* 2006c, van Abeelen *et al.* 2012).

The cardiovascular system has also been shown to be affected by famine exposure *in utero*. Increased resting blood pressure was most often observed when the mothers had consumed a small ratio of protein to carbohydrates, rather than a low macronutrient level overall (Roseboom 2001). Famine during late gestation resulted in decreased vessel lumen diameter in the offspring (Painter *et al.* 2007), while exposure to famine *in utero* during early gestation increased the risk for coronary artery disease in individuals in their 50's, and the average age at which these individuals experienced coronary artery disease was 47 years of age as opposed to 50 years of age in individuals who had not been exposed to famine (Painter *et al.* 2006b). In addition, people born to mothers who experienced famine during early pregnancy exhibited higher blood pressure increases during psychological stressors such as public speaking (Painter *et al.* 2006a). Higher

increases in SBP in response to stress has been associated with future development of hypertension (Carroll *et al.* 2012).

c) Low Birth Weight, Developmental Plasticity and the "Thrifty Phenotype"

The nutritional environment while *in utero* is very important in determining the subsequent development of the offspring. This is due to reduced availability of nutrients *in utero* (Harding 2001). In a retrospective study used to examine the effect of genetics versus the effect of *in utero* environment on the birth weight of babies in cases of oocyte donation, the size of the baby was determined by the size of the mother who developed the fetus *in utero* rather than by the size of the mother who donated the oocyte (Brooks *et al.* 1995). The DOHaD hypothesis (mentioned earlier) predicts that the maternal environment a fetus is exposed to can cause it to have a higher incidence of certain diseases later in life [Reviewed in (Calkins & Devaskar 2011, Poston 2011)]. This is due to a concept called "developmental plasticity," which refers to multiple phenotypes resulting from one genotype in response to different environmental cues [reviewed in (Calkins & Devaskar 2011), Jirtle and Skinner, 2007)].

Programming occurs when the phenotypic changes acquired in response to a specific environment become permanent. This can be beneficial when the subsequent environment is similar to the *in utero* environment and the programmed phenotype allows the individual to perform well in the subsequent environment [reviewed in (Brenseke *et al.* 2013)]. However, the changed phenotype can become maladaptive if the subsequent environment does not match the original environment to which the individual was programmed. For example, an individual who experiences low nutrition *in utero* can

develop a “thrifty phenotype” where the development of organs like the pancreas and kidney are stunted in favor of developing vital organs like the brain, which can then predispose the individual to diseases like diabetes and hypertension when the individual has high nutrient availability later in life [(Hales 1992) reviewed in (Brenseke *et al.* 2013)].

d) Animal Models of Undernutrition

Due to a limited number of detailed birth records in humans, animal models are often used to study the effects of maternal undernutrition on offspring development. One commonly-used model of animal undernutrition is that in which a low protein diet is given to the mother. A maternal low protein diet supplied only during oocyte development in mice had adverse outcomes on the offspring including reduced arterial function in males and increased SBP in males and females (Watkins *et al.* 2008). A study in sheep also revealed that a diet with low amino acid content resulted in decreased methylation of two imprinted genes in the offspring when compared to those exposed to higher amino acid content *in utero* (Lan *et al.* 2013). Abnormal methylation of imprinted genes can affect the ability of the offspring to develop, as proper imprinted gene expression is essential for the development of the fetus and placenta (Fowden *et al.* 2006).

A study of low protein diet in mice by Watkins *et al*. (2011) revealed adverse effects on the cardiovascular and metabolic health of the offspring. Adverse effects were observed in that study regardless of whether the diet was provided while the oocyte was developing, during preimplantation development, or throughout the entire gestation. One

effect shown in the study was increased SBP at one year of age in all of the low protein diet groups. Also, maternal consumption of a low protein diet during gestation resulted in decreased female offspring body weight compared to controls, and low protein diet during embryo development resulted in female offspring with increased body weight (Watkins *et al.* 2011). It has also been observed in rats that females exposed to low protein diet *in utero* show a preference for high fat foods, suggesting a possible link between maternal consumption of a low protein diet and diet-induced obesity in the offspring (Bellinger *et al.* 2004). Amino acids in protein help with antioxidant synthesis, so decreased availability of amino acids can lead to greater oxidative damage [reviewed in (Brenseke *et al.* 2013)].

While protein deficiency is a common animal model of undernutrition, diets with global nutrient restriction (Gilbert *et al.* 2005, Tchoukalova *et al.* 2013), methyl-deficiency (Maloney *et al.* 2011), and low polyunsaturated fatty acids [PUFA's;(Armitage *et al.* 2003, Mathai *et al.* 2004)] have also been used to represent maternal undernutrition. An effect of greater oxidative damage on offspring development has been demonstrated in a model of maternal nutrient restriction in rats (Franco Mdo *et al.* 2004). When male rats from undernourished mothers were supplemented with an essential cofactor for NO, they had less ROS production and improved endothelial function, suggesting that oxidative damage is involved in the development of endothelial dysfunction with maternal undernutrition (Franco Mdo *et al.* 2004).

In baboons, a thirty percent reduction in global nutrients resulted in decreased body weight and increased expression of adipogenesis-related genes in male fetuses (Tchoukalova *et al.* 2013). Global maternal nutrient restriction in sheep increased MAP

in the lambs and increased ACE expression (Gilbert *et al.* 2005). A methyl-donor deficient maternal diet in rats resulted in male offspring with higher glucose levels and insulin resistance at 8 months of age when compared to controls (Maloney *et al.* 2011). When female rats consumed a PUFA-deficient diet, the offspring displayed increased MAP (Armitage *et al.* 2003) as well as increased appetite signaling (Mathai *et al.* 2004) as adults.

In summary, the initial observation that low birth weight was associated with later disease state led to interest in studying the effect that maternal nutrition has on the health of the offspring. Records from several human birth cohorts have provided insight on low birth weight, and the records from the Dutch Famine provide a glimpse on the effects of maternal undernutrition in humans. However, animal studies of maternal undernutrition are used to investigate the effects that certain dietary deficiencies, including low protein diets and methyl donor deficiencies have on the development of the offspring. Together, these studies have provided the initial insight that the environment in which a fetus develops can affect the disease state of the offspring in adulthood. Due to the increasing epidemic of obesity (Kelly *et al.* 2008), the DOHaD hypothesis has been expanded to include the effects of maternal obesity and overweight (Armitage *et al.* 2008). This will be discussed in the following section.

2.5 Obesity

a) Prevalence

It is estimated that by 2030 almost sixty percent of adults world-wide will be overweight or obese. Body mass index; (weight in kg divided by height in m^2) is used as a measure of overweight and obesity. Overweight is defined as a BMI greater than twenty-five and obesity is a BMI greater than thirty in adults. The rate of obesity is highest in developed and developing countries (Kelly *et al.* 2008). Over 200 million men and 300 million women world-wide were considered obese in 2008 (WHO 2013e). Two-thirds of adults in the United States are overweight and one-third are obese (NIH 2012). Also, 64% of women in the United States are either overweight or obese (NIH 2012).

Obesity has also become an increasing problem in children. World Health Organization (WHO) statistics showed that globally there were over forty-two million children under the age of five who were overweight in 2010 (WHO 2013c). For children, overweight is at or above the 85th percentile, while obesity is at or above the 95th percentile of body weight (NIH 2012). Obesity is associated with increased risk for other health problems including hypertension, certain cancers, and T2DM (NIH 2012).

b) Effect of Obesity and Overweight on Oocyte Competence

Maternal obesity and overweight can have adverse effects on female reproductive function by its effects on the oocyte (Igosheva *et al.* 2010, Luzzo *et al.* 2012, Machtinger *et al.* 2012). In humans and in mice, using oocytes from a non-obese female for *in vitro* fertilization (IVF) and embryo transfer into an obese female resulted in less adverse developmental effects than if the oocyte was from an obese female (Luke *et al.* 2011a,

Luzzo *et al.* 2012). This suggests that poor oocyte quality is predictive of adverse outcomes *in utero*. An inflammatory environment induced by obesity has been observed in the ovaries of obese women, providing an altered environment for oocyte development (Robker *et al.* 2009).

A pro-inflammatory environment can result in increased oxidative stress by the production of ROS [Reviewed in (Fernandez-Sanchez *et al.* 2011)]. A mouse model of maternal obesity demonstrated that oocytes from obese dams had abnormal mitochondrial distribution and function, and that this resulted in increased production of ROS (Igosheva *et al.* 2010). Increased levels of ROS can affect oocyte developmental potential (Jiao *et al.* 2013). In addition, oocytes from obese women are more likely to have abnormal meiotic spindles and misaligned chromosomes during the metaphase II (MII) phase of meiosis than oocytes from women with normal BMI's [18.5-24.9; (Machtinger *et al.* 2012)]. Also, higher levels of nonesterified fatty acids (NEFA) that can be found in obesity have been shown to upregulate genes involved in energy metabolism and oxidative stress in bovine oocytes (Van Hoeck *et al.* 2013).

c) Effect of Obesity and Overweight on Preimplantation Embryo Development

The pro-inflammatory environment in obesity can affect the uterus. The uteri from obese dams in a rat maternal obesity model had increased expression of pro-inflammatory genes at day 4.5 post-coitum (Shankar *et al.* 2011). Shankar *et al.* (2011) also showed that male preimplantation embryos had increased expression of genes involved in pro-inflammatory immune response and decreased expression of antiapoptotic genes (Shankar *et al.* 2011).

Embryo development can also be affected by maternal obesity. It has been shown that embryos from obese females are more likely to have delayed development, chromosomal abnormalities and are more likely to be degraded than those from their non-obese counterparts (Binder *et al.* 2012, Luzzo *et al.* 2012). Further, Van Hoeck *et al.* (2011) showed that bovine embryos exposed to high NEFA during maturation had reduced viability as demonstrated by reduced oxidative metabolism and increased apoptosis in day 7 blastocysts (Van Hoeck *et al.* 2011).

d) Adverse Effects of Obesity on Post-implantation Development

The abnormalities during preimplantation development associated with an environment surrounded by adipose tissue can affect the development of the fetus *in utero*. The obese environment in humans has been associated with adverse outcomes during pregnancy including pre-eclampsia, intrauterine death, neural tube defects, gestational diabetes mellitus, the need for emergency C-sections, as well as a risk for having children born as large (>90th percentile in weight) for gestational age [LGA;(Sebire *et al.* 2001, Di Cianni *et al.* 2005, Chu *et al.* 2007, Rasmussen *et al.* 2008)]. The effects of low birth weight have been previously described, but high birth weight can also have adverse effects on a person's future disease state, including increased risk for obesity (Sorensen *et al.* 1997). However, in mouse models, maternal obesity is associated with decreased *in utero* development and lower birth weight in the offspring (Luzzo *et al.* 2012). Although mice offspring from mothers exposed to excess fatty acids tend to be born small, they catch up and quickly surpass the size of mice born to mothers with normal fatty acid levels (Jungheim *et al.* 2011).

The placenta is responsible for transporting nutrients to the growing fetus. The placenta has been shown to be affected by maternal obesity (Liang *et al.* 2010, Hayes *et al.* 2012). In mice, diet-induced obesity resulted in oxidative stress in the placenta, adversely affected trophoblast invasion, and resulted in more cellular necrosis in the placenta (Liang *et al.* 2010). Obese dams in a rat model had increased rates of fetal death, most likely caused by the altered vasculature found in the placentas of these dams (Hayes *et al.* 2012).

These *in utero* effects can also affect the future disease state of the offspring. Children born to obese mothers are more likely to develop metabolic syndrome (MS) with symptoms including high blood pressure, lowered glucose tolerance and increased body weight (Boney *et al.* 2005). Women who are obese also have offspring with higher incidences of obesity (Nelson *et al.* 2010, Poston 2011, Morandi *et al.* 2012). Also, in a prospective study in women in Australia, maternal high BMI was associated with offspring high BMI, and this association was more pronounced than the association between paternal BMI and offspring BMI (Lawlor *et al.* 2007).

Rodent models of maternal obesity have revealed increased body weight in the offspring (Samuelsson *et al.* 2008, Franco *et al.* 2012) and impaired development of skeletal muscles (Bayol *et al.* 2005). A study in mice found increased body weight and body fat mass in thirty-week-old male offspring born to obese mothers even though the offspring consumed a normal diet after weaning (Torrens *et al.* 2012). Males from obese dams also have been shown to exhibit glucose intolerance (impaired glucose tolerance) and increased inflammatory adipokines, which are cytokines released by adipose tissue (Magliano *et al.* 2013). Hyperleptinemia and hyperinsulinemia have also been observed

in offspring from rodent maternal obesity models (Shankar *et al.* 2010, Franco *et al.* 2012)**.** Hyperleptinemia can result in increased rates of obesity and increased blood pressure in the offspring as adults (Trevenzoli *et al.* 2007).

Maternal Obesity/Overweight and Cardiovascular Abnormalities in the Offspring

A maternal overweight or obese environment can also affect vascular functioning and development of hypertension in the offspring (Samuelsson *et al.* 2008, Samuelsson *et al.* 2010, Torrens *et al.* 2012). In mice, prenatal exposure to maternal obesity resulted in increased SBP, decreased NO production and decreased vasorelaxation to acetylcholine in the femoral arteries in male offspring at thirty weeks of age (Torrens *et al.* 2012). It was also found that the generation of NADPH-dependent O_2^- was increased in the liver of these males. Increased ROS have been associated with endothelial dysfunction (Chinen *et al.* 2007).

While not as many studies have been performed in humans as in animal models, associations have still been made between a maternal overweight and obesity and cardiovascular outcomes in the offspring. An Australian cohort study in five-year-old children revealed that maternal BMI was one of the factors that was associated with an increased SBP in the children (Lawlor *et al.* 2004). Another study found that an increase in maternal BMI was associated with increased leptin, SBP, and BMI in the children, regardless of whether or not the mother had diabetes (West *et al.* 2011).

e) Maternal Consumption of a High Fat Diet

In some studies the goal is not to examine the effect of maternal obesity, but rather to examine how a mother consuming a high fat (HF) diet during gestation and/or

lactation can affect the offspring. A study in mice found that global DNA methylation of imprinted genes important for proper *in utero* development was lower in the placentas of female offspring when the mothers consumed a HF diet during pregnancy (Gallou-Kabani *et al.* 2010). Maternal overnutrition during the last month of pregnancy in sheep affected fetal development as demonstrated by increased levels of glucose and insulin in fetal plasma, as well as increased expression of genes involved in adipogenesis (Muhlhausler *et al.* 2007).

High fat diet consumption during pregnancy can also affect the cardiovascular outcome in the offspring. Maternal consumption of a diet containing 21% (w/w) fat during pregnancy and lactation in rats decreased the ability of the offspring aortas to relax in response to the potent vasodilator acetylcholine (Kelsall *et al.* 2012). ROS levels have also been shown to be increased in the hearts of mice offspring that were exposed to a HF diet both prenatally and postnatally (Turdi *et al.* 2013).

Increased maternal gestational weight gain which can occur as a result of HF diet consumption during pregnancy can also affect offspring development. In a retrospective cohort study in humans, children at seven years of age were more likely to be overweight if their mother had gained more than the recommended amount of weight during pregnancy, showing that diet during pregnancy is important (Wrotniak *et al.* 2008). Another study from an Australian birth cohort found that increases in maternal gestational weight gain tended to increase SBP in the children at 21 years of age (Mamun *et al.* 2009).

f) Effect of High Fructose Consumption

High fructose consumption during pregnancy can also affect offspring development. High fructose corn syrup is a sweet corn based syrup made by using glucose isomerase to convert starch from corn into glucose, and then converting this glucose into fructose (Bray *et al.* 2004). Due to its sweet flavor, high fructose corn syrup is now often used to replace sugar in commonly consumed foods and drinks [*i.e.* baked goods and carbonated beverages; (Hanover & White 1993)]. In approximately the past thirty years, the amount of fructose that is consumed by children in the United States has increased 10-20% (Marriott *et al.* 2009).

Male rats born to mothers that consumed fructose in their drinking water during lactation had increased body weight, increased insulin and leptin levels, and consumed more food than control rats (Alzamendi *et al.* 2010). Another study found that rats born to mothers who had consumed 50% fructose during pregnancy were hyperglycemic at birth (Jen *et al.* 1991). Higher fasting insulin levels (Rawana *et al.* 1993) and increased plasma leptin and glucose (Vickers *et al.* 2011) have also been observed in rats born to mothers that consumed fructose.

Fructose is sometimes included in obesity models to represent a "Western diet" where high levels of sugars are consumed. The consumption of fructose in adolescents increases cardiometabolic risk factors, and this is shown to be mediated by visceral adiposity (Pollock *et al.* 2012). Also, increased blood pressure and plasma insulin and triglycerides were shown in rats that consumed a high fructose diet for two weeks (Hwang *et al.* 1987). Another rat study showed that consuming a HF diet with fructose

increased leptin in rats and increased SBP and endothelial dysfunction (Panchal *et al.* 2011).

2.6 Assisted Reproductive Technologies

a) Introduction

Infertility affects around 10% of females in the United States (CDC 2013). To help overcome this infertility, couples may seek the use of assisted reproductive technologies (ART). In 2010, there were 61,564 infants born (slightly more than 1% US births) that were conceived with the use of ART (CDC 2012). ART procedures include non-invasive techniques such as ovarian hyperstimulation and intrauterine insemination, embryo culture and IVF or invasive procedures such as intracytoplasmic sperm injection (ICSI). For clinics that are members of the Society for Assisted Reproductive Technologies (SART), there were 154,412 ART cycles performed in 2011 (SART 2013). The use of ART is increasing, with 107,587 cycles performed in 2001 and 147,260 in 2010 (CDC 2012).

b) ART Procedures

Ovarian Hyperstimulation

Ninety-nine percent of the cycles performed in 2011 in SART clinics used ovarian hyperstimulation (SART 2013). In ovarian hyperstimulation, exogenous gonadotropins are administered to increase the number of oocytes ovulated in a single reproductive (menstrual) cycle. This is done to provide a greater number of mature eggs to be used in

the subsequent ART procedure (Zegers-Hochschild *et al.* 2009). The Center for Disease Control (CDC) does not consider ovarian hyperstimulation and ART procedure (CDC 2012), but we consider the use of exogenous gonadotropins ART in our laboratory because we have observed effects of the gonadotropins alone on gene expression and global DNA methylation in oocytes (Almamun 2011). In mice, ovarian hyperstimulation is known as superovulation (SO), and is commonly performed by administering five international units (IU) eCG followed by 5IU hCG approximately 48 hours later (Luo *et al.* 2011). The eCG has follicle stimulating hormone (FSH) activity, and regulates granulosa cell differentiation and proliferation, while the hCG has luteinizing hormone (LH) activity, and induces ovulation (Nagy A 2003).

In humans, ovarian stimulation is used not only to recruit multiple follicles to obtain more oocytes for the ART procedures, but also to remove the need to detect the LH surge in order to obtain the oocytes at the correct stage of maturity (Davis & Rosenwaks 2001). Often gonadotropin releasing hormone (GnRH) agonists are used to create a surge in endogenous LH and FSH, and this surge results in the downregulation of the endogenous gonadotropins due to desensitization in the anterior pituitary (Davis & Rosenwaks 2001). By downregulating endogenous gonadotropins, the exogenous gonadotropins determine the timing of the cycle. The ovarian follicles are stimulated daily by administration of FSH in the form of human menopausal gonadotropin (hMG), purified urinary FSH or recombinant FSH, starting at day 2 or 3 of the menstrual cycle. The hCG administration in humans is timed carefully to be performed when the mean size of the lead follicles is above 16mm in diameter. The oocytes for the subsequent

ART procedures are collected between 34 and 36 hours after hCG administration (Davis & Rosenwaks 2001).

IVF and ICSI

In IVF, the embryo is formed by culturing the sperm and oocyte in fertilization medium and allowing the sperm to naturally fertilize the oocyte. IVF was used for 99% of the ART cycles performed in 2010 (CDC 2012). Intracytoplasmic sperm injection (ICSI) is a specific type of IVF that involves manually injecting a single sperm directly into the cytoplasm of the oocyte (CDC 2012). Sixty-six percent of the 154,412 ART cycles performed in 2011 involved ICSI (SART 2013). There have been adverse outcomes in the offspring associated with the process of IVF such as increased blood pressure and peripheral body fat in children (Ceelen *et al.* 2007, Ceelen *et al.* 2008, Ceelen *et al.* 2009), as well as the more invasive ICSI procedure including abnormal retinal vascularization in children (Wikstrand *et al.* 2008).

Embryo Culture

Embryo culture is an ART procedure which allows the embryo to develop outside of the normal maternal environment. All culture media commercially used for human ART include the inorganic ions Na^+, K^+, Cl^-, Ca^{2+}, Mg^{2+} and SO_4^{2-} and the presence of these ions has not changed since the original media used for mouse embryo culture in the 1950s [reviewed in (Baltz 2012)]. The original successful mouse embryo culture media was Whitten Medium (Whitten 1956). Whitten discovered that adding glucose as a carbon source and bovine serum albumin (BSA) to a physiological saline medium allowed 8-cell stage mouse embryos to develop to the blastocyst stage [reviewed in

(Biggers 1998)]. However, it was soon realized that an ideal medium had not yet been created because many embryos arrested at the two-cell stage (Goddard 1983).

The two cell arrest is known as the two-cell block, and it coincides with the time of major zygotic genome activation in the mouse (Goddard 1983). Subsequently, adding EDTA as a chelator was found to help overcome the two-cell block (Abramczuk J 1977). EDTA acts as a chelator by binding with high affinity to Cu^{2+} and Zn^{2+} ions that can be harmful in too high of concentrations [reviewed in (Baltz 2012)]. Since that time, many culture media have been developed to try to more closely represent the natural oviductal fluid for the development of the embryo [reviewed in (Baltz 2012)], although no medium can perfectly mimic the maternal environment. The shortfalls of culture media when compared to oviductal and uterine fluids can have adverse effects on embryo development and subsequent development of the offspring (Rinaudo & Schultz 2004, Pfeifer *et al.* 2012).

A major inconsistency between embryo culture and the maternal environment is that embryo culture is often performed in high oxygen tension while the normal environment in which an embryo develops is actually hypoxic. The normal oxygen content is 8% and 1.5% in the oviduct and uterus, respectively, whereas many culture protocols involve oxygen tension of around 20% [*i.e.*the oxygen content of air; (Pfeifer *et al.* 2012)]. The adverse effects of high levels of oxygen will be discussed in a later section.

Embryo Transfer

In human embryo transfer, embryos are produced *in vitro* and are transferred either into the woman who provided the oocyte for the ART procedure (autologous), or into another woman facing infertility (donor). Embryos are most-often transferred on day 3 after egg retrieval (CDC 2012). In 68.5% of cycles, in 2010, women used their own oocytes for the ART procedures (CDC 2012). Women undergoing embryo transfer can also receive either fresh or frozen embryos. For fresh embryo transfer, ovarian stimulation is used to increase the number of oocytes available for the subsequent ART procedure. Therefore, in an autologous and fresh transfer, the embryos are transferred into a hormonally-stimulated environment. However, in frozen and donor embryo transfer, the embryo is transferred without ovarian stimulation (Kalra *et al.* 2011).

c) Adverse Consequences Associated with ART

ART procedures take place at a time when the developing embryo is very susceptible to the environment because they occur at a time when the embryonic genome is being activated (Goddard 1983). These procedures can cause an altered maternal environment that results in aberrant effects as early as the oocyte and early embryo.

One way that ART can affect the maternal environment is through altered hormone levels. Estradiol levels in women undergoing ovarian stimulation can be up to 20 times higher than normal physiological levels (Kalra *et al.* 2011). In humans, the amount of interleukin-6 (IL-6) released into the culture medium—a measure of the ability of the embryo to develop and implant—was lower from women who had undergone SO (Yu *et al.* 2012). Also, the uterine environment was shown to be affected in a mouse SO

model when embryos were less likely to implant in superovulated recipients than in naturally ovulated recipients (Ertzeid & Storeng 2001). Similar results were found in humans when women who had undergone ovarian hyperstimulation for an IVF cycle had lower implantation rates than those who underwent unstimulated IVF (Paulson 1990).

Another way ART can create an unfavorable maternal environment is through increased oxidative damage. ART has been associated with increased oxidative damage in mouse ovaries and oocytes, possibly through its effects on the mitochondria (Chao *et al.* 2005, Ge *et al.* 2012). The mitochondria, which are maternal in origin (Wagner 1972), are involved in the production of ROS; therefore, if the mitochondria are altered in the oocyte (the source of embryonic mitochondria) then they will not be able to support the development of the embryo (Dumollard *et al.* 2009). Multiple cycles of ovarian stimulation are not uncommon, as approximately 45% of the women who used ART with fresh, non-donor eggs in 2010 had undergone ART cycles before (CDC 2012). It has been shown that increased number of cycles of ovarian stimulation can induce changes in the mitochondria that increase oxidative damage in the resulting embryos (Chao *et al.* 2005).

ART has also been shown to induce epigenetic and gene expression changes in the oocyte and early embryo. SO alone has been shown to decrease global DNA methylation levels in mouse oocytes (Almamun 2011). DNA methylation is an epigenetic mark associated with regulation of gene expression. One study in mouse preimplantation embryos showed that even the highest quality embryo cultures used presently resulted in aberrant expression of genes involved in embryo development and oxidative stress regulation (Pfeifer *et al.* 2012). Another study in preimplantation mouse

embryos showed misregulation of 114 genes after culture in Whitten's medium, and 29 genes after culture in KSOM supplemented with amino acids when compared to *in vivo* embryos (Rinaudo & Schultz 2004). A study by Denomme *et al*. (2011) suggested that SO affects the embryo's ability to maintain gene imprints (Denomme *et al.* 2011). Imprinted genes are genes that are only expressed from one parental allele, and whose correct expression is required for proper growth and development of the fetus and the placenta (Fowden et al. 2006).

The use of ART and the change in environment during embryo development can result in aberrant programming of critical physiological systems and development. Along with effects on the preimplantation embryo, ART procedures can affect the subsequent fetal development of the offspring. The procedure of ovarian stimulation alone can cause an increased risk for low birth weight (Ertzeid & Storeng 1992, Kalra *et al.* 2011). In addition, in single births from single pregnancies using fresh, non-donor oocytes, 11.7% of infants were born pre-term, and 8.6% had low birth weight (CDC 2012). A difference in birth weight in humans born from IVF from different culture media has been observed, even when accounting for infertility (Dumoulin *et al.* 2010, Nelissen *et al.* 2012). ART has also been associated with adverse outcomes *in utero* including greater dysplasia, multiple gestations, risk of monozygotic twinning, and increased rates of congenital heart defects [(Schachter 2001, Katalinic *et al.* 2004, Olson *et al.* 2005) reviewed in (Zollner & Dietl 2013)]. Adverse effects of ART can also be seen with the increased risk of loss-of-imprinting (LOI) disorders including Beckwith-Wiedemann Syndrome and Angelman Syndrome in humans, as well as the related Large Offspring Syndrome (LOS) in ruminants (Young 1998, Maher 2003, Horsthemke & Ludwig 2005, Chen *et al.* 2013).

Adverse metabolic outcomes have been observed in offspring conceived through ART. In human retrospective studies, ART was associated with higher peripheral body fat (Ceelen *et al.* 2007). They also found that study participants conceived using IVF had a higher fasting glucose level (Ceelen *et al.* 2008). In a similar study, girls that were conceived with the use of ICSI had increased peripheral adiposity at 14 years of age when compared to age matched controls that were spontaneously conceived (Belva *et al.* 2012). This could have possible implications for a relationship between ART and diabetes later in life. According to Chen *et al*., children conceived through ART also have higher levels of insulin and higher levels of triglycerides (Chen 2011).

ART and Cardiovascular Abnormalities in Offspring

The cardiovascular system has also been shown to be affected with the use of ART. Since the first person conceived with the use of ART [Louise Brown; (Edwards *et al.* 1980)] is in her mid-thirties and the onset of CVD is generally later in life, it cannot yet be determined whether people who were conceived using ART develop CVD at an increased rate. However, studies have been conducted in children and young adults to examine possible precursors to CVD, including increased blood pressure and abnormal vascularization.

One study examined retinal vascularization in a Swedish population of children conceived by ICSI (Wikstrand *et al.* 2008), as decreased retinal vascularization has been associated with an increased risk of death from coronary heart disease (Liew *et al.* 2011). Wikstrand *et al.* (2008) looked at the number of branching points in the vessels in the eye to represent vascularization. One observation that they made was that children conceived using ICSI had a lower median number of branching points than in the control children

who were conceived naturally. Specifically, the boys conceived using ICSI had significantly fewer branching points than the boys conceived naturally. This effect was not observed in girls (Wikstrand *et al.* 2008).

Ceelen *et al*. have performed retrospective studies in children and young adults conceived with the use of IVF in the Netherlands. As mentioned previously, some of the adverse outcomes they observed in the children were increased peripheral body fat and higher fasting glucose levels (Ceelen *et al.* 2007, Ceelen *et al.* 2008). In relation to cardiovascular development, they also found a significant difference in systolic and diastolic blood pressure in participants conceived through in IVF as opposed to participants conceived naturally (Ceelen *et al.* 2007). In a later study, they compared children conceived using IVF to children that were born spontaneously to subfertile parents in order to control for infertility. They found that children conceived using IVF who experienced rapid growth during early childhood had higher blood pressure in the follow-up measurement at 8-18 years old, and that this was not observed in the controls (Ceelen *et al.* 2009).

Animal models have also been used to examine the effects of ART on the cardiovascular system. A study in mice showed that ART (*i.e.* SO, embryo culture and embryo transfer) can also affect the RAS (Watkins *et al.* 2007). Specifically, an increase in ACE was seen in the study. ACE is responsible for cleaving Angiotensin I into the active form, AngII (a vasoconstrictor). When the RAS is overactive (as in the case of increased ACE), hypertension can occur (Kaplan 2002). Since prolonged vasoconstriction results in higher levels of ROS and MMPs, leading to higher blood

pressure (Martinez-Lemus *et al.* 2011), it is possible that ART might affect the blood pressure of offspring through the RAS and the production of ROS and MMPs.

In summary, these studies point to the cardiovascular system as a system that is susceptible to changes in the maternal environment. They show that different forms of ART including SO, embryo transfer, and IVF have been associated with adverse cardiovascular outcomes in children including increased blood pressure and abnormal vascularization.

2.7 Maternal Obesity and Infertility and ART

The previous sections have shown how perturbations in the maternal environment can affect the offspring, both *in utero* and in their future development. Maternal obesity is highly prevalent in today's society, and has been associated with adverse effects on the oocytes and embryos (Igosheva *et al.* 2010) as well as with pregnancy complications (Sebire *et al.* 2001). As with ART and maternal undernutrition, maternal obesity can also result in adverse health in the offspring as adults (Nelson *et al.* 2010, West *et al.* 2011). While ART and maternal obesity are often studied alone as suboptimal maternal environments, they often coincide in reality as will be discussed in this section.

a) Maternal Obesity and Infertility

Obesity is associated with an increased incidence of infertility (Grodstein *et al.* 1994, Spandorfer *et al.* 2004, van der Steeg *et al.* 2008). It has been estimated that women who were obese at 18 years of age are nearly three times more likely to be

infertile than women with lower BMI's [20-21.9; (Rich-Edwards *et al.* 1994)]. An increased BMI correlates with decreased ability to conceive, even in women who ovulate regularly (Jensen *et al.* 1999, van der Steeg *et al.* 2008). However, women who are obese are also more likely to have decreased ovulation. Increased risk for anovulation, or the lack of release of an oocyte in a menstrual cycle, is found in women who are overweight or obese (Grodstein *et al.* 1994, Spandorfer *et al.* 2004). Ovulatory dysfunction accounted for approximately 7% of the cases of infertility present in clinics in 2010 (CDC 2012).

Obese women also have an increased time to conception (Lake *et al.* 1997, Jensen *et al.* 1999). With the rise in obesity, more overweight and obese women are utilizing ART (Robker 2008). Obese (Radavelli-Bagatini *et al.* 2011) and HF diet (Wu *et al.* 2010) mouse models have also revealed increased anovulation. In a mouse model of diet induced obesity, obese mice were more likely to show anovulation and were more likely to be deemed infertile as determined by failure to breed for over 20 consecutive days (Bermejo-Alvarez *et al.* 2012). Consumption of a HF diet has also resulted in abnormal estrous cyclicity in rats (Balasubramanian *et al.* 2012).

b) Reduced ART success with Maternal Obesity

The adverse effects of obesity during ART procedures can first be observed during ovarian stimulation and oocyte collection. Increased amounts of gonadotropins are needed in ART cycles for obese women (Depalo *et al.* 2011, Valckx *et al.* 2012, Zander-Fox *et al.* 2012), and women who are obese have greater rates of IVF cancellation, as measured by a lack of response to exogenous gonadotropin stimulation (Spandorfer *et al.* 2004, Luke *et al.* 2011a). Further, less oocytes are able to be collected

from obese or morbidly obese (BMI ≥40) women undergoing ART (Spandorfer *et al.* 2004, Dokras *et al.* 2006, Valckx *et al.* 2012, Zander-Fox *et al.* 2012). Wittemer *et al.* (2000) showed that women who were overweight had fewer good quality MI and MII oocytes when undergoing IVF than women of normal weight [BMI 20-25; (Wittemer *et al.* 2000)]. Decreased oocyte quality could have an adverse effect on fertilization rates. For example, Shah *et al.* (2011) showed that women undergoing IVF alone or an IVF procedure including ICSI had less fertilized oocytes if their BMI was ≥35 (Shah *et al.* 2011).

Embryo number and quality can also be affected in obese women undergoing ART procedures (Metwally *et al.* 2007, Zhang *et al.* 2010, Valckx *et al.* 2012). Luke *et al.* (2011) found that as the BMI of women undergoing IVF increased, the proportion of blastocyst-stage embryos were decreased (Luke *et al.* 2011b). Valckz *et al.* (2012) have also shown reduced blastocyst formation during IVF for women with increasing BMI's (Valckx *et al.* 2012). Other studies have revealed that the embryos that result from obese women undergoing IVF treatments are lower quality than women with a normal BMI (Metwally *et al.* 2007, Zhang *et al.* 2010).

Reduced oocyte competence and embryo development have also been demonstrated in animal models of obesity or maternal overnutrition. Fewer oocytes from mice consuming a HF diet were able to be fertilized *in vitro* (Wu *et al.* 2010). Wu *et al.* (2010) further showed that the cumulus oocyte complexes from obese females had increased lipid content and increased expression of stress marker genes (Wu *et al.* 2010). Further, embryos from overfed ewes who consumed more than twice the amount of food than control ewes had decreased morula and blastocyst rate in culture when compared to

embryos from control ewes (Grazul-Bilska *et al.* 2012). In sheep, high levels of PUFAs decreased the development of fertilized oocytes to the blastocyst stage in culture (Wonnacott *et al.* 2010). Retarded embryo development in culture due to obesity has also been observed in mice (Minge *et al.* 2008, Binder *et al.* 2012). Wakefield *et al.*(2008) further demonstrated that feeding female mice excess PUFAs decreased the ability of their embryos to develop to the blastocyst stage in culture after *in vivo* fertilization and SO (Wakefield *et al.* 2008).

Decreased implantation rates have been observed when embryos were transferred into morbidly obese women (Zander-Fox *et al.* 2012) and women with BMI >25 (Depalo *et al.* 2011) when compared to women with normal body weights (BMI 19-24.9). A similar study that controlled for infertility by transferring an embryo from a healthy, fertile donor into a healthy, fertile recipient found a significant decrease in implantation rate with increasing BMI of the embryo recipients. (DeUgarte *et al.* 2010). However, many studies have also shown no change in implantation with obesity (Fedorcsak *et al.* 2001, Dokras *et al.* 2006). Despite this discrepancy, women undergoing IVF who are obese have displayed decreased pregnancy rates (Lake *et al.* 1997, Maheshwari *et al.* 2007, Zander-Fox *et al.* 2012).

c) Adverse Pregnancy Outcomes with Obesity and ART

Adverse outcomes due to obesity in ART pregnancies are increased risk for pre-eclampsia, gestational diabetes, pre-term deliveries and an increased need for cesarean section deliveries (O'Brien *et al.* 2003, Dokras *et al.* 2006, Bhattacharya *et al.* 2007, Dickey *et al.* 2012, Sauber-Schatz *et al.* 2012, Zander-Fox *et al.* 2012). Obesity during

ART procedures can also have adverse effects on the pregnancy. In a large study performed with women undergoing ART in England, women with BMI's ≥27 had a 33% decreased chance of delivering a live birth (Lintsen *et al.* 2005). Increased risk of miscarriage has been observed in obese women undergoing ART procedures (Dokras *et al.* 2006, Bhattacharya *et al.* 2007, Maheshwari *et al.* 2007). There is also a greater miscarriage rate in obese women receiving donor oocytes from non-obese individuals implying reduced endometrial receptivity (Bellver *et al.* 2007). Wen *et al*. (2010) found that children conceived through IVF or ICSI were at a higher risk for congenital heart defects, and that this risk increased when the mothers had a BMI of 30 or above (Wen *et al.* 2010). Also, women who are obese and use IVF are more likely to deliver macrosomic offspring (Dokras *et al.* 2006, Zander-Fox *et al.* 2012).

RATIONALE FOR THESIS

Previous research has shown that the maternal environment an individual is exposed to during *in utero* development can affect the subsequent disease state of the individual. Maternal obesity or the use of ART have been associated with adverse outcomes in the offspring including increased adiposity and blood pressure which are both associated with the development of CVD. Obese women have an increased risk for infertility, and the number of obese women who seek ART to conceive is increasing. Little is known about the effect that these two maternal environments have on the offspring when both are present simultaneously or what mechanism is involved in the increased blood pressure found in studies involving ART or maternal obesity.

High blood pressure (hypertension) has been associated with inward remodeling of the vasculature. Previous research in our laboratory suggests that the matrix metalloproteinases (MMPs) are involved in this inward remodeling. MMP activity can be increased by NADPH Oxidase (NOX)-derived reactive oxygen species (ROS), and decreased by the endogenous tissue inhibitors of matrix metalloproteinases (TIMPs).

We hypothesize that obesity and ART independently and synergistically adversely affect the cardiovascular health and body weight of the offspring. We further hypothesize that these two suboptimal maternal environments are associated with higher blood pressure in the offspring, and that this is mediated by the MMPs, TIMPs and NOXs. The research presented in Chapter III of this thesis tested these hypotheses in a mouse model.

CHAPTER 3

Effects of the Use of Assisted Reproduction and High Caloric Diet Consumption on Body Weight and Cardiovascular Health of Juvenile Mouse Offspring

3.1 Abstract

Maternal obesity and the use of assisted reproductive technologies (ART) are two suboptimal maternal environments that can lead to offspring obesity and cardiovascular disease. We hypothesized that these environments independently and synergistically adversely affect the offspring's weight and cardiovascular performance at ~7 weeks of age. Mice were fed either 24% fat and 17.5% high fructose corn syrup (HF) or maintenance chow (5% fat; LF). Dams were subdivided into no-ART and ART groups. ART embryos were cultured in Whitten's medium and transferred into pseudopregnant recipients consuming the same diet as the donor. Offspring were fed the same diet as the mother. Body weights were measured weekly and mean arterial pressure (MAP) was collected through carotid artery catheterization at sacrifice (55 ± 0.5 days old). Expression of genes involved in cardiovascular remodeling was measured in thoracic aorta using qRT-PCR, and levels of reactive oxygen species (ROS) were measured intracellularly and extracellularly in mesenteric resistance arteries. ART resulted in increased body weight at weaning, but this effect decreased over time and diet was the predominant determinant of body weight by sacrifice. Males had greater MAP than females ($p=0.002$) and HF consumption was associated with greater MAP regardless of sex ($p<0.05$). Gene expression was affected by sex ($p<0.05$) and diet ($p<0.1$). Lastly, the use of ART resulted in offspring with increased intracellular ROS ($p=0.05$). In

summary, exposure to an obesogenic diet affects weight, MAP, and gene expression while ART increases oxidative stress in mesenteric resistance arteries of juvenile offspring, no synergistic effects were observed.

3.2 Introduction

Developmental priming and fetal programming have emerged as important hypotheses that partly explain the surge in obesity and cardiovascular disease (CVD) currently observed worldwide (WHO 2013a, WHO 2013b). These hypotheses, collectively known as developmental origins of adult disease, assert that the uterine environment has profound effects on the body composition and cardiovascular performance of the offspring in later life (Hales 1992, Lawlor *et al.* 2004).

Recent research has shown that maternal obesity, over-nutrition, and diabetes increase the incidence of glucose intolerance, arterial endothelial dysfunction, arterial stiffness, hypertension, and CVD in the offspring in humans and mice (Wildman *et al.* 2003, Samuelsson *et al.* 2008, Samuelsson *et al.* 2010, Kelsall *et al.* 2012, Torrens *et al.* 2012, Magliano *et al.* 2013). In mice, maternal obesity can result in progenies with increased body weight (Samuelsson *et al.* 2008, Franco *et al.* 2012, Magliano *et al.* 2013). Further, Torrens *et al.* showed that offspring exposed to maternal obesity had decreased production of the vasodilator nitric oxide (NO) , a blunted vasodilatory response to acetylcholine in the femoral artery, as well as increased systolic blood pressure at 30 weeks of age (Torrens *et al.* 2012). Maternal high caloric intake in mice

has also been linked to impaired vascular responses and increased blood pressure of the offspring during adulthood (Samuelsson *et al.* 2008).

The use of Assisted Reproductive Technologies (ART) has been associated with adverse outcomes in children (Olson *et al.* 2005, Kallen *et al.* 2010, Wen *et al.* 2010). Studies have shown that the use of *in vitro* fertilization (IVF) results in an increased risk for congenital malformations such as cardiac septal defects, neural tube defects and cleft palate (Olson *et al.* 2005, Reefhuis *et al.* 2009, Kallen *et al.* 2010). In addition, Wen *et al.* found that the risk for congenital heart defects with the use of ART was amplified when the mother was obese (Wen *et al.* 2010).

Retrospective studies in human (Wikstrand *et al.* 2008, Ceelen *et al.* 2009, Sakka *et al.* 2010) have also pointed at the method of conception as a causative factor for abnormal cardiovascular parameters in children during postnatal development. For example, it has been demonstrated that children conceived by the use of ART have increased systolic and diastolic blood pressure, increased vascular dysfunction, and decreased retinal vascular branching (Ceelen *et al.* 2008, Wikstrand *et al.* 2008, Sakka *et al.* 2010, Scherrer *et al.* 2012). Similarly, in mice, an association exists between ART and an elevated systolic blood pressure (Watkins *et al.* 2007). Further, increased angiotensin converting enzyme (ACE), which cleaves angiotensin I into the vasoconstrictor angiotensin II, has also been observed in mice offspring conceived via ART (Watkins *et al.* 2007). Lastly, the use of ART has been associated with increased adiposity in children (Ceelen *et al.* 2007, Belva *et al.* 2012). For example, children conceived by IVF and ICSI have higher levels of peripheral adiposity than age matched naturally conceived controls at 8-18 years of age (Ceelen *et al.* 2007, Belva *et al.* 2012).

Cardiovascular disease is the number one cause of death. In 2008 CVD accounted for 30% of deaths worldwide (WHO 2013b). Obesity and hypertension are two important contributors to the high incidences of CVD mortality worldwide (WHO 2013b). The development of hypertension has been proposed to involve the matrix metalloproteinases (MMPs) and reactive oxygen species [ROS;(Martinez-Lemus & Galinanes 2011)]. ROS can mediate the expression and activation of MMPs (Siwik *et al.* 2001, Martinez-Lemus *et al.* 2011, Zhang *et al.* 2013). In the vasculature, ROS are mainly produced by the enzyme NADPH oxidase [NOX; (Cai *et al.* 2003)]. MMPs are involved in processes such as the degradation of the extracellular matrix (ECM), the cleavage of extracellular receptors (Rodrigues *et al.* 2010), disruption of cellular adhesions (Yang *et al.* 2007) and shedding of vasoactive factors within the ECM (Hao *et al.* 2004). Specifically, MMP2 and MMP9 have been implicated in vascular remodeling, and MMP7 might also be involved as it appears to play a role in hypertension (Lehoux *et al.* 2004, Wang *et al.* 2009, Martinez-Lemus *et al.* 2011). Tissue Inhibitors of MMPs (TIMPs) are endogenous inhibitors of the MMPs, and an imbalance between TIMPs and MMPs has been implicated in pathological conditions (Gomez *et al.* 1997).

Maternal obesity and the use of ART often coincide because many women of reproductive age face infertility, and the number of them who attend ART clinics is rising (Robker 2008). It is clear that obesity and ART are each predisposing children to CVD. However, given the short history of the use of ART, no studies have yet addressed the threat of CVD from the combination of the two suboptimal maternal environments, despite the large number of children who have been exposed to it. In the current study we hypothesize that obesity and ART independently and synergistically adversely affect the

cardiovascular health and body weight of the offspring. We further hypothesize that these two suboptimal developmental environments are associated with higher blood pressure in the offspring, and that this is associated with expression of the MMPs, TIMPs and NOXs in the vasculature. To test these hypotheses, two suboptimal developmental environments were used; namely, those that exist with an obesogenic environment and with the use of ART. We sought to determine what effect these two environments, either alone or in combination, would have on cardiovascular health markers in juvenile mice offspring (~7 weeks of age). The measures of cardiovascular health chosen in this study were MAP, the expression of genes implicated in poor cardiovascular health and vascular remodeling (*Mmp2*, *Mmp9*, *Timp1*, and *Nox2*), and levels of intracellular and extracellular ROS. We also measured offspring body weight weekly until sacrifice, as high body weight has been associated with adverse cardiovascular outcomes (NIH 2012).

3.3 Materials and Methods

a) Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Missouri under protocol number 7501. The strain of the females used for this experiment was NSA (CF1; Harlan Laboratories, Indianapolis, IN, USA) while the strain of the studs and vasectomized males was B6D2F1/J (The Jackson Laboratory, Bar Harbor, ME, USA). Females were between 6 and 10 weeks of age, and were housed under 12 hour (h) light/dark cycle between 20°C and 23.3°C in groups until

conception when they were individually caged. Mice were given *ad libitum* access to water and the appropriate diet.

b) Diet

Experimental animals were fed either a "high fat, high fructose" (HF) or "low fat, no fructose" (LF) diet. The HF group were fed a diet containing 24% fat and 17.5% high fructose corn syrup from TestDiet 58Y1 (TestDiet ; St. Louis, MO, USA) *ad libitum*. The calories (kcal%) provided by the HF diet are as follows: protein = 17.6%, carbohydrate = 36% and fat = 46.4%. The females in the LF group were fed a diet containing 5% fat (measured by ether extraction; Lab Diets 5001 PMI Nutritional International St. Louis, MO, USA) *ad libitum*. The calories (kcal%) provided by the LF diet is a follows: protein = 28.5%, carbohydrate = 58% and fat = 13.5%. The LF females were given Lab Diet 5008 (6.5% fat) during pregnancy and lactation. The calories (kcal%) provided by the LF pregnancy diet are as follows: protein=26.8%, carbohydrate=56.4% and fat=16.7%. The HF diet administered prior to and during pregnancy and lactation was TestDiet 58Y1 (46% kcal fat with corn syrup). Note: the terms "maternal and offspring consumption of HF diet" and "exposure to an obesogenic diet" will be used interchangeably.

c) Control (no ART) Groups

The control females were fed the HF or LF diet at least 3 weeks prior to being paired with proven fertility B6D2F1/J males. Three weeks were chosen because this is the approximate time required for oocyte development in mice (Eppig *et al.* 2002). Copulation was confirmed by visual observation of a copulatory plug. Pregnant females

were placed in a separate cage and were allowed to carry the pregnancy to term. Twelve total control females were paired with fertile males (6 HF and 6 LF), but one HF female did not become pregnant. After birth, the litters were culled to 8 pups (4 males and 4 females, when possible), to help control for the smaller litter sizes in the ART groups.

d) Assisted Reproduction Groups

Three ART procedures were used in this experiment, namely, superovulation, embryo collection and culture, and embryo transfer in order to simulate three procedures commonly used in human ART.

Superovulation

Females of 6-10 weeks of age fed either HF or LF diet for three weeks before superovulation received an intraperitoneal (IP) injection of 5 IU equine chorionic gonadotropin (eCG; Calbiochem La Jolla, CA, USA) followed by 5 IU of human chorionic gonadotropin (hCG; Sigma St. Louis, MO, USA) 45 h later. The injections were given to increase the number of oocytes ovulated by each animal. Superovulated females were then co-caged overnight with B6D2F1/J intact males.

Embryo Collection and Culture

Two-cell embryos were harvested from the oviduct approximately 46 h post-hCG injection. Oviducts were flushed with warm bicarbonate-free minimal essential medium (Earle's salt) supplemented with 3 mg/ml polyvinylpyrrolidone (PVP) and 25 mM Hepes (MEM+PVP [pH 7.3]; Sigma St. Louis, MO, USA). Embryos were washed from debris with three consecutive washes in MEM+PVP and once in Whitten's medium. Whitten's medium was prepared as previously published by us (Negron-Perez *et al.*

2013). Whitten's medium has previously been shown to support development to term (Ecker *et al.* 2004, Sommovilla *et al.* 2005), although it is known to be a suboptimal culture medium (Rinaudo & Schultz 2004). We purposely used this medium to determine whether the cardiovascular measures examined in the current study would be adversely affected by a suboptimal culture medium, as such findings could direct the focus of future studies in more favorable culture media. The embryos were cultured at 37°C in Whitten's medium in an atmosphere of 5% CO_2 in air at a density of 1 embryo per ~3.5 µl of medium. Embryos were cultured for 3 days (or 116 h post-hCG) at which time zona-enclosed blastocysts were transferred to the uteri of day 2.5 (d2.5) pseudopregnant NSA (CF1) females.

Embryo Transfers

Females designated as embryo recipients [NSA(CF1)] were fed HF or LF diets at the same time as the embryo donors prior to receiving the superovulation protocol described above. Immediately after the hCG injection, the females were co-caged overnight with vasectomized B6D2F1/J males. Females which showed a copulatory plug the following morning (denoted as d0.5 of pseudopregnancy) were selected as potential embryo recipients. A plane of anesthesia was reached by an IP injection of the 2.5% (w/v) Avertin stock at 0.014 ml/g body weight (Nagy A 2003). Twenty blastocyst-stage embryos produced as described above were transferred (ten/uterine horn) to embryo recipients on d2.5 of pseudo-pregnancy according to standard procedures (Nagy A 2003). Pseudopregnant recipients were consuming the same diet as the embryo donor. Pregnant recipients were returned to their cages and allowed to carry their pregnancies to term.

Forty-five transfers were performed (22 LF and 23 HF). Nearly 27% of transfers resulted in pregnancy (determined by birth of offspring; Supplementary Table 1).

e) Pregnancy, Lactation and Weaning

All females were maintained on the diet they had been consuming prior to becoming pregnant until their role in the experiment was completed at weaning. Offspring were weaned from the mothers at 22 days post-partum. They were separated by sex and placed on the same diet that the mothers had consumed until sacrifice.

f) Measurement of Offspring Body Weights

Body weight was recorded using a standard scale (Mettler Toledo; Columbus, OH, USA) five days after birth, and every week thereafter until sacrifice. Control litters were culled to eight (4 males and 4 females when possible) at the 5-day weighing to control for the larger litter sizes in the control groups. No identification marks were used prior to weaning; therefore, weights were recorded separately for males and females from each litter and averaged each week during the first three weeks. Each individual received an identifying ear punch at weaning, and weights were recorded by individual from that point on.

g) Mean Arterial Pressure Measurement

Offspring male (n=37) and female (n=31) mice 55.5 ± 0.5 (n=68) days old were anesthetized by inhalation of 5% isoflurane until visible loss of consciousness. At that point, mice were placed in dorsal recumbency and maintained under anesthesia at 3% (v/v) through a cone covering the nose and mouth until surgical plane anesthesia was confirmed by loss of spinal reflexes. The right carotid artery was cannulated with a

polyethylene catheter (PE-10) filled with phosphate-buffered physiological saline solution containing 100 U/mL of heparin. The catheter was connected to a pressure transducer in order to measure mean arterial pressure (MAP) with the use of a PowerLab 4/30 Data Acquisition System (ADInstruments Colorado Springs, CO,USA). Following catheterization, the level of anesthesia was lowered to 2% (v/v) for 10 min., and the average systolic and diastolic pressures recorded during the final minute were used to calculate MAP. It should be noted that three animals had bleeding during catheterization, and were excluded from the MAP analysis as blood loss can affect pressure.

h) Mesenteric Resistance Artery and Thoracic Aorta Collection

Subsequent to MAP measurement, animals were euthanized under anesthesia. A portion of the mesentery was excised and pinned flat in a refrigerated (4 °C) dissecting chamber containing physiological saline solution (PSS) of the following composition (in mmol/L): 145.0 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 1.0 NaH_2PO_4, 5.0 dextrose, 3.0 3-(N-morpholino) propanesulfonic acid (MOPS) buffer, 2.0 pyruvate, 0.02 EDTA, and 0.15 bovine serum albumin (BSA), pH 7.4. Small pieces (~3mm length) of second order mesenteric resistance arteries were isolated and stored at -70°C for subsequent analysis of ROS using high performance liquid chromatography (HPLC). A small section (~5mm length) of the thoracic aorta was also collected and stored at -70°C for quantitative RT-PCR analysis. Due to time constraints for the processes performed, tissues were only collected for two animals per day. In order to keep the age average similar between the ART and No ART groups, 34 of the 36 ART offspring and 34 of the 87 No ART controls were collected and used for the remainder of the study. The collected offspring were selected to have at least one male and one female for each mother. Two or three offspring

were collected from all of the control females except for the last LF control female (three males and three females were collected) and the last two HF control females (two males and two females were collected). All ART-conceived pups were collected except for two HF males who had advanced too far in age by the time of collection.

i) Dihydroethidium Incubation of Single Mesenteric Resistance Arteries for Detection of ROS

Isolated pieces of mesenteric resistance arteries were taken from the -70°C freezer, placed in 99 µL 1x phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) with 100 µM DTPA (Sigma-Aldrich St. Louis, MO, USA) and incubated in a 37°C water bath for 10 min. The 100 µM DTPA was prepared as previously described (Buettner 2008). Briefly, 0.1967 g DTPA was added to 1.7 mL 1M NaOH to make the DTPA go into solution more easily. The flask was then filled to approximately 40 mL with ultrapure H_2O. The solution was sonicated to bring the DTPA into solution. Then, the solution was adjusted to a pH of 7.1 using a 1M HCl solution before bringing the solution to a final volume of 50mL with ultrapure H_2O. A 5 mM stock solution of dihydroethidium (DHE) was made by dissolving 1 mg DHE (Molecular Probes-Life Technologies Grand Island, NY, USA) in 634 µL DMSO (Sigma St. Louis, MO, USA). The resulting solution was separated into 15 µL aliquots and lyophilized for 2 h. The lyophilized DHE aliquots were stored at -20°C until use. On each day of the experiment, the lyophilized DHE was resuspended in 15 µL pure HPLC grade acetonitrile. After the initial incubation, 1 µL of a 5 mM DHE stock in acetonitrile was added to the solution bathing the vessel to bring the final concentration to 50 µM DHE. Ambient light was avoided to diminish excess oxidation of the DHE. The resistance

artery was incubated with DHE at 37°C in the water bath for 1h, with gentle agitation every 10 min. The vessel was then removed and washed twice, consecutively, in 400 µL 1xPBS.

j) Fluorescence Confocal Microscopy for Detection of Intracellular ROS

After being washed in 1xPBS, the vessels were placed on a glass slide in a drop of 1xPBS and covered with a 1.5 thickness coverslip. Two random areas were chosen to be imaged for each vessel. Images were obtained using a Leica True Confocal Scanning-Spectral Photometric 5 DMI 6000 Confocal Laser Scanning Microscope (Leica TCS SP5). A 3D image was obtained for each section at a photomultiplier gain of 800 V using a 20x dry lens. DHE was detected using a multi-photon excitation of 800 nm and an emission detection in the range of 600-700 nm. DHE has previously been used to detect superoxide in arterioles (Martinez-Lemus *et al.* 2011), as it forms a red fluorescent product upon oxidation (Owusu-Ansah *et al.* 2008). Laser scanning was performed using a resonator set at 8000 Hz to reduce photo bleaching. Z-sections were set at 0.5 µM to cover the entire vessel. All images were taken at the same excitation power and detection perameters. Analysis of relative DHE fluorescence was performed using Imaris 7.6.1 software over an area of 150x150x20 pixels for each vessel. Values for DHE fluorescence were averaged over the two areas of the vessel that were imaged. Readings for intracellular ROS were excluded if there was discrepancy in the relative DHE fluorescence between the two areas on the vessel from the same individual.

k) High Performance Liquid Chromatography (HPLC)

HPLC was performed using 50 µL of the original DTPA+1xPBS+DHE solution used to bathe the resistance artery in order to detect the oxidation of DHE outside the cells, which is a measure of extracellular ROS production. A C8 reverse phase column (Phenomenex Torrence, CA, USA) was used to separate the two byproducts of DHE oxidation, 2-hydroxyethidium (2-OH-Et) and ethidium (Et). The phase solutions used for product separation were Solution A (ultrapure water), Solution B (99.9% v/v water with 0.1% v/v TFA), and Solution C (99.9% v/v acetonitrile with 0.1% v/v TFA; Fischer Pittsburgh, PA, USA). The samples were placed in a Waters 2590 Separation Module and 2-OH-Et and Et fluorescence were detected using a Waters 474 Scanning Fluorescence detector set at an excitation of 510 nm and emission of 595 nm. Each day before running samples, the column was cleaned by running 99.9% v/v acetonitrile with 0.1% v/v TFA for 20 min. at a flow of 1 ml/min., followed by 100% ultrapure water at a flow of 0.5 ml/min. for 20 min. This cleaning was repeated after every 4 or 5 samples to stop the pressure in the column from increasing. For each sample being run, a gradient of acetonitrile (v/v) in water was used to elute the samples. The gradient was run at a flow of 0.4 ml/min. with an increase in acetonitrile from 10% to 46% in the first 10 min., an increase again to 100% from min. 20 to 25, and ending with the original 10% acetonitrile from 25 to 35 min. to clean the column. The 2-OH-Et eluted at 16.19 ± 0.03 min. and the Et eluted at 16.61 ± 0.03 min. (See Fig. 3.4A for a representative example). The Empower program (Waters, Build #1154) was used to extract the areas under the 2-OH-Et and Et peaks detected by the fluorescence detector. One vessel for a LF No ART

control male was lost during transfer to the DHE incubation step, so this observation is not included.

l) Homogenizing Vessels for Protein Measurement

An Omni Bead Ruptor 24 Homogenizer (Omni International Kennesaw, GA, USA) was used to pulverize the resistance arteries. After the confocal image was taken, the vessels were placed in a 0.5mL tube with a cap (Omni International Kennesaw, GA, USA) with 100 µL pure HPLC grade acetonitrile and 50 mg of 0.1 mm glass beads (Omni International Kennesaw, GA, USA). The program for homogenization was performed at 22°C. There were 3 cycles, each at a speed of 8 m/s for 45 sec., with a 3 sec. wait time between cycles. The tubes were centrifuged briefly after homogenization to ensure the beads were at the bottom of the tube, and 60 µL of the supernatant was recovered. This supernatant was sonicated for 1 min. to further lyse the tissue. Then, the solution was centrifuged at 2000xg for 10 min., and 50 µL of the supernatant was removed. The remaining 10 µL was used for protein measurement using a Micro BCA Kit.

m) Protein Assays using MicroBCA Kit

Each sample was diluted 1:4 (v/v) in pure HPLC grade acetonitrile (Fischer Pittsburgh, PA, USA) prior to protein detection. The Micro BCA^{TM} Protein Assay Kit (Pierce Rockford, IL, USA) was used to detect the concentration of protein for each sample. A standard curve with bovine serum albumin (BSA) was generated using a blank (pure acetonitrile) and the concentrations from 1 µg to 200 µg/mL in acetonitrile. The working reagent was prepared in accordance with manufacturer's instructions.

Reactions were prepared by mixing 4 µL of the BSA standard or diluted sample with 4 µL of the working reagent. The reactions were incubated at 60°C for 1 h, and then allowed to cool to room temperature. A Nanodrop spectrophotometer was used to measure the absorbance of each sample at 562 nm. A standard BSA curve was performed each day, and the sample absorbances were plotted against the standard curve to determine the concentration in µg/mL.

n) RNA Extraction from Thoracic Aorta and RT-PCR

RNA isolation was performed on thoracic aorta segments using the DynaBEADS mRNA DIRECT KIT (Invitrogen, Grand Island, NY, USA). The DynabeadOligo (dT)25 beads were equilibrated according to the manufacturer's instructions. For RNA and DNA isolation, thoracic aorta segments were placed in 200 µL lysis buffer (100 mM Tris-HCl, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM dithiothreitol), pulverized with a DNase-RNase free pestel and then sheared by passing through a 20 gauge needle followed by a 22 gauge needle. Isolation of mRNA separated from the beads was then performed following manufacturer's specifications. The final wash of the beads was performed in 20 µL sterile water, and then heated at 80°C for 2 min. to separate the mRNA from the beads. The water with the mRNA was quickly removed from the beads, and 3 µL was used for the creation of a 60 µL cDNA reaction using 100 U Superscript II Reverse Transcriptase (Invitrogen, Grand Island, NY, USA) and the following (1X First-Strand Buffer, 10 mM DTT, 0.8 mM dNTPs, 0.5 µg random primers, 44 U RNasin). The reaction was incubated at 42°C for 1 h followed by 95°C for 10 min. One microliter of the resulting cDNA was used for each qRT-PCR reaction. Procedures were followed using manufacturer's instructions, thus a template quantification step was not used.

o) Quantitative Real-Time PCR for Gene Expression in Thoracic Aorta

The TaqMan Gene Expression Assays (Applied Biosystems Life Technologies Grand Island, NY, USA) used are shown (Table 3.1). No DNase treatment was performed as cDNA preparation was performed per the manufacturer's instructions. However, as an extra precaution and to ensure no DNA amplification, all TaqMan probes used were intron-spanning. After reverse transcription, cDNA (1 µL) underwent real-time PCR amplification with 10 µL 2X TaqProbe qPCR Mastermix-low ROX (BEQPCR-PL; MidSci St. Louis, MO, USA), 8 µL sterile water, 1 µL 20X TaqMan Gene Expression Assays probe [(Life Technologies Grand Island, NY, USA); see Table 3.1 for probe information]. The reactions were performed in triplicate using a 7500 Real Time PCR Machine (Applied Biosystems Grand Island, NY, USA) with the following cycles: 50°C-2min.; 95°C-1min.; 40 cycles of 95°C-15sec.; 60°C 1min. Genes analyzed were *Mmp2* (Mm00439498_m1), *Mmp7* (Mm00487724_m1), *Mmp9* (Mm00442991_m1), *Timp1* (Mm00441818_m1) and *Cybb* (*Nox2*; Mm01287743_m1). The average threshold cycles (C_T) for the cDNA were normalized to the expression level of beta-2 microglobulin (*B2m*; Mm00437762_m1). *B2m* is a gene involved in immune response as a member of the major histocompatability complex class 1, and is not involved in energy metabolism (Shertzer *et al.* 2013). *B2m* has been previously shown to be stable in high-fat diet induced oxidative stress in adipose tissue (Bailey-Downs *et al.* 2013), and in response to oxidative stress in the brain (Shertzer *et al.* 2013) and lung (Shimada *et al.* 2009). A C_T above 35 was considered to be not expressed, and measurements with standard deviations above 0.5 were discarded and repeated. Two LF male ART offspring were unable to be analyzed for gene expression due to complications

during mRNA isolation. Five or six No ART controls were selected randomly from each group (HF males, LF males, HF females, LF females) to be used for gene expression analysis.

p) Statistical Analysis

The logarithms of offspring body weights were regressed on the ART effect (pregnancy through ART or not), diet effect (HF or LF), sex effect (female or male), time effect (weaning, 4^{th} week, 5^{th} week, 6^{th} week, and 7^{th} week since birth), as well as interaction effects including sex by diet, ART by time, and diet by time. Mothers are random effects in the model to capture the correlations between offspring that have the same mother. Since repeated measurements for the same offspring were taken at all time points, compound symmetry correlation structure was used to model the correlations among these observations. The model was constructed and chosen by biological considerations and diagnostic statistics. The main effects and interaction effects were all statistically significant at level 0.05. The compound symmetry correlation structure for modeling the repeated measurements on the same offspring was chosen for its highest likelihood and lowest Akaike information criterion (AIC), Bayesian information criterion (BIC) statistics. The studentized residual plot and normal quantile-quantile (QQ) plot suggest a consistent normality assumption, and do not indicate any obvious outliers.

At the time of sacrifice, correlations were computed and tested between the logarithms of body weights, intracellular ROS, extracellular ROS (2-OH-Et and Et), and the differences in cycle thresholds of *Mmp2*, *Mmp9*, *Timp1* and *Nox2*.

Table 3.1 TaqMan probes used for quantitative RT-PCR analyses in mouse thoracic aorta

Name (NCBI accession no.)	Sequence	Amplicon Length (bp)
B2m (NM_009735.3)	CGGCCTGTATGCTATCCAGAAAACC	77
Mmp2 (NM_008610.2)	ACCAGATCACATACAGGATCATTGG	62
Mmp7 (NM_010810.4)	GGAACAGGCTCAGAATTATCTTAGA	128
Mmp9 (NM_013599.2)	TCCAGTACCAAGACAAAGCCTATTT	76
Timp1 (NM_001044384.1, NM_011593.2)	CTGCAACTCGGACCTGGTCATAAGG	90
Cybb [*Nox2* (NM_007807.4)]	AGTGAACACCCTAACACCACAATAG	63

NCBI= National Center for Biotechnology Information. No.= number. Bp= base pairs.

We also examined the associations between the diet, ART, and sex effects with MAP and gene expression. The significance of each of the three effects (diet, ART and sex) was tested within fixed levels of the other two. For example, the association between diet and MAP (or gene expression) was tested within fixed levels of ART and sex.

3.4 Results

a) Effects of ART and Diet on Body Weight from Weaning to Sacrifice

Increased offspring body weight has been observed separately in models of maternal obesity and ART (Ceelen *et al.* 2007, Samuelsson *et al.* 2008, Nelson *et al.* 2010, Belva *et al.* 2012, Morandi *et al.* 2012). We sought to determine whether the combination of ART and high fat diet consumption by the mothers and offspring would amplify the increase in body weight expected in the offspring. The use of ART resulted in offspring with increased body weight when compared to no ART controls (No ART; $p<0.0001$) at weaning (*i.e.* 22 days of age; Fig. 3.1A). However, this effect diminished over time as the No ART group began to catch up in growth and resulted in non-significant body weight differences at sacrifice (*i.e.* 55 ± 0.5 days; $p=0.1052$). Additionally, maternal and offspring consumption of a HF diet resulted in increased body weight of the offspring from weaning ($p<0.0001$) until sacrifice (Fig. 3.1B**;** $p<0.0001$). Male offspring weighed significantly more than females from weaning until sacrifice ($p<0.0001$; Fig. 3.1C). No effect of the interaction between diet and ART was observed in this study ($p>0.05$; data not shown). However, there was an interaction between diet and sex. Males in the HF group had a greater increase in body weight when compared

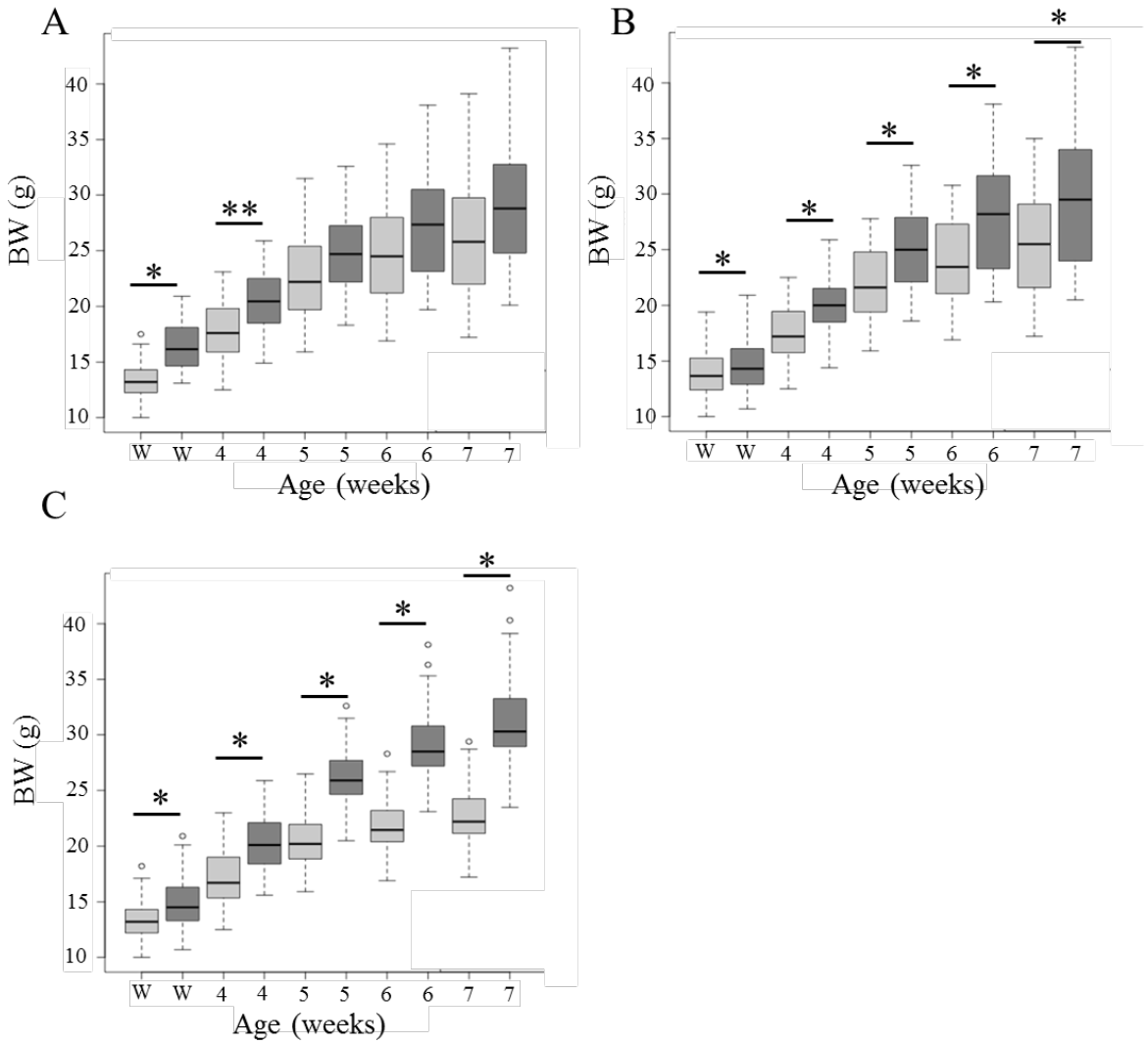


Figure 3.1. Effects of ART, diet and sex on mouse offspring body weight from weaning until seven weeks of age. **A.** Body weights for all offspring conceived by natural conception (No ART; n=87; light grey bars) or with the use of ART (ART; n=36; dark grey bars). **B.** Body weights for all offspring conceived by from mothers consuming a maintenance chow (LF; n=68; light grey bars) or a diet containing 24% fat and 17.5% high fructose corn syrup (HF; n=55; dark grey bars). **C.** Body weights of females (n=60; light grey bars) and males (n= 63; dark grey bars). Comparisons were made at weaning (22 days), week 4 (26 days), week 5 (33 days), week 6 (40 days) and week 7 (47 days). The middle bar in a box shows the median (50%) body weight. The upper and lower borders of a box show the 1st and 3rd quartiles (25% and 75%, respectively) of a group body weight. The bars above or below the dash lines mark the body weights that are 2.5 times of a quartile away from the median. Dots outside of bars are even further away from the median. ART = assisted reproductive technologies. BW = body weight. Solid black lines with asterisks above are used to demarcate statistical differences between groups at given times after birth. * = $p<0.0001$, ** = $p<0.05$.

to the LF males at sacrifice than the HF females did when compared to the LF females ($p<0.03$; 34.03 ± 0.71 g vs. 28.77 ± 0.41 g and 24.12 ± 0.48 g vs. 21.91 ± 0.37 g, respectively).

b) Effects of Sex and Diet on Mean Arterial Pressure

Hypertension is another common risk factor for the development of CVD (WHO 2013b). We used mean arterial pressure (MAP; 1/3 systolic + 2/3 diastolic pressure) as a measure of blood pressure in the offspring. Overall, males had a higher MAP than females at sacrifice ($p=0.002$; Fig. 3.2A). This increased MAP was associated with an increased body weight at seven weeks of age in males when compared to females ($p<0.0001$; Fig. 3.1C). Further, MAP was positively correlated with body weight at sacrifice ($p<0.02$; Table 3.2). Diet also had an effect on offspring MAP ($p<0.05$) after taking into account ART and sex effects. When offspring consumed a HF diet after developing in a HF maternal environment, they had higher MAP than offspring that had been exposed to a LF diet throughout (Fig. 3.2B). We did not observe increased blood pressure due to ART ($p>0.05$, Supplemental Fig. 3.1).

c) Gene Expression in Response to Diet and Sex

Altered activities of MMP2, MMP7, MMP9, TIMP1 and NOX-derived ROS have been associated with CVD (Gomez *et al.* 1997, Lehoux *et al.* 2004, Wang *et al.* 2009). Further, MMP2 and 9 have been suggested to be involved in ROS-dependent vascular remodeling associated with hypertension (Martinez-Lemus *et al.* 2011). We examined gene expressions of *Mmp2, Mmp7, Mmp9, Timp1* and *Nox2* in the thoracic aorta of offspring from the different treatment groups to determine if these possible markers of

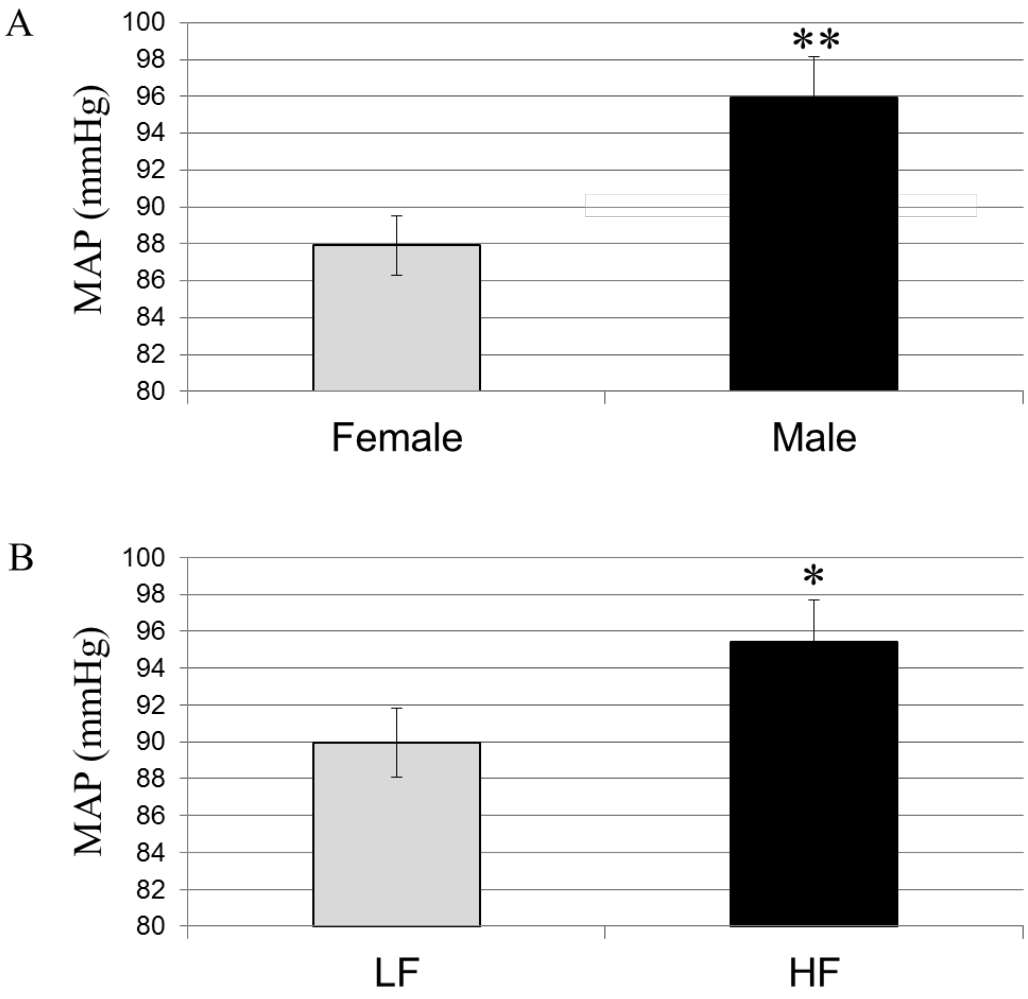


Figure 3.2. Mean arterial pressure (MAP) in offspring at sacrifice. Carotid artery catheterization was performed to measure MAP as a representation of blood pressure in the offspring immediately prior to sacrifice (55 ± 0.5 days). A. MAP in female (n=29) and males (n=33), offspring. **B.** MAP in LF (n=37) and HF (n=25) offspring. The bars represent the mean ± the S.E.M. LF=maternal and offspring consumption of a maintenance chow. HF=maternal and offspring consumption of a diet containing 24% fat and 17.5% high fructose corn syrup. *$p<0.05$. **$p<0.01$.

Table 3.2 Table of significant correlations between measurements in mouse offspring.

	Mmp2	*Mmp9*	*Timp1*	*Nox2*	2-OH-Et	Et	SacBW	MAP
Mmp2		**0.71722** **<0.0001** **53**	**0.74208** **<0.0001** **53**	**0.37357** **0.0059** **53**	**-0.36414** **0.0080** **52**	**-0.27963** **0.0447** **52**	**0.31696** **0.0208** **53**	0.22528 0.1120 51
Mmp9	**0.717** **<0.0001** **53**		**0.63446** **<0.0001** **53**	**0.54996** **<0.0001** **53**	**-0.38716** **0.0046** **52**	**-0.36276** **0.0082** **52**	0.25921 0.0609 53	0.11085 0.4387 51
Timp1	**0.74208** **<0.0001** **53**	**0.63446** **<0.0001** **53**		**0.47868** **0.0003** **53**	**-0.42027** **0.0019** **52**	**-0.29326** **0.0349** **52**	0.17788 0.2026 53	0.15661 0.2742 51
Nox2	**0.373357** **0.0059** **53**	**0.54996** **<0.0001** **53**	**0.47868** **0.0003** **53**		**-0.33790** **0.0143** **52**	-0.25759 0.0652 52	-0.00719 0.9539 53	0.22264 0.1163 51
2-OH-Et	**-0.36414** **0.0080** **52**	**-0.38716** **0.0046** **52**	**-0.42027** **0.0019** **52**	**-0.33790** **0.0143** **52**		**0.84805** **<0.0001** **67**	0.00958 0.9387 67	**-0.26383** **0.0399** **61**
Et	**-0.27963** **0.0447** **52**	**-0.36276** **0.0082** **52**	**-0.29326** **0.0349** **52**	-0.25759 0.0652 52	**0.84805** **<0.0001** **67**		0.09509 0.4440 67	-0.13643 0.2944 61
SacBW	**0.31696** **0.0208** **53**	0.25921 0.0609 53	0.17788 0.2026 53	-0.00719 0.9539 53	0.00958 0.9387 67	0.09509 0.4440 67		**0.29531** **0.0198** **62**
MAP	0.22528 0.1120 51	0.11085 0.4387 51	0.15661 0.2724 51	0.22264 0.1163 51	**-0.26383** **0.0399** **61**	-0.13643 0.2944 61	**0.29531** **0.0198** **62**	

The logarithms of the ΔCT for matrix metalloproteinase 2 and 9 (Mmp2 and 9), tissue inhibitor of matrix metalloproteinase 1 (Timp1), and NADPH Oxidase 2 (Nox2) expression in thoracic aorta, extracellular 2-hydroxyethidium (2-OH-Et) and ethidium (ET) in mesenteric arterioles, body weight at sacrifice (SacBW) and mean arterial pressure (MAP) were compared using correlational analysis. The values in each cell from top to bottom represent the Pearson correlation coefficient, p-value and number of measurements; respectively. Boldface = significance at $p<0.05$. ΔCT= difference in cycle threshold.

cardiovascular health were affected by the two treatments alone or in combination. Offspring who were exposed to an obesogenic diet had lower expression of *Mmp9* and a tendency towards lower expression of *Mmp2* when compared to offspring in the LF group ($p<0.05$ and $p<0.1$, respectively; Fig. 3.3A and C). Further, males had decreased expression of *Mmp2* and the inhibitor *Timp1* ($p<0.05$ and $p<0.04$, respectively; Fig. 3. 3B and D) when compared to females. No difference in expression of *Nox2* was observed between treatment groups ($p>0.05$, data not shown). Also, the use of ART did not show a significant difference in the expression of any of the genes assayed in this study ($p>0.05$, Supplemental Fig. 3.2). *Mmp7* expression was not detected in the thoracic aorta of the offspring from any treatment.

Several correlations with gene expression were observed in the offspring. The expression of all of the genes analyzed (*Mmp2*, *Mmp9*, *Timp1* and *Nox2*) were positively correlated with each other ($p<0.01$; Table 3.2). The differences in cycle thresholds of *Mmp2* also had a positive correlation and *Mmp9* tended to have a positive correlation with body weight at sacrifice ($p= 0.02$ and $p=0.06$, respectively; Table 3.2). Therefore, as the body weight increased, the expression of *Mmp2* decreased and *Mmp9* tended to decrease.

d) Effect of ART on ROS in Offspring Mesenteric Resistance Arteries

Previous research has shown increased oxidative stress, which can occur due to increased ROS, in obesity as well as with the use of ART (Chao *et al.* 2005, Torrens *et al.* 2012). We examined the levels of intracellular or extracellular ROS in mesenteric resistance arteries of the experimental offspring. Offspring produced by ART had

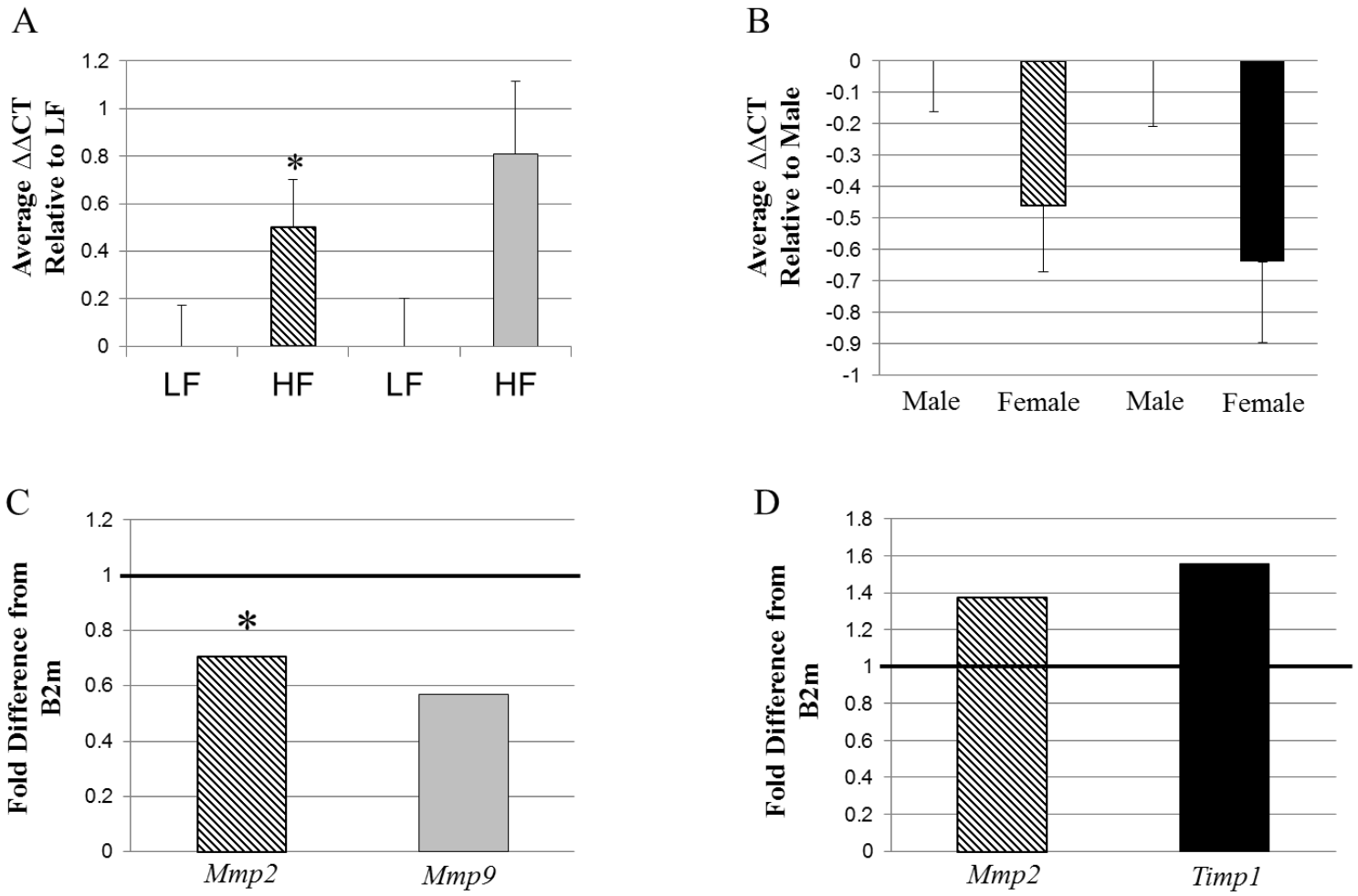

A
Average ΔΔCT Relative to LF
1.2
1
0.8
0.6
0.4
0.2
0
*
LF
HF
LF
HF
B
Average ΔΔCT Relative to Male
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
-0.9
-1
Male
Female
Male
Female
C
Fold Difference from B2m
1.2
1
0.8
0.6
0.4
0.2
0
*
Mmp2
Mmp9
D
Fold Difference from B2m
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Mmp2
Timp1

Figure 3.3

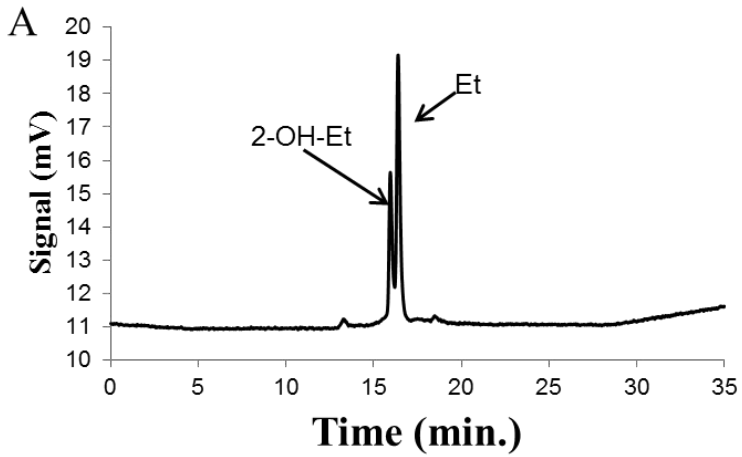
Figure 3.3. Quantitative RT-PCR assessment of the expression of genes potentially involved in inward remodeling in offspring thoracic aortas. All gene expressions were normalized to the expression of *B2m* (ΔC_T) and then averaged within the respective groups. **A**. The relative difference in cycle thresholds ($\Delta\Delta$CT) between the HF and LF treatment groups for *Mmp2* and *Mmp9*. **B.** The relative difference $\Delta\Delta$CT between females and males for Mmp2 and *Timp1*. The error bars represent the S.E.M. The fold differences between treatments groups for each gene analyzed were then calculated. **C** = fold difference calculation of data presented in **A**. **D** = fold difference calculation of data presented in **B**. The solid black line across "1" demarcates "LF" for the two bars in **C** (diet effect) and "males" in **D** (sex effect). *Mmp2* = diagonal line bar; *Mmp9* = gray bar; *Timp1* = black bar. *MMp2* - HF vs. LF, females vs. males; n=24 and 29, n=24 and 29, respectively. *Mmp9* - HF vs. LF n=24 and 29, respectively. *Timp1* - females vs. males; n=24 and 29, respectively. All differences are significant at $p<0.05$, except for the asterisk (*) which represents a trend at $p<0.1$. LF=consumption of maintenance chow by mothers and offspring. HF=maternal and offspring consumption of a diet with 24% fat and 17.5% high fructose corn syrup. ΔC_T = change in cycle threshold. *B2m*= beta 2-microglobulin.

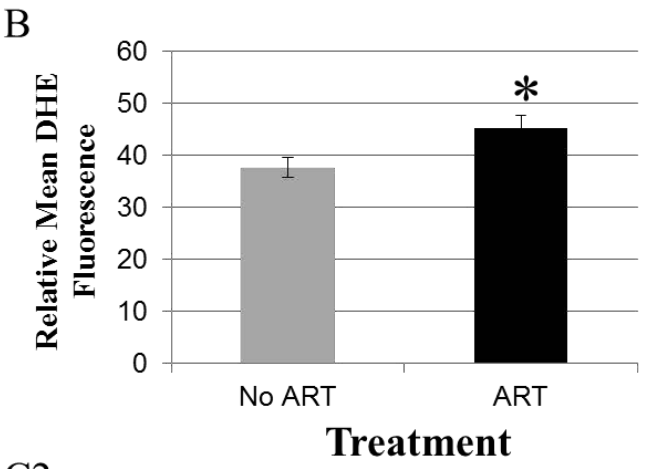
increased intracellular ROS in mesenteric resistance arteries ($p=0.05$; 45.2 ± 2.47 and 37.67 ± 1.97, respectively; Fig. 3.4B-C2). There was no significant difference in intracellular ROS between the HF and LF groups ($p>0.05$; 41.96 ± 2.35 and 40.94 ± 2.27, respectively) or between the males and females ($p>0.05$; 39.57 ± 2.08 and 43.64 ± 2.57, respectively). Further, we did not observe a significant difference in the oxidation level of DHE outside the cells indicating that extracellular ROS did not differ in the mesenteric resistance arteries between treatment groups ($p>0.05$, data not shown).

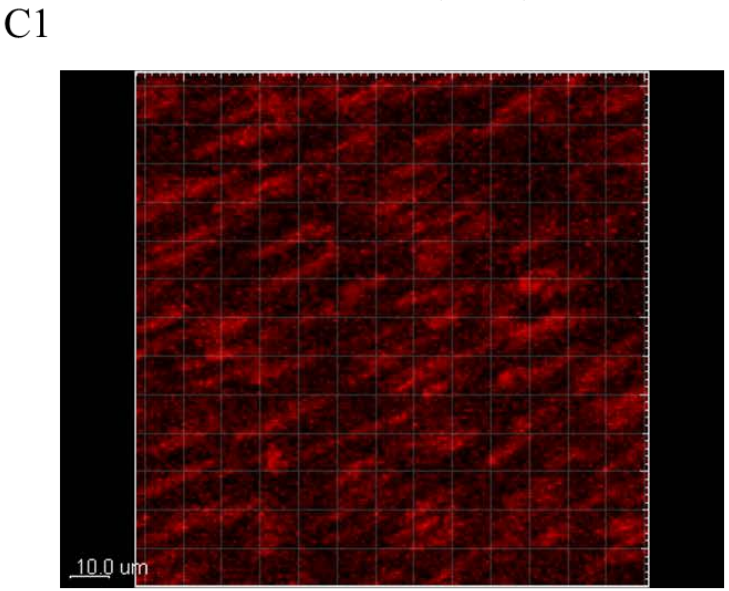
2-OH-Et and Et as measured by HPLC in the extracellular solution containing DHE are a measure of extracellular superoxide (O_2^-) production and non-specific ROS production, respectively. Intracellular ROS did not correlate significantly with either extracellular 2-OH-Et or Et ($p>0.05$, data not shown). When extracellular 2-OH-Et was increased, Et was also increased ($p<0.0001$; Table 3.2). Extracellular 2-OH-Et was negatively correlated with the differences in cycle threshold of *Mmp2*, *Mmp9*, *Timp1* and *Nox2* ($p<0.01$, $p<0.01$, $p<0.01$ and $p<0.02$, respectively; Table 3.2), meaning that as the expression of each of the four genes was increased, extracellular superoxide was also increased. Extracellular Et was negatively correlated with the differences in cycle threshold of all of the genes analyzed (Table 3.2). Also, there was a negative correlation between extracellular 2-OH-Et and MAP (Table 3.2).

3.5 Discussion

Both ART (Ceelen *et al.* 2007, Belva *et al.* 2012) and maternal diet-induced obesity (Samuelsson *et al.* 2008, Nelson *et al.* 2010, Morandi *et al.* 2012) have been shown to produce offspring with increased body weight or increased adiposity. Our


A
Signal (mV)
Time (min.)
2-OH-Et
Et


B
Relative Mean DHE Fluorescence
No ART
ART
Treatment
*


C1
10.0 um

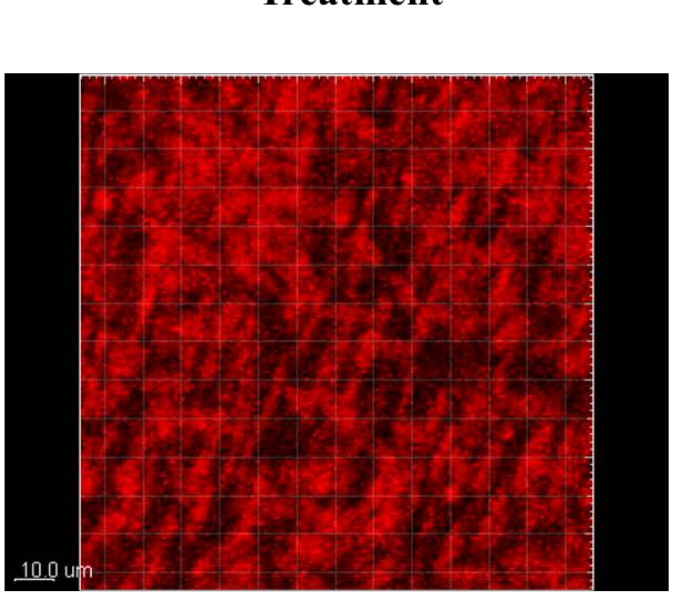

C2
10.0 um

Figure 3.4

Figure 3.4. Detection of extracellular and intracellular ROS in mesenteric resistance arteries of offspring. A. Two fluorescent byproducts of dihydroethidium (DHE) were detected by high performance liquid chromatography (HPLC) in offspring (55 ± 0.5 days) mesenteric resistance arteries. Resistance arteries were incubated with 50 µM DHE for one hour. 2-hydroxyethidium (2-OH-Et; specific byproduct of superoxide and DHE) eluted at 16.19 ± 0.03 min and the ethidium (Et; less specific byproduct) eluted at 16.61 ± 0.03 min. Areas under each curve were determined using the Empower program (Waters, Build #1154) and were normalized to the concentration of protein in each vessel before comparisons between treatments. Protein amounts were with a Micro BCA^{TM} Protein Assay Kit. **B.** Determination of levels of intracellular ROS in offspring mesenteric resistance arteries using confocal microscopy for relative DHE fluorescence. Intracellular DHE fluorescence (red) was measured by confocal microscopy. Gain was set at 800 V. Data represent the group average ± S.E.M. of DHE fluorescence over the average of two areas of the mesenteric resistance arteries for offspring conceived naturally (No ART; gray, n=32) or with the use of ART (ART; black, n=31). **C.** Shown is a confocal micrograph of a longitudinal section of a mesenteric resistance artery from a naturally conceived LF male (**C.1**) and an ART conceived LF male (**C.2**). Red fluorescence results from the oxidation of DHE. Fluorescence was quantified with the Imaris 7.6.1 software. ART = assisted reproductive technologies. DHE = dihydroethidium. ROS = reactive oxygen species. *p=0.056.

study supported these previous findings, as both ART and diet affected offspring body weight. Maternal and offspring consumption of a HF diet resulted in offspring with higher body weight than the LF controls, and this effect persisted and increased from weaning (22 days) until sacrifice (55 ± 0.5 days). However, the effect of ART that we observed on increased body weight in the offspring decreased over time after weaning. This is similar to findings from a previous study (Scott *et al.* 2010), where female mouse offspring conceived using IVF had increased body weight at three weeks of age when compared to no ART controls, but not from four weeks of age until eight weeks of age. Further, one retrospective ART study in humans showed increases in child adiposity rather than body weight (Ceelen *et al.* 2007), while the other showed increases in adiposity and body weight (Belva *et al.* 2012). However, the study by Belva *et al.* (2012) involved intracytoplasmic sperm injection (ICSI) which is a more invasive ART procedure compared to those used in our study. Future studies examining the effect of ART on offspring should look at adiposity in addition to body weight and also the type of ART procedure used to further clarify this issue.

Maternal and offspring diet had a greater effect on body weight in the male offspring compared to females. Males from the HF group weighed significantly more when compared to the LF males than the HF females did when compared to LF females. This finding is supported by previous research that exhibited pronounced adverse metabolic outcomes in males born to an obese mother (Torrens *et al.* 2012, Magliano *et al.* 2013). To our knowledge, no studies have previously been performed to examine the effect of ART and a HF developmental environment together on juvenile offspring body weight. Our study did not show a significant ART and diet interaction, suggesting that

the adverse effects on offspring body weight from these two environments are not amplified by the combination of ART and HF diet consumption, at least at the age examined.

It has previously been reported that increased body weight can predispose an individual to adverse cardiovascular outcomes including hypertension and an increased risk for CVD (NIH 2012, Iguchi *et al.* 2013, Simoes-Silva *et al.* 2013). Data from our study support this previous research. First, males had increased body weight when compared to females from weaning until sacrifice, and this was accompanied by an increased MAP in males compared to females at sacrifice. The difference in MAP between males and females is also supported by previous research showing that estrogens can have a protective effect on blood pressure response [reviewed in (Xue *et al.* 2013)]. Further, MAP had a positive overall correlation with body weight at sacrifice, showing that individuals who were heavier had higher MAP.

Previous research has also shown increased blood pressure in offspring born to mothers consuming a high fat diet (Samuelsson *et al.* 2008, Torrens *et al.* 2012). Results from our study support these findings, as HF diet consumption (both by the dam and the offspring) resulted in increased MAP in the offspring.

In humans, an association has previously been made between the use of ART and increased systolic and diastolic blood pressure in children (Ceelen *et al.* 2008, Sakka *et al.* 2010). These retrospective studies involved children at ages from eight to 18 years old (Ceelen *et al.* 2008) and from four to 14 years old (Sakka *et al.* 2010) which we expect are comparable to the seven weeks of age juvenile mice that we studied. Further, an effect of ART on offspring blood pressure has also been observed in mice (Watkins *et*

al. 2007). Our study, however, did not show increased MAP due to ART. There are several differences between the Watkins *et al.* (2007) work and ours that could partially explain the difference in results. For example, in our study we studied juvenile mice (~ 7 weeks of age) while they used older mice (*i.e.* 15 and 21 weeks of age). In addition, the strain of mice used varied between studies [NSA (CF1) females and B6D2F1/J males vs (CBA X C57/BL6)F1 females and MF1 males for our and their studies, respectively] and the embryo culture medium was different (Whitten's Medium vs T6 Medium, for our and their studies, respectively). Finally our measurement of MAP combines both systolic and diastolic pressure, while in the study by Watkins *et al.* (2007) only differences in systolic blood pressure were measured.

Interestingly, while offspring who were exposed to an obesogenic diet had increased MAP, they had lower expression of *Mmp2* and *Mmp9* when compared to offspring in the LF group. This is counterintuitive to previous studies that have suggested MMP2 and MMP9 to be involved in vascular remodeling associated with hypertension (Lehoux *et al.* 2004, Martinez-Lemus *et al.* 2011). However, in the study by Lehoux *et al.* (2004) MMP9 was shown to be involved in outward remodeling rather than inward remodeling (Lehoux *et al.* 2004), so increased MMP9 has been suggested to initially provide a compensatory response to increased pressure (Martinez-Lemus & Galinanes 2011). Therefore, it is possible that the decreased *Mmp9* expression found in the offspring with increased MAP is representative of a decreased ability of these offspring to compensate for their increased MAP. It should be noted that our interpretation of the results are based on measurements of signal for gene expression of

the enzymes. Future experiments will determine whether protein expression and enzymatic activity align with our current findings.

Further, while males had increased MAP compared to females, they displayed decreased expression of *Mmp2*. However, they also displayed decreased expression of the inhibitor *Timp1*. Increased *Timp1* expression has previously been shown to inhibit the activity of MMP2 and MMP9 (Zacchigna *et al.* 2004); therefore, it is possible that decreased *Timp1* expression in the males is allowing for more MMP2 and MMP9 activity when compared to the females.

An ART effect was observed with levels of intracellular ROS in the offspring mesenteric resistance arteries. Superovulation, embryo culture and embryo transfer were used in this experiment in order to simulate three of the procedures commonly used in human ART. In this initial study, we chose to use Whitten's Medium to culture the embryos as this medium is known to be suboptimal for embryo development even though it is adequate to support development to full term (Ecker *et al.* 2004, Sommovilla *et al.* 2005). Future studies will determine if similar effects are observed with various culture conditions. Offspring who were conceived using ART had increased levels of ROS in their mesenteric resistance arteries when compared to the offspring who were conceived naturally. While this increase was not accompanied by a significant change in MAP, this could predispose the offspring to greater vascular dysfunction and remodeling, as increased levels of ROS have been associated with reduced bioavailability of NO and vascular remodeling (Katakam *et al.* 2005, Martinez-Lemus *et al.* 2011). The increased levels of intracellular ROS in the ART group also occurred without a significant change in *Nox2* expression. This could be an artifact of the different tissues used for ROS

quantification and gene expression analysis. Alternatively, the increased ROS levels could be due to other producers of ROS [*i.e.* NOX1, NOX4, xanthine oxidase and nitric oxide synthase; (Cai *et al.* 2003, Bedard & Krause 2007, Demarco *et al.* 2010)]. Extracellular ROS levels were not significantly affected by any of the treatments.

We chose to study ROS levels in the resistance arteries, as the microvasculature is where most of the inward remodeling has been implicated in hypertension (Korsgaard *et al.* 1993). Due to limited amounts of tissue from the microvasculature, we performed gene expression analysis in the aorta. Dysfunction in the aorta has also been implicated in adverse cardiovascular health in rodents, and studies have been performed examining the MMPs and TIMPs in the aorta (Allaire *et al.* 1998, Khan *et al.* 2012). While we acknowledge that the aorta (a large conduit vessel) and the resistance arteries (resistance vessels) are not the same, and we cannot make strong causative conclusions in our findings between the two tissues, we can still use our findings as possible markers of adverse cardiovascular health in the offspring. Previous studies have also used both large conduit vessels and small resistance vessels to examine cardiovascular health in rodents (Beyer *et al.* 2008, Davidson *et al.* 2010, Sakurada *et al.* 2010, Agbor *et al.* 2012).

In summary, our study has shown an effect of ART on offspring body weight at weaning in a rodent model. We have also shown an effect of the use of ART on the level of ROS in mesenteric resistance arteries in the offspring. Maternal and offspring consumption of a HF diet resulted in increased body weight, MAP and misregulated gene expression in the offspring aortas. We further found a relationship between elevated extracellular ROS from mesenteric resistance arteries and increased gene expression of *Nox2*, *Mmp2*, *Mmp9*, and *Timp1* in the thoracic aorta. Knowledge is lacking on what

effect the combination of ART and HF diet have on offspring body weight and cardiovascular health, but our data suggest that the two developmental environments do not have an additive effect on body weight, MAP, gene expression in thoracic aorta and ROS in mesenteric resistance arteries in juvenile mice. The experimental design used in the present study does not allow us to differentiate if the adverse cardiovascular outcomes observed were the result of exposure to an obesogenic diet prenatally or postnatally. Future research will examine this question. Further, as CVD in humans often does not present until adulthood (Jousilahti *et al.* 1999), follow-up studies will also determine if an increased risk for adverse cardiovascular outcomes resulting from ART or obesogenic diet alone or in combination is evident later in life.

3.6 Declaration of interest

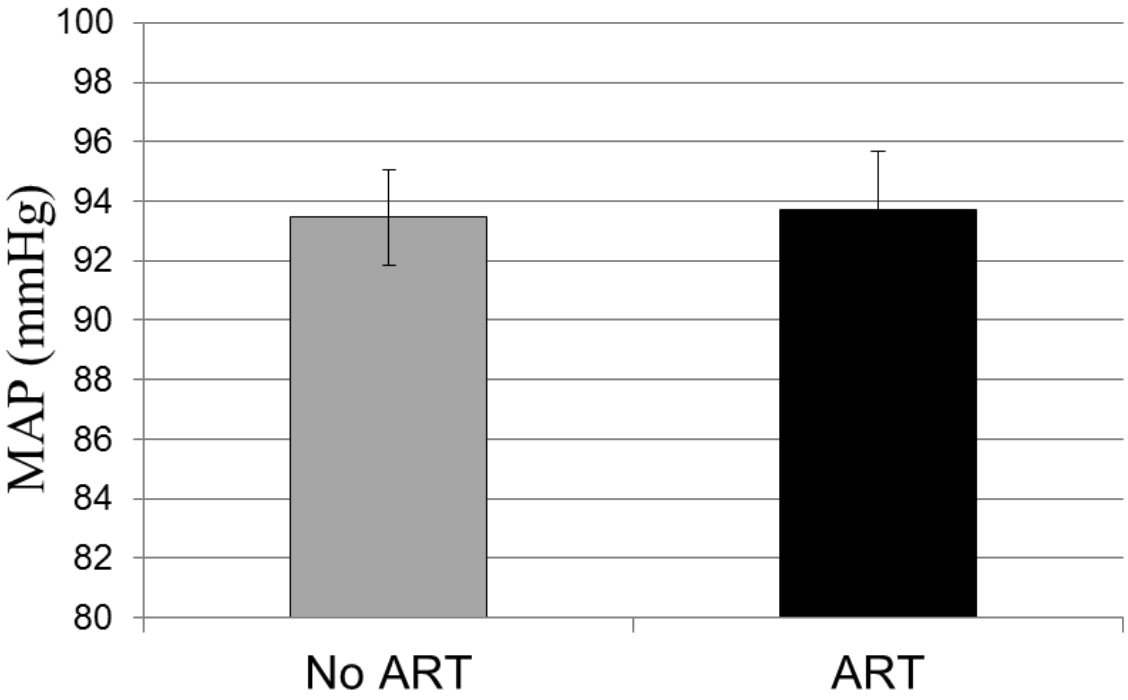
None declared.

3.7 Funding

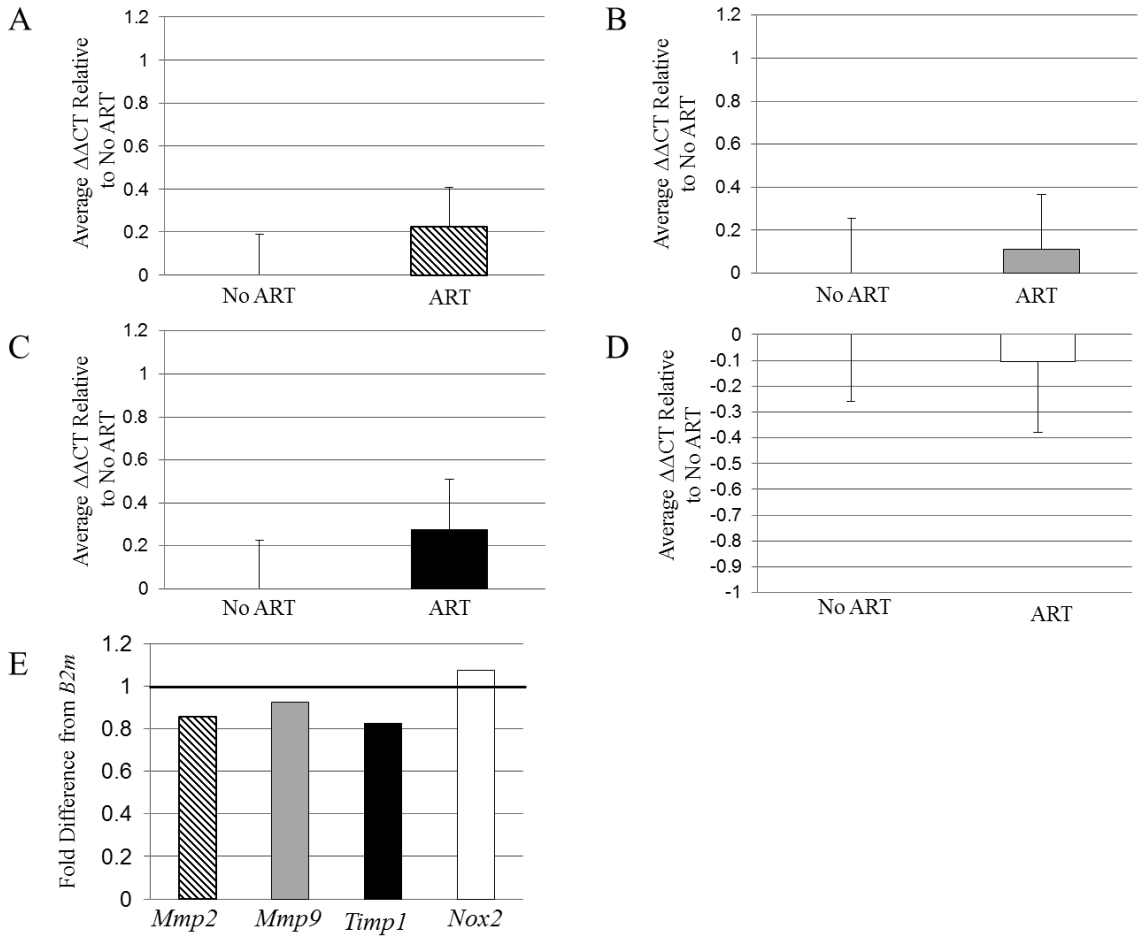
ALS is funded by a Miller Fellowship granted by MU Division of Animal Sciences. RMR is funded by F21C**.** LAM-L is funded by grant R01 HL088105 from the National Heart, Lung and Blood Institute.

3.8Acknowledgements

We would like to thank Matthew Sepúlveda from the Rivera laboratory for his valuable assistance with embryo transfer surgeries, and Guiling Zhao from the Martínez-Lemus laboratory for her help with isolation of the mesenteric resistance arteries.



Supplementary Figure 3.1. Mean arterial pressure (MAP) in ART offspring and no ART controls at sacrifice. Carotid artery catheterization was performed to measure MAP as a representation of blood pressure in the offspring immediately prior to sacrifice (55 ± 0.5 days). Group averages were compared between the naturally-conceived controls (No ART; grey bar; n=30) and the ART-conceived offspring (ART; black bar; n=29). Data are presented as mean ± the S.E.M.



Supplementary Figure 3.2. Quantitative RT-PCR assessment of the expression of genes potentially involved in inward remodeling in thoracic aortas of ART offspring and no ART controls. All gene expressions were normalized to the expression of *B2m* and then averaged within the respective groups. The relative difference in cycle thresholds (ΔΔCT) between the ART (n=32) and no ART (n=21) treatment groups for *Mmp2* (**A**), *Mmp9* (**B**), *Timp1* (**C**) and *Nox2* (**D**). The no ART group serves as the control. **A**, **B**, **C** and **D** = mean ± S.E.M. The fold differences between treatments groups for each gene analyzed were then calculated. The solid black line across "1" demarcates "No ART" for the four bars in **E**.

Supplementary Table 3.1. Births and number of offspring resulting from embryo transfers

Diet	Total Embryo Transfers	Litters Born	Live Litters	Avg. # of Live Offspring Per Litter
LF	22	7	6	3.3
HF	23	5	3	5.3

LF=consumption of maintenance chow by mothers and offspring. HF=maternal and offspring consumption of a diet with 24% fat and 17.5% high fructose corn syrup.

CHAPTER 4

General Discussion

The DOHaD hypothesis has revealed that the maternal environment in which fetal development occurs can affect the offspring's subsequent disease state in adulthood (Barker *et al.* 1989, Gillman *et al.* 2007). Two suboptimal maternal environments for fetal development are those that result from the use of ART, or that result from maternal obesity or consumption of a HF diet. The use of ART is on the rise with infertility affecting approximately 10% of couples (CDC 2012). Furthermore, obesity is also becoming more common, and it is estimated that by 2030, 60% of adults will be overweight or obese (Kelly *et al.* 2008). Both of these increasingly common maternal environments can result in adverse cardiovascular and metabolic outcomes in the offspring (Ceelen *et al.* 2007, Watkins *et al.* 2007, Ceelen *et al.* 2008, Nelson *et al.* 2010, Torrens *et al.* 2012). However, little is known about what effect these two environments can have on the offspring when they occur simultaneously. Knowledge on the combined effects ART and maternal obesity would be beneficial because they often coincide. Obese women have increased rates of infertility; therefore, the use of ART by obese women is increasing (Robker 2008).

CVD is the number one cause of death world-wide (WHO 2013b). However, the mechanisms that result in the development of CVD are largely unknown. With the DOHaD hypothesis and the ability of the aforementioned maternal environments to adversely affect cardiovascular health, we wanted to use ART and consumption of a HF

diet in a mouse model to examine the effects on possible markers of adverse cardiovascular health in the offspring.

Further with the increasing prevalence of obese women pursuing ART to conceive, we wanted to examine the effects of ART and consumption of a HF diet combined. To represent the use of ART in our model, we used superovulation, embryo culture in Whitten Medium, and embryo transfer. For the effect of diet, we fed females a diet with 24% fat and 17.5% high fructose corn syrup during oocyte maturation (~3 weeks), pregnancy, lactation and weaning. For the group combining ART and HF diet, embryos were transferred into superovulated pseudopregnant recipients consuming the same diet as the embryo donors. All offspring were maintained on the same diet as their mother.

We first measured body weight weekly for the offspring, as increased body weight can increase the risk for CVD (NIH 2012). Hypertension has also been associated with increased risk for CVD (NIH 2012), so we examined MAP in the offspring at sacrifice. We further examined the expression of genes that have been implicated in vascular remodeling and adverse cardiovascular health (*Mmp2*, *Mmp9*, *Timp1*, and *Nox2*) in offspring aortas at sacrifice. The last measure of cardiovascular health that we chose for our study was levels of ROS which have been previously shown in our lab to increase activity of MMPs in inward vascular remodeling in rat cremaster arterioles (Martinez-Lemus *et al.* 2011). We measured ROS both intracellularly and extracellularly in offspring mesenteric arterioles.

We first wanted to examine the effect of exposure to an obesogenic diet (HF) on the offspring cardiovascular health (Results summarized in **Fig. 4.1A**). An association

has been found in both human and mouse models between exposure to maternal high fat diet consumption while *in utero* and increased offspring body weight (Samuelsson *et al.* 2008, Nelson *et al.* 2010, Morandi *et al.* 2012). Exposure to a high fat *in utero* environment has also been associated with adverse cardiovascular outcomes in the offspring including increased blood pressure (Samuelsson *et al.* 2008, Torrens *et al.* 2012). Our data support the previous findings, as offspring exposed to an obesogenic diet were heavier from weaning until sacrifice, and also had a higher MAP when compared to the low fat diet controls. MAP also had a positive correlation with body weight at sacrifice (SacBW).

Based on previous research, we hypothesized that an increase in MAP could be associated with the MMPs, TIMPs and NOX-produced ROS. Previous findings in our lab and others have implicated MMP2 and MMP9 in the vascular remodeling associated with hypertension (Lehoux *et al.* 2004, Martinez-Lemus *et al.* 2011). However, increased MAP was not correlated with the expression of *Mmp2* or *Mmp9*. Future studies should examine the activity of the MMPs in case the activity is increased without a change in expression. Alternatively, this could mean that there are other pathways involved in the increase in MAP.

ROS are involved in activating the MMPs in vascular remodeling (Martinez-Lemus *et al.* 2011); therefore, we were not surprised that our study showed that as extracellular ROS decreased the expression of the MMPs decreased as well. NOX (*i.e.* NOX2) is the main producer of ROS in the vasculature (Cai *et al.* 2003), and we found that as NOX2 expression decreased, the production of extracellular ROS also decreased.

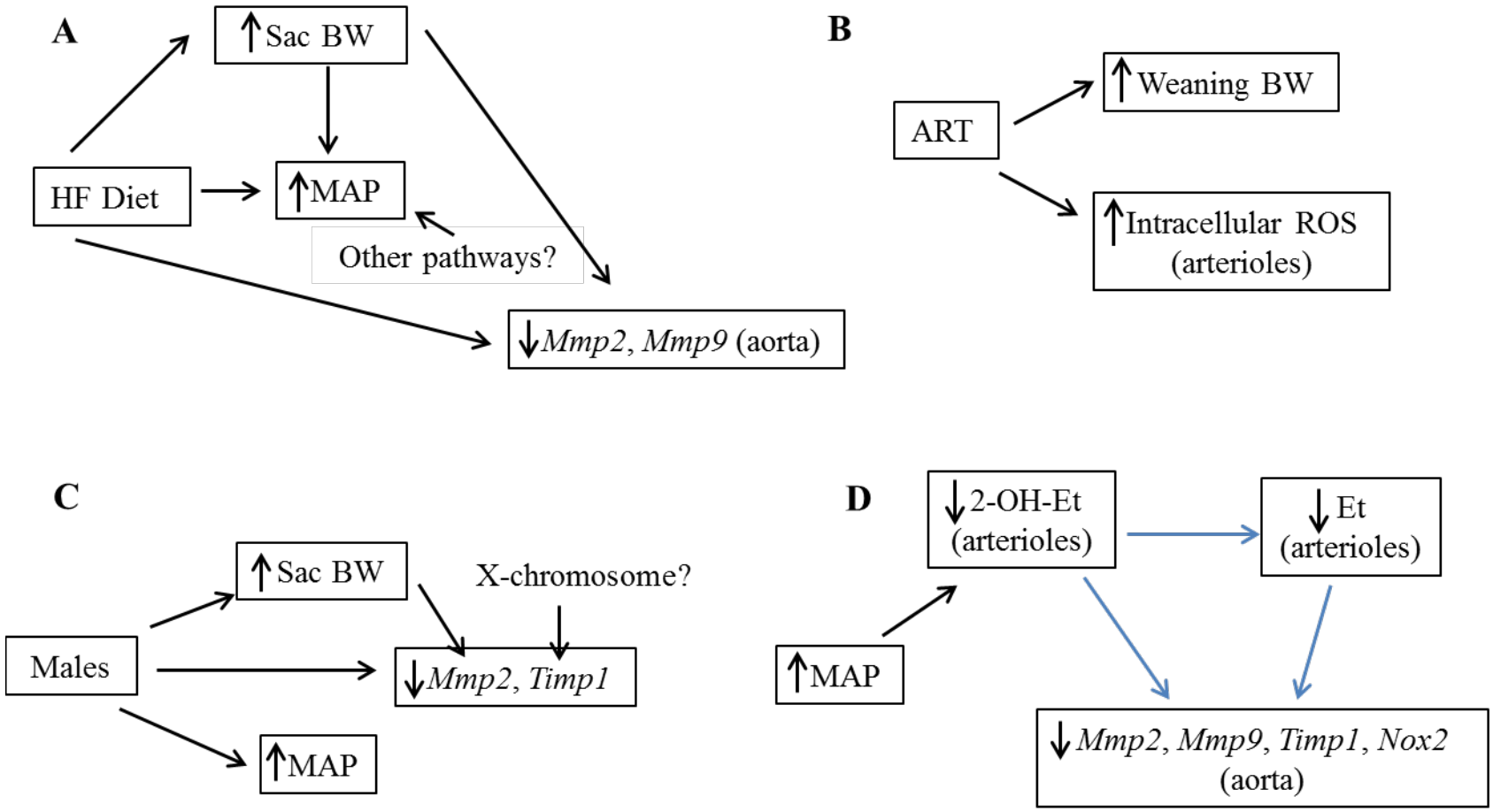

A
↑Sac BW
HF Diet
↑MAP
Other pathways?
↓*Mmp2*, *Mmp9* (aorta)
B
ART
↑Weaning BW
↑Intracellular ROS
(arterioles)
C
↑Sac BW
X-chromosome?
Males
↓*Mmp2*, *Timp1*
↑MAP
D
↓2-OH-Et
(arterioles)
↓Et
(arterioles)
↑MAP
↓*Mmp2*, *Mmp9*, *Timp1*, *Nox2*
(aorta)

Figure 4.1

Figure 4.1 Summary of significant effects on offspring cardiovascular health. The effects of maternal and offspring consumption of a HF diet are summarized in **A**. Exposure to an obesogenic diet (HF diet) resulted in increased body weight at sacrifice (SacBW; 55 ± 0.5 days) as well as an increase in MAP in the offspring, indicative of adversely affected cardiovascular health. The HF group also had decreased expression of *Mmp9* and a trend for decreased *Mmp2* expression, which we cannot explain at this time. An increase in SacBW correlated with increased MAP and decreased *Mmp2* expression and a trend toward decreased *Mmp9* expression. There was no correlation between MAP and expression of *Mmp2* or *Mmp9*, so we can speculate that other pathways might be involved to increase MAP. **B** summarizes the significant effects of ART on offspring cardiovascular health. Offspring body weight in the ART group at weaning (22 days) was increased compared to the No ART controls, but this effect diminished over time. However, intracellular ROS were increased in the ART-conceived offspring mesenteric arterioles at sacrifice indicating oxidative stress. This could potentially lead to increased vascular remodeling or endothelial dysfunction. **C** shows the gender effect on offspring cardiovascular health. Males had increased MAP and SacBW compared to females, as well as decreased *Mmp2* and *Timp1* expression. We speculate that decreased expression of *Timp1* in males could be due to the ability of *Timp1* to escape X-chromosome inactivation in females, leading to increased expression in the females. **D** represents correlations between MAP and extracellular 2-OH-Et, and between 2-OH-Et and gene expression. An increase in MAP correlates with an increase in 2-OH-Et, without a change in *Nox2* expression. Extracellular 2-OH-Et and Et are positively correlated. Further, they both correlate positively with expression of all of the genes analyzed. Blue lines= the association between 2-OH-Et and Et, and between both 2-OH-Et and Et with gene expression. The blue lines serve to separate these associations from the association between MAP and 2-OH-Et, as MAP is not significantly correlated with either Et or the gene expressions. 2-OH-Et= 2-hydroxyethidium. Et= ethidium. MAP= mean arterial pressure.

A finding that is more puzzling is that with increased SacBW the expression of *Mmp2* was lower, even though increased SacBW was positively correlated with increased MAP. While we did not analyze the correlation between SacBW and *Mmp2* expression in males and females separately, it is possible that the correlation between SacBW and lower *Mmp2* expression was driven by the males because they weighed more and had less *Mmp2* expression than the females. However, we cannot prove causality based on this speculation. Further, exposure to an obesogenic diet resulted in increased MAP accompanied by a lower *Mmp9* expression. MMP9 has been proposed to be involved in a compensation mechanism to induce outward remodeling in response to high vascular pressure (Lehoux *et al.* 2004, Martinez-Lemus & Galinanes 2011), but it is possible that the lower *Mmp9* in the HF group with higher blood pressure is showing a failed ability to compensate for the higher blood pressure.

Secondly, we wanted to examine how ART could affect the cardiovascular health in the offspring (results demonstrated in **Fig. 4.1B**). Previous research has shown increased body weight or adiposity with the use of ART in both human and mouse models (Ceelen *et al.* 2007, Scott *et al.* 2010, Belva *et al.* 2012). In our study, we found that offspring conceived using ART were heavier at weaning (Weaning BW) than the no ART controls, but this effect of ART on body weight diminished over time. This is similar to the finding in Scott *et al.* (2010) where female mice conceived with the use of IVF were heavier at three weeks of age when compared to no ART controls, but were no longer heavier from four weeks until eight weeks of age (Scott *et al.* 2010).

Previous studies have shown increased oxidative stress with the use of ART at the level of the ovary, oocyte and embryo (Chao *et al.* 2005, Ge *et al.* 2012). We further

showed an increase in intracellular ROS in the second order mesenteric arterioles from our ART conceived offspring when compared to those that were naturally conceived. This increase was not accompanied by an increase in *Nox2* expression in the offspring. One possible explanation for this is that ROS can be produced by other enzymes [*i.e.* NOX1, NOX4, xanthine oxidase, and nitric oxide synthase; (Cai *et al.* 2003, Bedard & Krause 2007, Demarco *et al.* 2010)]. An increase in intracellular ROS without an increase in *Nox2* expression could also be an artifact of using arterioles for ROS measurement and aorta for gene expression analysis.

Determining the effect of gender was not one of the main goals of our study; however, we could not ignore that gender might have an effect (results summarized in **Fig. 4.1C**). Males had increased body weight when compared to females, and this was accompanied by an increase in MAP. This result could be an effect of the positive correlation between MAP and SacBW mentioned earlier. Alternatively, the effect of gender on MAP could be a true gender effect, as it has previously been shown that estrogen in females can have a protective effect on blood pressure response [reviewed in (Xue *et al.* 2013)]. Males also had decreased expression of *Mmp2* and *Timp1*. The lower expression of *Mmp2* in males could be due to the correlation between increased SacBW and lower *Mmp2* expression mentioned previously. We can speculate that the lower expression of *Timp1* might be a true gender effect because *Timp1* is located on the X-chromosome and has the ability to escape X-chromosome inactivation (Anderson & Brown 1999). X-chromosome inactivation is a mechanism leading to equal expression of X-linked genes between males and females, so if the *Timp1* is escaping this inactivation, it could explain why females would have higher expression than males.

As increased levels of ROS can increase the expression of the MMPs (Zhang *et al.* 2013), it is possible that the negative correlation we observed between 2-OH-Et and Et with the difference in cycle threshold of the MMPs was due to a ROS-dependent increase in gene expression (**Fig. 4.1D)**. Further, NOX (*i.e.* NOX2) is the main producer of O_2^- in the vasculature (Cai *et al.* 2003), so it is not surprising that we observed increased levels of 2-OH-Et and a trend toward increased levels of Et (from downstream products of O_2^-) when *Nox2* expression was increased.

Interestingly, extracellular levels of 2-OH-Et (representative of O_2^- levels) were decreased with an increase in MAP without a significant decrease in *Nox2* expression or the production of downstream products of O_2^- (represented by Et). We can speculate that the offspring with increased MAP might be compensating in a way that would reduce superoxide levels. This could be accomplished by decreased activity of NOX2 as NOX is involved in O_2^- production in the vasculature, or perhaps by decreasing the bioavailability of O_2^- with increased SOD (Li *et al.* 2001) or increased NO production which would favor the production of peroxynitrite (Frears *et al.* 1996). Future studies should look at the levels of scavengers such as SOD or producers of NO such as nitric oxide synthase to see if this compensation might be occurring.

In summary, the findings from this study have provided a gateway for future research. Previous studies combined with our study show that ART and consumption of an obesogenic diet can adversely affect the offspring. These effects range from increased oxidative damage and misregulated gene expression, to metabolic differences in the children conceived using ART. While we did not observe a combined effect of ART and consumption of an obesogenic diet in juvenile offspring cardiovascular health (~7

weeks), we did observe effects of each environment independently on markers of adverse cardiovascular health. Our study was performed in mice so the findings can only be suggestive to humans, but our results together with other animal and human studies may be used to caution obese individuals planning to conceive or individuals considering assisted reproduction of possible outcomes.

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APPENDIX 1

Design of Bisulfite Assays for Section of Promoter Region in *Mmp2, Mmp7, Mmp9, Mmp14* and *Timp1* in Mouse

While our experimental offspring were being produced, we wanted to design primers to analyze the methylation patterns in the promoter regions of genes implicated in vascular remodeling [*i.e. Mmp2, Mmp7,* and *Mmp9* (Wang *et al.* 2009, Martinez-Lemus *et al.* 2011)], an inhibitor of the MMPs [*Timp1* (Itoh & Nagase 1995)] and another MMP that has been associated with increased body fat [*Mmp14 (Chun et al. 2010)*]. Proper DNA methylation in the promoter region of genes is important for regulating gene expression (Nagae *et al.* 2011); therefore, misregulated methylation in the promoter region of a gene could change its transcriptional activity. DNA methylation in mammals primarily occurs in a CpG context (Razin & Riggs 1980) . I will refer to these CpG occurrences as CpG sites. Due to limited time, DNA methylation analysis has not been performed in our experimental tissues (from Ch. 3), but it will be done in the future.

The tissues used for the design of the bisulfite sequencing primers were frozen aortas from CF1 x B6D2F1/J offspring previously produced in our laboratory. DNA isolation and bisulfite mutagenesis of the DNA was performed as described in (Chen *et al.* 2013). Gene promoters, except for *Mmp9*, were identified using the promoter database website (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home) and were then aligned to NCBI sequences for each appropriate gene. The promoter for *Mmp9* was identified directly through NCBI. A region designed to overlap both the 5' and 3' end of the promoter region was chosen as a region of interest, and this region was used to design

primer pairs for bisulfite-converted DNA using the Methyl Primer Express Software v1.0, except for *Mmp9* and *Mmp14* for which I used Bisearch Primer Design and Search Tool (http://bisearch.enzim.hu/?m=search). Several primer pairs were attempted, but the ones that produced the cleanest bands on 1% agarose gels were optimized as shown below and sequenced to verify that the right product had been amplified. *Note: the agarose gel pictures except for *Mmp14* and *Timp1* (**Fig. A1.1**) are shown in C7 tail bisulfite converted DNA, but all primers were also tested in CF1 x B6D2F1/J progeny aorta bisulfite DNA.

Table A1.1 PCR conditions for amplification of promoter regions in mouse bisulfite-converted DNA

Gene Symbol	PCR Annealing Tm (°C)	PCR size (bp)	Primer [] µM	$MgCl_2$ [] mM	# Cycles	2nd PCR	Taq Polymerase
Mmp2	60	569	0.3	4	45	yes	Go Taq Promega
Mmp7	59.2	512	0.3	4	45	yes	Go Taq Promega
Mmp9	61	526	0.3	4	45 (1st) 40 (2nd)	yes	Go Taq Promega
Mmp14	57.3	281	0.3	4	45 (1st) 40 (2nd)	yes	Go Taq Promega
Timp1	59.7	362	0.3	4	45	no	Go Taq Promega

[]= concentration, Tm= temperature (°C), 2nd PCR= 2nd PCR with the template from the first PCR and the same primers

Table A1.2 Primer pair information for promoter regions in bisulfite-converted mouse DNA

Gene Symbol		Bisulfite Primers (5'-3')	NCBI accession # Reference assembly (mus musculus)	Primer Location in Reference Sequence (bp)	Chromosome
Mmp2	Forward Reverse	TTTTGGGTAAGGTAATGGTATATGA TTATCCTCTAACTCAACCCCAT	NM_008610	396-422 990-1012	8
Mmp7	Forward Reverse	GAAAGTGTGTAAATGAGTTATTTATTT TACTCTCCAATTTCTAAATTAAAAAA	NM_010810	701-728 1165-1191	9
Mmp9	Forward Reverse	AGAGAAGTTTGGGAGAATATTTA CAAACAAAAACTTCATCCTTC	AF403768	177-200 683-703	2
Mmp14	Forward Reverse	GGAAAAGAGAAGAGTAAATAGAT AATTACTACCTTAAACCCAA	NM_008608	739-762 999-1019	14
Timp1	Forward Reverse	GGAATATGGGAAATTTGAGAGGTA CAAACCCCAATTCTACCAAAA	NM_011593	498-513 830-851	X

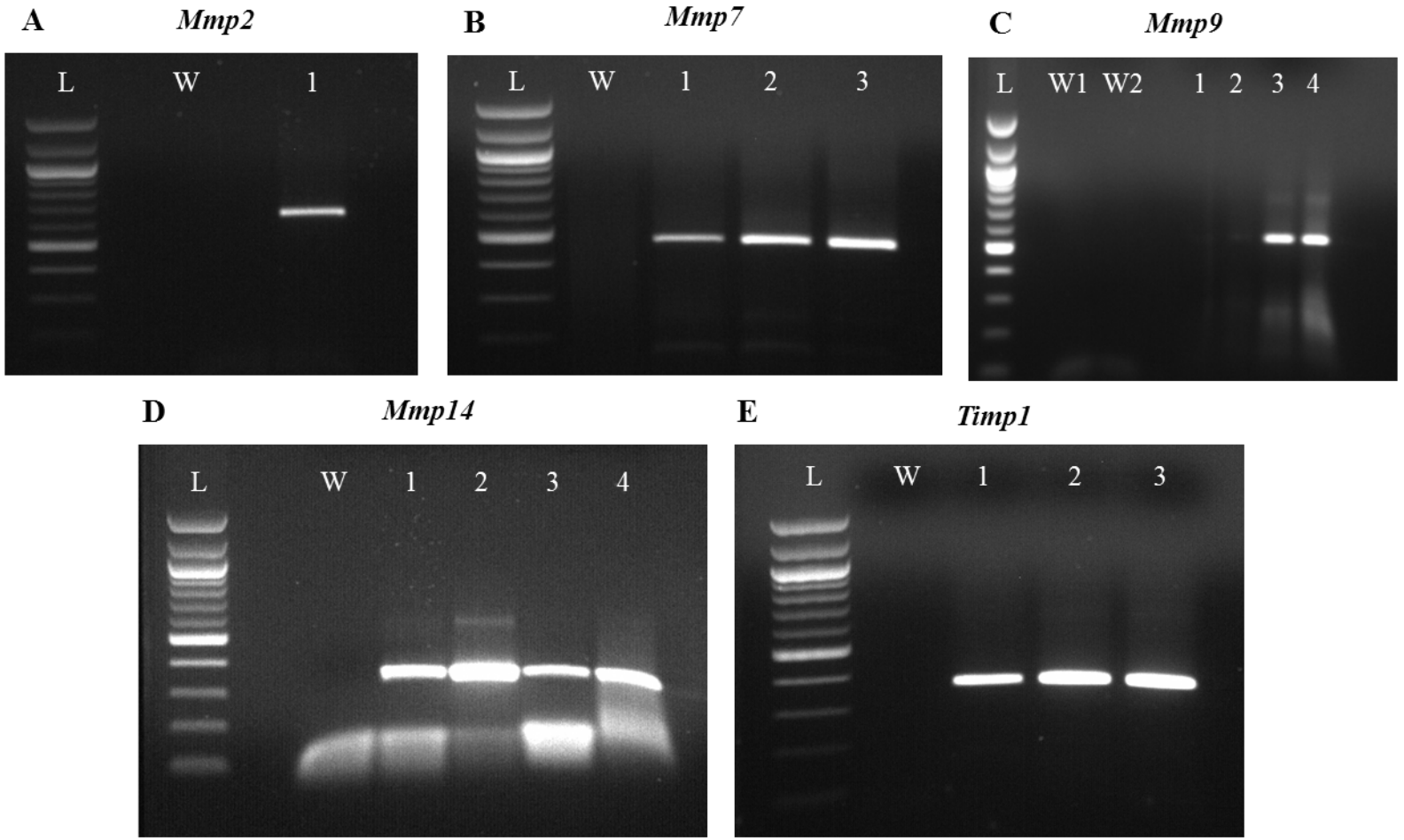
A
Mmp2
L
W
1
B
Mmp7
L
W
1
2
3
C
Mmp9
L
W1
W2
1
2
3
4
D
Mmp14
L
W
1
2
3
4
E
Timp1
L
W
1
2
3

Figure A1.1.

Figure A1.1 Amplification of mouse bisulfite converted DNA. Bands represent the amplicon of portions of various promoter regions (*i.e. Mmp2*, *Mmp7*, *Mmp9*, *Mmp14* and *Timp1*) resolved on a 1% agarose gel. DNA was isolated from tail except when noted. **A** a 569 bp amplicon containing 10 CpG sites for the *Mmp2* promoter region is shown. W = negative control (water). 1 = C7 tail bisulfite-converted DNA. **B** shows a 512 bp amplicon containing 8 CpG sites for the *Mmp7* promoter region. 1 = C7 tail bisulfite-converted DNA with 0.2 mM dNTPs. 2 = C7 tail bisulfite-converted DNA with 0.4 mM dNTPs. 3 = C7 tail bisulfite-converted DNA with 0.6 mM dNTPs. **C** is a 526 bp amplicon containing 10 CpG sites for the *Mmp9* promoter . W1 = negative control (water) from 1st PCR. W2 = negative control (water) from 2nd PCR. 1 = C7 tail bisulfite-converted DNA with 0.09 μM primers from 1st PCR. 2 = C7 tail bisulfite-converted DNA with 0.09 μM primers from 2nd PCR. 3 = C7 tail bisulfite-converted DNA with 0.3 μM primers from 1st PCR. 4 = C7 tail bisulfite-converted DNA with 0.3 μM primers from 2nd PCR. We varied the amount of primers and tried a single PCR or a nested PCR with the original PCR product as template to see which condition would help us get enough product for sequencing. **D** shows a 281 bp amplicon containing 27 CpG sites for the *Mmp14* promoter region. Lanes 2, 3, 4 and 5 = CF1 x B6D2F1/J aorta bisulfite-converted DNA. A 362 bp amplicon containing 14 CpG sites for the *Timp1* promoter region. **E**. 1= C7 tail bisulfite-converted DNA. 2 and 3= CF1 x B6D2F1/J aorta bisulfite-converted DNA. L = NEB Ladder N3231S. W = water control (*i.e.* blank). dNTPs= deoxynucleotide triphosphates.

VITA

Angela Louise Schenewerk was born on June 1, 1989 in Kirksville, Missouri. She obtained a Bachelor of Arts in Biology at Westminster College in Fulton, Missouri in May of 2011. During her undergraduate studies she developed her interest in comparative human and animal health through her experiences working in a human cadaver lab, traveling to Belize for a summer to learn about the wildlife, and working at a summer camp with over 100 species of animals. In August of 2011, Angela began her Master of Science studies in developmental epigenetics under Dr. Rocío Rivera in the Division of Animal Sciences and Dr. Luis A. Martinez-Lemus in the Department of Medical Pharmacology and Physiology and the Dalton Cardiovascular Research Center at the University of Missouri. Upon completion of her M.S. degree, Angela hopes to pursue a career in cancer research or scientific writing.