THE IMPACT OF ADVERSE MATERNAL ENVIRONMENTS ON RESISTANCE ARTERY FUNCTION AND MECHANICAL CHARACTERISTICS IN OFFSPRING

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By

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

THE IMPACT OF ADVERSE MATERNAL ENVIRONMENTS ON RESISTANCE ARTERY FUNCTION AND MECHANICAL CHARACTERISTICS IN OFFSPRING

presented by Francisco I. Ramirez-Perez, a candidate for the degree of Doctor of Philosophy, and hereby certify that, in their opinion, it is worthy of acceptance.

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“What makes the desert beautiful, 'said the little prince,'

is that somewhere it hides a well...”

— Antoine de Saint-Exupéry, The Little Prince

I want to dedicate my dissertation to my parents, Sandra and Ignacio, it was them who taught me to dream and they were my first love. Thank you, Mom and Dad, we have shared a lot of good and sad times together. I'm sure it was hard for you two to let me go when I started school as a kid, and it was harder to see me leave home in pursuit of my dreams. It was your example of kindness and hard work that helped me to grow as a person. To my sisters, Sandra, Alejandra, Selene and Donaji, I'm deeply grateful for your love and for always being there for me when I needed you the most, thank you. To my nephews, Logan, Gaara, Carlos and Ethan, it has been hard to be away from you but I'm happy to know that we are close regardless of the distance, I love you all. To my grandparents, Francisco and Magdalena, thank you for your love, support and for all those beautiful childhood memories.

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ABSTRACT

Cardiovascular disease is one of the leading causes of death worldwide. Maternal obesity, gestational diabetes mellitus (GMD) and assisted reproductive technologies (ART) have been associated with cardiovascular deficiencies in offspring.

Obese women often suffer from infertility and use ART to achieve a pregnancy. Children of mothers that undergo ART or experience GDM have a higher risk of developing hypertension, but little is known about the mechanisms that control this process. Here, I report that in offspring, the interaction between a high fructose and high fat diet (also known as western diet, WD) and ART exhibited impaired endothelial-dependent dysfunction in mesenteric resistance arteries, this was determined by the presence of reduced acetylcholine vasodilation. Arteries from WD-ART male mice had greater wall cross-sectional area (CSA) and wall-to-lumen ratio (W/L) compared to their respective ART control, indicative of vascular hypertrophic remodeling.

Another adverse maternal environment during pregnancy is GDM, with 2-10 % of pregnancies in the US being affected. To study this model, we designed a two-by-two experiment array using male mice, with main effects genotype and diet. In resistance mesenteric arteries of wild type (WT) offspring fed a WD experienced enhanced vasodilation to acetylcholine. Furthermore, in offspring of hyperleptinemic dams WD reduced vasodilation to insulin. Offspring of hyperleptinemic dams had stiffer arteries regardless of the diet. Therefore, we conclude that while maternal hyperleptinemia was

beneficial to offspring vascular health fed a standard diet (SD), it had detrimental effects when fed a WD. The results of these two projects suggest that an adverse maternal environment (i.e. ART or GDM) in combination with a WD favors the development of endothelial dysfunction and arterial stiffening in resistance mesenteric arteries of offspring.

CHAPTER 1

INTRODUCTION

Cardiovascular disease has been increasing to become the second leading cause of death worldwide, accounting for 30% of all deaths. In 2008, 10% of men and 14% of women in the world were obese and two of the main factors are sedentary lifestyle and high-fat, energy-dense diets. In the United States, recent reports suggest that around 80 million (one out of three) of adults have high blood pressure or hypertension, 21 million have been diagnosed with diabetes and it is speculated that over 8 million undiagnosed cases. Furthermore, around 24 million children (one out of three) ages 2 to 19 are overweight or obese.

Among all factors that play a role in the increase of cardiovascular disease (CVD) we are primarily interested on obesity, hypertension and diabetes. Notably, hypertension is one of the most prevalent causes of death in the United States, and it involves vascular remodeling and stiffening. Physical inactivity and high fructose and high fat diet (also known as western diet, WD) are the cornerstone of an obesogenic environment for parents and children, with parental lifestyle habits having direct impact in their children's health. An additional side effect of obesity is infertility, and the use of assisted reproductive technologies (ART) is a common alternative to this problem. Individually maternal obesity and the use of ART are associated with unfavorable cardiovascular outcomes in offspring, and their combination is expected to further increase the damage. Another common adverse maternal environment associated with metabolic and/or CVD outcomes in children, including hypertension, is gestational diabetes mellitus (GDM). A key feature of GDM is maternal hyperleptinemia, yet there is limited information on the effects of maternal

hyperleptinemia on vasoreactivity, arterial remodeling and stiffening of the offspring's vasculature.

At the vascular level, arterial remodeling and stiffening is an earlier biomarker of CVD. Moreover, arterial stiffening known to increase as the number factors involved on CVD increases. Therefore, it is important to determine if the mechanisms that regulate vascular stiffness on the vasculature while exposed to adverse maternal environments has the potential to provide the basis for treatments. Allowing to alleviate not only arterial remodeling process but reducing arterial stiffness and having a positive overall effect decreasing the CVD risk. Furthermore, this knowledge could provide a window for the development of new treatments to prevent and if possible, reverse the effects of arterial stiffening.

Chapters 2 will address the effect of the first adverse maternal environment studied in this dissertation, ART. This chapter will focus on the functional and structural changes of mesenteric resistance arteries from male and female offspring. Vascular remodeling and stiffening are known as earlier biomarkers of CVD. Finally, we will present the result of biomarkers measured from plasma.

Chapter 3 will present and discuss the effects of GDM on mesenteric resistance arteries from young and adult male mice using the animal model db/+. In this chapter we will further analyze the changes of vascular remodeling using confocal microscopy. Confocal microscopy will allow us to quantify and study the morphology and wall composition of the vascular walls, an insight into the structural changes that lead to the remodeling and stiffening of the vessels.

These two chapters provide a description of the effects that two adverse maternal environments have on the cardiovascular structure and health of offspring. The results obtained in this dissertation are based on animal model developed to emulate, as close as possible, the conditions found in humans.

Finally, in Chapter 4, I summarize the additional projects that I worked during my doctoral research.

CHAPTER 2

EFFECTS OF THE USE OF ASSISTED REPRODUCTIVE TECHNOLOGIES AND AN OBESOGENIC ENVIRONMENT ON OFFSPRING RESISTANCE ARTERY FUNCTION AND DIABETES BIOMARKERS [1]

2.1 Introduction

Obesity has become a major health problem worldwide [1,2]. Excessive body weight in the form of accumulated adipose tissue is accompanied by adverse pathophysiological conditions. Of these conditions, hypertension and type 2 diabetes mellitus (T2DM) are particularly important for their association with cardiovascular disease and the incidence of life threatening cardiovascular events such as myocardial infarction and stroke [1]. The epidemic proportions at which obesity is increasing are associated with an increased consumption of hypercaloric diets rich in fats and carbohydrates, as well as with more sedentary life styles [3,4,5]. In addition to these factors, evidence is increasing that indicates exposure to an obesogenic environment during embryonic and fetal development programs individuals for obesity, hypertension and T2DM later in life [6,7,8,9,10,11,12,13]. This developmental programming of adult

[1] The research included in this chapter was originally published in PLoS One. Ramirez-Perez, F. I. et al. Effects of the use of assisted reproductive technologies and an obesogenic environment on resistance artery function and diabetes biomarkers in mice offspring, PLoS One, 2014 Nov 11;9(11):e112651. doi: 10.1371/journal.pone.0112651.

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diseases is hypothesized to participate in perpetuating the incidence of obesity and cardiovascular disease across generations [5].

An additional consequence of the obesity epidemic is that many women of reproductive age are overweight and have difficulties getting pregnant [14]. Many of these women pursue the use of assisted reproductive technologies (ART) to address their infertility. The use of ART has been associated with an increased incidence of developmental abnormalities including cardiovascular malformations that is further increased when mothers are obese [15,16,17]. This suggests that ART has the potential of influencing the developmental programming of adult diseases. However, little is known about the interactions that ART and maternal obesity may have on the development of adult diseases in offspring.

In order to emulate the increasing association between the use of ART and obesity in women with the incidence of obesity, hypertension and T2DM in the offspring, we recently developed a mouse model of this condition [18]. We have already reported that, in this model, consumption of a western diet (WD) high in fat and carbohydrates by both mother and offspring resulted in augmented mean arterial pressure at seven weeks of age. We also reported that the use of ART resulted in increased body weight at weaning, as well as in the presence of an increased production of reactive oxygen species (ROS) by the resistance arteries of the juvenile offspring [18].

Presence of an increased production of ROS or an imbalance in the redox status of tissues can cause oxidative stress, which in turn has been associated with vascular dysfunction and the development of T2DM [19,20]. In particular, ROS can cause vascular endothelial dysfunction, which is associated with decreased vasodilation efficiency, a

hallmark of both hypertension and diabetes. An increased production of ROS, in conjunction with obesity, is indicative of a pro-inflammatory state that has been associated with changes in biomarkers related to orexia, glucose metabolism and the development of T2DM. In the present study we investigated the effects of the use of ART in the context of an obesogenic environment from embryonic development to early life on the vasoconstriction and vasodilation properties of mesenteric resistance arteries, as well as on the presence of T2DM associated biomarkers in juvenile mice. We report that a significant interaction exists between an obesogenic environment and the use of ART that affects the endothelial function and structure of mesenteric resistance arteries, while having only a small effect on T2DM associated biomarkers in juvenile mice offspring.

2.2 Methods

2.2.1 Ethics statement

All animal procedures were performed in accordance to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. The Institutional Animal Care and Use Committee at the University of Missouri approved all animal procedures used in the study. Animal use protocol was 7501.

2.2.2 Mice and ART

Female mice used for reproductive purposes were of the NSA strain (CF1; Harlan Laboratories, Indianapolis, IN, USA). Males used for reproductive purposes were B6D2F1/J mice (The Jackson Laboratory, Bar Harbor, ME, USA). Dams were split in two groups to receive different diets beginning three weeks before mating with males. The

control diet (CD) group of dams was fed chow containing 6.5% fat (LabDiet 5008). The western diet (WD) group received feed containing 24% fat and 17% high fructose corn syrup (TestDiet 58Y1, St. Louis, MO, USA) as previously described [18]. Each group of dams was divided into two subgroups. In the No ART subgroup, dams were mated naturally and allowed to carry their pregnancies from beginning to end. In the ART subgroup, female mice 6-10 weeks old were superovulated with an intraperitoneal injection of equine chorionic gonadotropin (5 IU) followed by human chorionic gonadotropin (5 IU) 45 hours later. Embryo donors were mated with intact males, while embryo recipient dams (surrogates) were mated with vasectomized males. Two-cell embryos were collected 46 hours following superovulation from the embryo donors and cultured in Whitten's medium for three days. Cultured embryos where then transferred to 2.5 days pseudopregnant recipients that received ten embryos per uterine horn. Pseudopregnant surrogate dams were fed the same diet as the embryo donors. We purposely chose to use Whitten's medium for embryo culture because this medium is known to provide suboptimal conditions that affect the molecular structure of the embryo [21,22], but supports full development to term [18,23,24]. In order to equalize litter sizes, pups were culled to constitute litters of four males and four females whenever possible. After weaning, mice were fed the same diet as their mothers and were euthanized at 7 weeks of age. We developed this protocol to emulate, as much as possible, those conditions found in human subjects, in that a woman undergoing ART is usually the recipient of her own embryos and children remain in the same environment after birth [25,26]. Thus, our experimental design created eight groups of offspring, namely, CD-No ART-males, CD-No ART-females, CD-ART-males, CD-ART-females, WD-No ART-males, WD-No ART-females, WD-ART-males and WD-

ART-females (Figure 1). The distribution of offspring per surrogate dam is shown in Table S1. As we are particularly interested in the mechanisms that program offspring exposed to ART and/or an obesogenic environment to hypertension and T2DM early in life, we euthanized mice at an age comparable to human puberty.

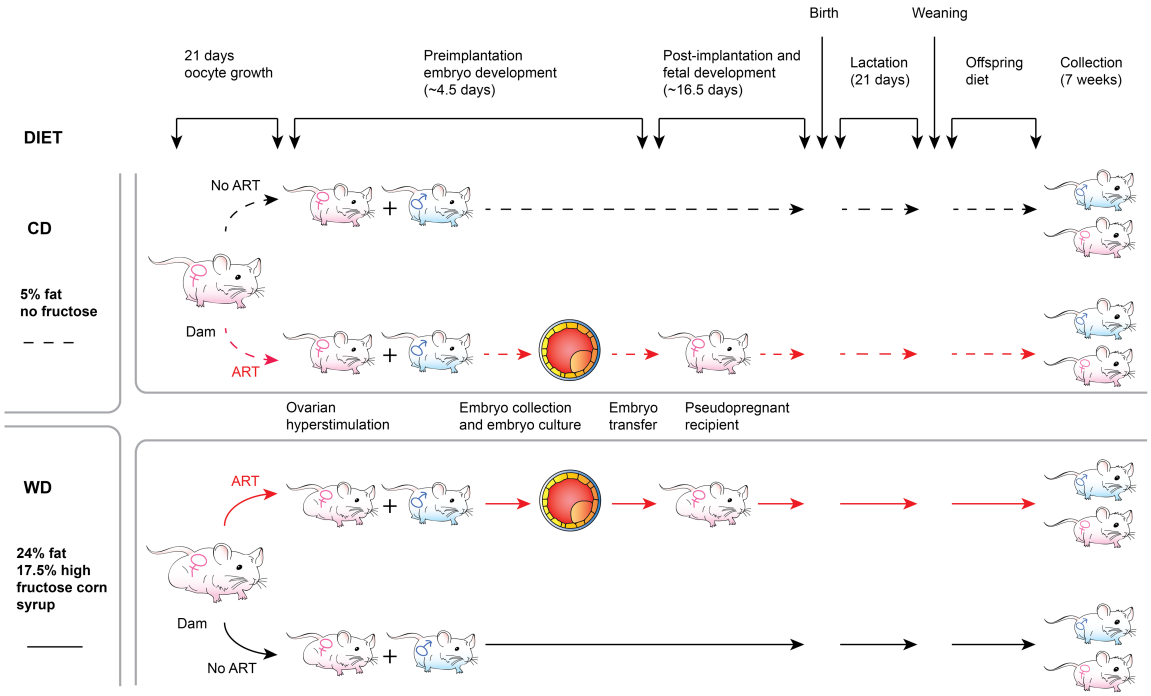

Figure 1. Diagrammatic representation of the experimental design. The design consisted of a factorial arrangement of diet effects (CD=Control Diet; WD=Western Diet), the effects of the use of assisted reproductive technologies (ART; No ART), and sex (males; females) on juvenile mice offspring.

2.2.3 Vessel isolation

Mesenteric arteries were collected immediately after euthanasia at 7 weeks of age. Two arteries were isolated from each mouse. The arteries were cannulated onto glass micropipettes, pressurized at 70 mmHg without flow, and warmed to 37 °C in commercial myograph chambers (Living Systems Instrumentation, Burlington, VT, USA) as

previously described [27]. The chambers contained physiological saline solution (PSS) with (in mM) 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), 2.0 pyruvate, and 0.02 EDTA at a pH of 7.4. The micropipettes were filled with PSS that contained 0.15 mM bovine serum albumin. To test for viability, cannulated arteries were allowed to stabilize for 40 min and then exposed to PSS in which NaCl was equimolarly substituted with 80 mM KCl. Only arteries that constricted more than 20% to this 80 mM K^+ solution were used in the analyses.

After the exposure to high K^+, the arteries were washed three times with fresh PSS and exposed to increasing concentrations of phenylephrine (10^{-8} to 10^{-4} M) to examine adrenergic-dependent vasoconstriction. Subsequently, arteries were washed and pre-constricted with 10^{-6} M phenylephrine. The arteries were then exposed to increasing concentrations of acetylcholine (10^{-9} to 10^{-5} M). After washing these agents from the arteries, they were again pre-constricted with 10^{-6} M phenylephrine and exposed to increasing concentrations of sodium nitroprusside (SNP, 10^{-8} to 10^{-4} M). All vasoactive agents were added in a cumulative fashion to the bath solution at increments of $10^{-0.5}$ M. Each concentration was maintained in the bath for 2 minutes. At the end of each experiment arteries were exposed to Ca^{2+}-free PSS with (in mM) 2 EGTA and 100 adenosine to obtain maximal passive diameter. Throughout the experiment, chambers were mounted on inverted microscopes with CCD cameras. Luminal diameter and wall thicknesses were recorded using a video caliper (Living Systems Instrumentation, Burlington, VT, USA) and a Powerlab data acquisition system (ADInstruments Inc, Colorado Springs, CO, USA).

The elastic characteristics of the arteries were determined at the end of each experiment. Vessels were exposed to consecutive changes in intraluminal pressure from 70 mmHg to 5, 10, 20, 30, 40, 60, 80, 100, 120, and back 70 mmHg while under passive conditions (Ca^{2+}-free PSS). Each pressure was maintained for 2 minutes while luminal diameter and wall thicknesses were recorded.

2.2.4 Plasma measurement of diabetes associated biomarkers

At euthanasia, blood from non-fasted mice was collected by cardiac puncture. Blood was allowed to clot and the plasma was collected and centrifuged at 3,000 x g for 15 minutes at 4°C to separate any cellular debris from the plasma. Plasma samples were frozen and kept at -70° C for subsequent analyses.

A volume of 50 µl of plasma from each mouse was used to measure the concentration of ghrelin, glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide-1 (GLP-1), glucagon, insulin, leptin, plasminogen activator inhibitor-1 (PAI-1), and resistin. The concentration of these biomarkers was measured using the Bio-Plex Pro Mouse Diabetes 8-Plex Assay Kit from Bio-Rad. Plasma samples were analyzed in a BioPlex 200 following instructions from the manufacturer.

2.2.5 Data analysis

Luminal diameters of arteries were normalized to maximal passive diameter or the level of initial vasoconstriction (phenylephrine-induced pre-constriction). The normalized data were then regressed to ART, diet, sex, intraluminal pressure, and their interactions. Important fixed factors were selected by controlling the statistical significance at level

0.10. According to experimental design, the offspring effect and mother effect were also included as random effects to incorporate covariances among observations. Correlated repeated measurements on the same experimental unit (each artery) were modeled by several different variance-covariance structures, and the best was chosen based on AIC (Akaike information criterion) and BIC (Bayesian information criterion) as well as biological interpretation. Models were implemented in SAS (version 9.4). Residual plots were carefully examined to check model assumptions. Proper Box-Cox transformations were applied to data to maintain normality and constant variance assumptions. Tukey-Kramer HSD (Honestly Significant Difference) test was applied to adjust multiple tests in pair wise comparisons. Statistical significance was considered at $P \leq 0.05$. All data are presented as means ± SEM. An extended version of the statistical analysis is included in Supporting Methods S1.

2.3 Results

2.3.1 Vasoconstriction to phenylephrine was greater in males than in females

No significant effects were found for diet, ART or their interaction with regard to maximal phenylephrine contraction or the half maximal concentration (EC50) of this vasoconstrictor. The only significant effect found for the vasoconstriction induced by increasing concentrations of phenylephrine was that of sex. Maximal constriction was achieved at a concentration of 10^{-5} M phenylephrine and was significantly ($P \leq 0.05$) greater in males than in females (Figure 2A). Exposure of vessels to concentrations of phenylephrine greater than 10^{-5} M was associated with paradoxical reductions in maximal constriction. In comparison, no significant differences were observed between arteries

from males or females on the constriction caused by KCl-induced depolarization (Figure 2B).

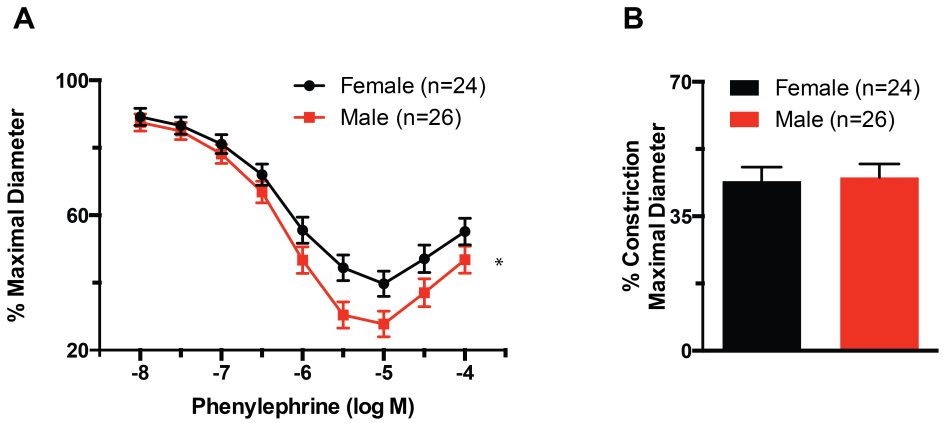

Figure 2. Phenylephrine-induced vasoconstriction is greater in male than in female mice. (A) Percent maximal diameter of mesenteric resistance arteries isolated from male and female mice, and exposed to incremental concentrations of phenylephrine. The maximal constriction to phenylephrine was significantly greater (*P≤0.05) in males than females. (B) Percent constriction after exposure to 80 mM KCl of mesenteric resistance arteries isolated from male and female mice. Data are means ± SEM.

2.3.2 Acetylcholine-induced vasodilation was significantly affected by sex and diet x ART interaction

Acetylcholine-induced vasodilation was significantly (P≤0.05) greater in mesenteric arteries isolated from females than in those isolated from males (Figure 3A). This was particularly noticeable at an acetylcholine concentration of $10^{-6.5}$ M. Acetylcholine-induced dilation was also significantly affected (P≤0.05) by the interaction between diet and ART (Figure 3C). Mesenteric resistance arteries from both males and females in the WD-No ART group had greater maximal vasodilations to acetylcholine than

those in the WD-ART group. In comparison, no differences in maximal acetylcholine-induced vasodilation were observed between the CD-ART and CD-No ART groups. Vasodilatory responses to SNP were not significantly different between any of the groups (Figure 3B,D)

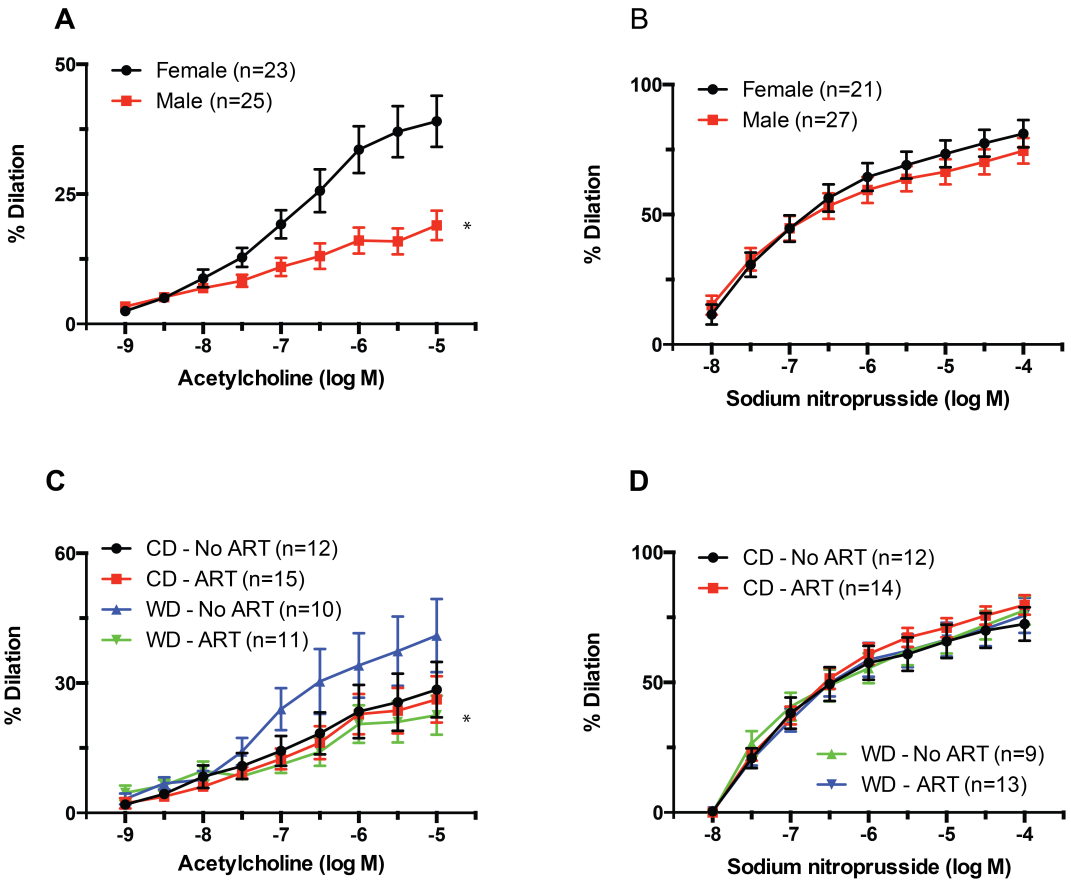

Figure 3. Effects of sex and diet x ART interaction on relaxation responses of mesenteric resistance arteries. (A) Percent dilation of mesenteric resistance arteries isolated from male and female mice, and exposed to incremental concentrations of acetylcholine. Maximal dilations to acetylcholine were significantly greater (*P≤0.05) in females than in males. (B) Percent dilation of mesenteric resistance arteries isolated from male and female mice, and exposed to incremental concentrations of SNP. (C) Percent dilation of mesenteric resistance arteries isolated from mice fed a WD or CD and obtained

by natural birth (No ART) or the use of ART, and exposed to incremental concentrations of acetylcholine. Maximal dilations to acetylcholine were significantly smaller (*P≤0.05) in arteries from WD-ART mice than in those from WD-No ART mice. (D) Percent dilation of mesenteric resistance arteries isolated from mice fed a WD or CD and obtained by natural birth (No ART) or the use of ART, and exposed to incremental concentrations of SNP. Data are means ± SEM.

2.3.3 Effects of diet and ART on vascular structure

A significant effect (P≤0.05) was found for the interaction between diet and ART on the media to lumen ratios (Figure 4A), and vascular wall cross-sectional areas (Figure 4C) of mouse mesenteric resistance arteries maintained under passive conditions. Arteries obtained from ART mice had greater wall to lumen ratios and wall cross-sectional areas than those obtained from No ART mice only when the animals were fed a WD. No differences between ART and No ART were observed in arteries from mice fed CD (Figure 4B,D). Not significant differences were observed either for the internal passive diameter of the arteries or their elastic characteristics, including stress-strain relationships and Young's modulus of elasticity between any of the groups (Figure S1).

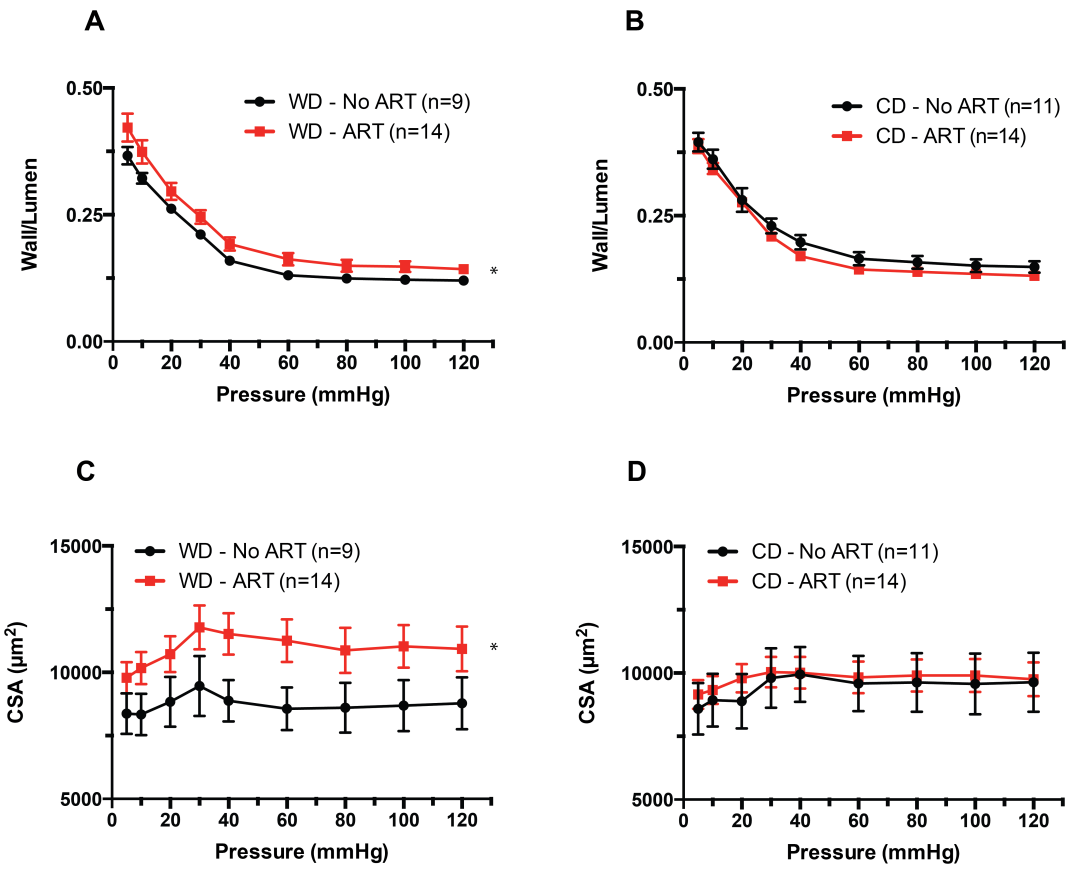

Figure 4. Effects of diet x ART interaction on arterial wall-to-lumen ratios and wall cross-sectional areas. (A) Wall to lumen ratios obtained under passive conditions (Calcium-free) and at different intravascular pressures in mesenteric resistance arteries isolated from mice fed WD or CD and obtained by natural birth (No ART) or the use of ART. Wall to lumen ratios were significantly greater (*P≤0.05) in ART-WD vs. No ART-WD arteries. (B) Wall cross-sectional areas (CSA) obtained under passive conditions (Calcium-free) and at different intravascular pressures in mesenteric resistance arteries isolated from mice fed WD or CD, and obtained by natural birth (No ART) or the use of ART. CSA were significantly greater (*P≤0.05) in ART-WD vs. No ART-WD arteries. Data are means ± SEM.

2.3.4 Diet had a significant effect on most of the diabetes related biomarkers measured

The concentrations of ghrelin, GIP, GLP-1, insulin, leptin, and resistin were all significantly greater (P≤0.05) in serum collected from mice in the WD group vs. the CD group irrespective of sex or ART status (Figure 5). Glucagon was the only biomarker measured that was not significantly affected by diet.

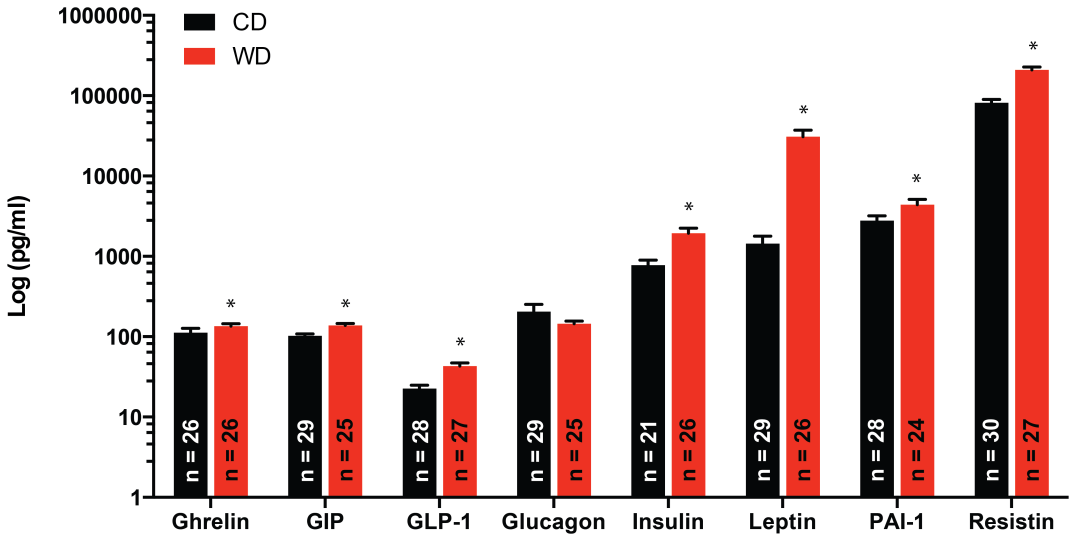

Figure 5. Effect of diet on the serum concentrations of diabetes biomarkers. Serum concentrations of ghrelin, GIP, GLP-1, glucagon, insulin, leptin, PAI-1 and resistin in mice fed WD or CD. Serum concentrations of ghrelin, GIP, GLP-1, insulin, leptin, PAI-1 and resistin were significantly greater (*P≤0.05) in WD vs. CD fed mice. Data are means ± SEM.

2.3.5 Effects of sex on the diabetes related biomarkers measured

Sex had a significant effect (P≤0.05) on the concentrations of insulin, leptin, and resistin, with males having consistently greater levels of these biomarkers in serum (Figure 6).

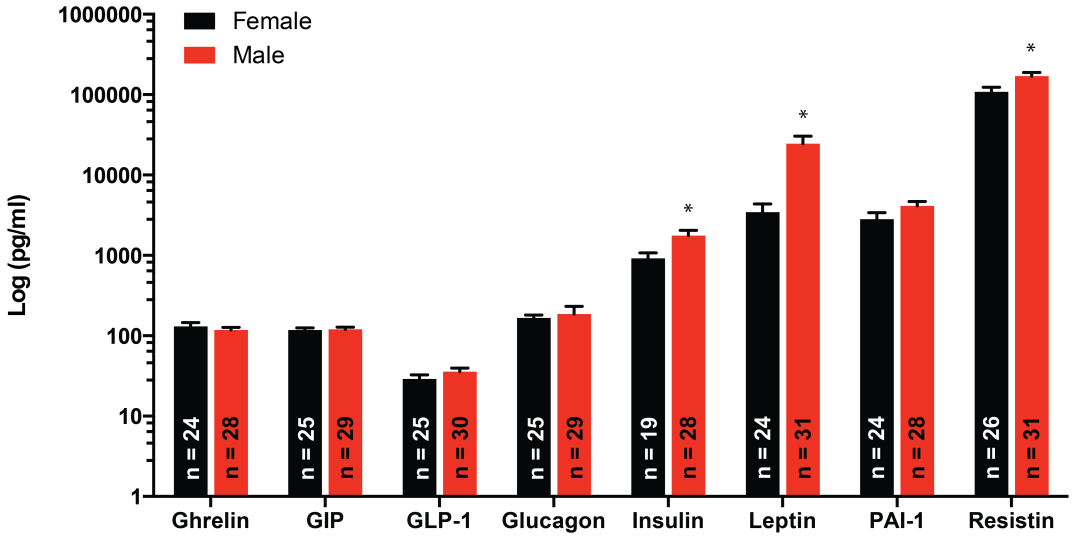

Figure 6. Effects of sex on the serum concentrations of diabetes biomarkers. Serum concentrations of ghrelin, GIP, GLP-1, glucagon, insulin, leptin, PAI-1 and resistin in male and female mice. Serum concentrations of insulin, leptin, and resistin were significantly greater (*P≤0.05) in male vs. female mice. Data are means ± SEM.

2.3.6 Leptin and PAI-1 were significantly affected by a diet x sex interaction

The interaction of diet x sex on leptin serum concentrations was manifested as a significantly greater difference between males and females when mice were fed the WD (Figure 7). In comparison, PAI-1 levels in serum were significantly lower in females than in males only in mice fed CD (Figure 7). No significant differences in PAI-1 serum levels were observed between sexes of mice fed WD. Consequently, WD increased serum levels of PAI-1 only in female mice.

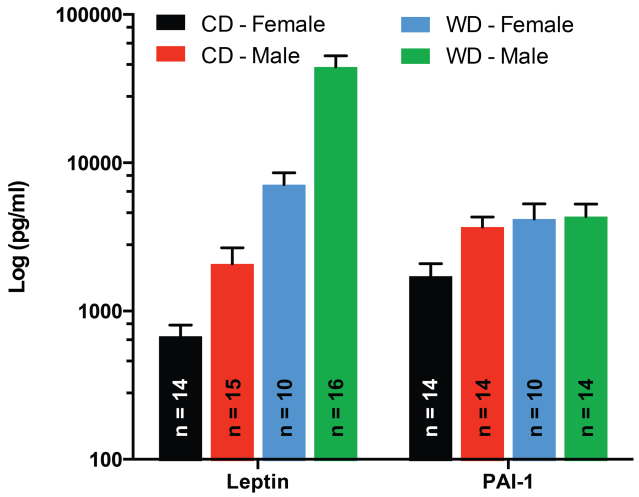

Figure 7. Effects of diet x sex interaction on the serum concentrations of diabetes biomarkers. Serum concentrations of leptin and PAI-1 in male or female mice fed a WD or CD. The increase in serum concentrations of leptin associated with consumption of WD was greater ($P \leq 0.05$) in male than female mice, while WD increased ($P \leq 0.05$) PAI-1 concentrations in females, but not in male mice. Data are means ± SEM.

2.3.7 Resistin was significantly affected by a diet x ART interaction

As mention above, WD increased overall resistin levels, but the increase was significantly greater in ART mice than in No ART mice, resulting in a significant ($P \leq 0.05$) diet x ART interaction (Figure 8). A trend interaction ($P=0.06$) in PAI-1 serum levels was also observed between diet and ART. PAI-1 tended to be increased in ART mice only when fed a WD. No effect of diet on PAI-1 was observed in mice of the No ART group (Figure S2).

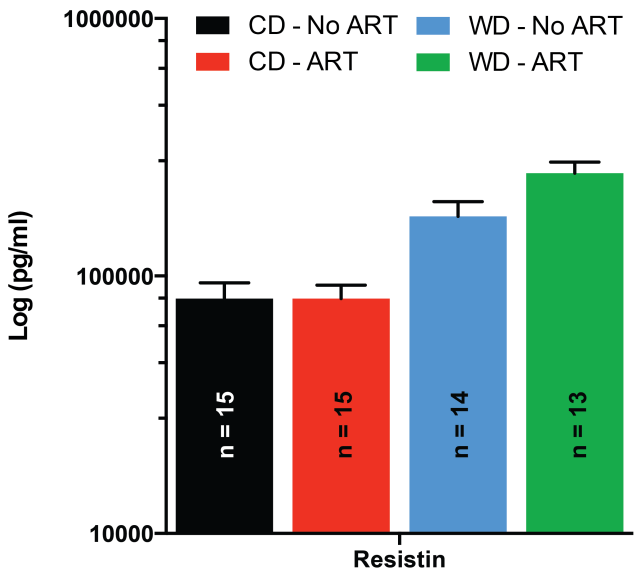

Figure 8. Effects of the diet x ART interaction on the serum concentrations of diabetes biomarkers. Serum concentrations of resistin mice fed a WD or CD and obtained by natural birth (No ART) or the use of ART. The increase in serum concentrations of resistin associated with consumption of WD was greater ($P \leq 0.05$) in ART than in No ART mice. Data are means ± SEM.

2.4 Discussion

The primary finding of the present study is that the use of ART in an obesogenic environment is associated with a reduced acetylcholine-induce dilation of mesenteric resistance arteries in juvenile mice offspring. We previously reported that the mesenteric resistance arteries of these same mice had an increased level of reactive oxygen species (ROS) in the vascular wall, independent of diet or sex effects [18]. Our present results suggest that exposure to an obesogenic environment tends to increase the ability of mesenteric resistance arteries to dilate more in response to endothelium-dependent acetylcholine stimulation, and that the use of ART under these obesogenic conditions

causes endothelial dysfunction. Because ROS reacts with nitric oxide (NO) and reduces its bioavailability, it is likely that the endothelial dysfunction we observed is associated with the well-known scavenging of NO by ROS [12,28]. We observed a numerical reduction in the transcription level of superoxide dismutase 1 (*Sod1*) in the blood vessels of ART mice (Figure S3). Therefore, it is possible that in the resistance arteries of No ART mice there are sufficient antioxidant mediators to prevent the manifestation of endothelial dysfunction, while in the ART mice even a marginal reduction in oxidant defenses allow ROS to reduce the bioavailability of NO, but this remains to be experimentally corroborated.

Our current results also indicate that the interaction of ART with an obesogenic environment was associated with hypertrophic remodeling of the mesenteric resistance vasculature in juvenile mice offspring. This was manifested as an increased media to lumen ratio of the arteries and an augmented cross-sectional area of the vascular wall. Although we observed no significant changes in passive luminal diameter in association with the obesogenic environment, overnutrition in mice has been previously associated with outward hypertrophic remodeling in db/db mice [29]. In the latter study, outward remodeling was deemed to have occurred as a consequence of persistent increased flow-induced vasodilation, which is NO dependent. Therefore, it is plausible that the increased vascular oxidative stress we found in the arteries of ART mice prevented the development of outward remodeling associated with the WD in our current study. The observation that endothelium-independent vasodilatory responses to SNP were not affected by WD or ART suggests that both the functional and structural changes we found in mesenteric resistance arteries are likely associated to vascular endothelial dysfunction.

The literature consistently indicates that, early in life, males are more susceptible than females to develop endothelial dysfunction and hypertension [30]. Accordingly we found that male mice had increased vasoconstrictor responses to phenylephrine and reduced vasodilator responses to acetylcholine compared to females. These results are consistent with our previous report in which we showed that these same male mice had greater mean arterial pressures than females at seven weeks of age [18].

Developmental programming of diseases such as hypertension and diabetes has been associated with oxidative stress. Our current finding that ART reduced the vasodilatory capacity of resistance arteries from mice in the WD group suggests that an interaction exists between an obesogenic environment and the use of ART to induce endothelial dysfunction. Our previous finding that the use of ART is associated with an increased level of ROS in the wall of resistance vessels further suggests that the mechanism responsible for this interaction may very well be oxidative stress. At this time there is no clear evidence on which mechanism(s) may be responsible for increasing ROS in the resistance vessels of ART mice and why only ART mice in the WD group showed functional endothelial dysfunction. We previously showed that the expression of the NADPH oxidase, NOX2, in the resistance arteries of these mice is not affected by ART or diet [18]. Our current results indicating that there is a marginal reduction of SOD1 in ART mice, suggest that ART may cause a reduction in vascular antioxidant capacity. Whether this reduction in SOD1 expression is sufficient to functionally increase vascular oxidation remains to be determined.

As we observed that the use of ART increased vascular ROS, and as obesity and diabetes are commonly accompanied by oxidative stress [31], we probed the plasma of

mice for a number of biomarkers commonly associated with glucose metabolism, diabetes or diabetes development. Not surprisingly, the obesogenic environment caused by feeding dams and offspring a WD resulted in increased plasma concentrations of GIP, GLP-1, insulin, leptin, and resistin. GIP and GLP-1 are incretins, gastrointestinal peptide hormones that increase insulin secretion in response to meal intake. Consistent with our findings, previous reports have indicated that both GIP and GLP-1 levels are increased in response to high-fat obesogenic diets [32,33]. An overwhelming body of literature also associates obesogenic WDs with hyperinsulinemia, hyperleptinemia, and insulin resistance [31,34,35]. As leptin is an anorexic cytokine produced by adipose tissue, it is considered that the hyperleptinemia observed in obese subjects is associated with reduced leptin sensitivity [36,37]. Contrary to our expectations, no significant interactions between diet and ART were observed to affect these biomarkers.

Reports on the levels of circulating resistin found in individuals with obesity and T2DM is controversial [38], but it is clear that resistin is associated with reduced insulin sensitivity [39,40]. Consequently, our finding that resistin levels were increased in WD fed mice is consistent with our observation that these mice were also hyperinsulinemic. We also found a significant diet x ART interaction indicating that the increased levels of resistin found in the WD fed mice were greater in animals obtained by ART than in those obtained via natural conception, gestation, and birth. It remains to be determined whether the association between ART and an obesogenic environment had any mechanistic relation with the increased levels of oxidative stress we found in the resistance vessels of ART mice.

Significant diet x sex interactions were observed for leptin and PAI-1. Leptin was significantly greater in males vs. female only in mice fed a WD. As mentioned above WD was associated with greater leptin levels in both sexes. In comparison, PAI-1 levels were significantly greater in males vs. females only in mice fed CD. Consumption of a WD increased PAI-1 serum concentrations only in females. Male offspring have been shown to be more severely programmed for obesity and cardiovascular disease than females when exposed to a maternal obesogenic environment [41,42]. Our observation that leptin levels increased more in males than in females when fed a WD is in accord with those reports. As for the differential effect of a WD on PAI-1 levels in male and females, our results suggest that a WD increased PAI-1 concentrations only in females. Previously, PAI-1 concentrations have been found to be correlated with body mass index, which is an indirect measure of adiposity in humans [43,44]. We did not measure adiposity in our current study, but we have previously reported that both male and female mice fed the WD were significantly heavier than those fed the CD [18]. Whether this increase in PAI-1 concentration in female mice fed a WD translates into a more prothrombotic phenotype remains to be determined. Additional studies are also needed to determine if the trend for an ART x diet interaction (P=0.06) we observed for PAI-1 affects thrombosis. That trend suggests that the use of ART may increase PAI-1 concentrations only in mice exposed to an obesogenic environment.

Our observation that plasma ghrelin was increased in mice fed a WD is contradictory to previous results indicating diet-induced obesity in mice is associated with low plasma levels of ghrelin [45,46], but consistent with the enlarged body weight of these mice. Increased serum levels of ghrelin are also contrary to the increased levels of insulin,

leptin and resistin we found, as previous reports indicate ghrelin reduces insulin secretion and is negatively correlated with leptin and resistin [47,48,49,50]. However, it is important to consider that the assay we used in this study measured total ghrelin and not only the active acylated form of this gastric peptide hormone [51]. Additional experiments will be needed to investigate the level of the acylated form of ghrelin, as well as the other measured biomarkers in fasted animals and at different times during feed intake and the development of obesity.

An important limitation of our study resides in the fact that our experimental design was not developed to interrogate the differential effects of exposure to an obesogenic environment during fetal development vs. exposure after birth. This study was designed to investigate the interaction between the use of ART and an obesogenic environment as it occurs in obese women using ART. An additional limitation of our study is that our experimental design does not allow for identification of the independent contributions of each of the components of our ART protocol in mice to specific effects attributed to ART on the measurements obtained in the offspring [52]. We have used the term ART in this study in reference to all three procedures combined in our protocol namely, superovulation, embryo culture and embryo transfer. Future experiments will be developed to determine the specific effects of each of these procedures on the offspring. Our major conclusion is that ART, as defined in this study, interacts with presence of an obesogenic environment to reduce the capacity of mesenteric resistance arteries to vasodilate in response to acetylcholine stimulation and to become remodeled. Further studies will be required to determine whether these interactions will influence the development of cardiovascular disease later in life.

2.5 Acknowledgments

The authors thank Guiling Zhao for her excellent technical support, and Laura Moon for technical assistance with the *Sod-1* qRT-PCR assay.

2.6 Supporting Methods

2.6.1 Statistical Analysis S1

Data Analysis for Artery Diameter Change with the Application of Vasoactive Agents

Before model fitting, samples that did not constrict at least 20% relative to the maximal passive diameter with the application of high K+ were removed from analysis. For each of the three applications of vasoactive agonists (phenylephrine, acetylcholine, and SNP), samples that did not constrict at least 10% relative to the maximal diameter with the initial application of phenylephrine were removed from the analysis of that vasoactive agent.

The offspring arterial diameters were modeled individually to determine which factors were significant when testing the elasticity of the sample with each of the vasoactive agents: phenylephrine, acetylcholine, and SNP. The diameters were normalized to represent a percent relative to their maximal diameter and initial constriction to account for the large variability amongst the arterial diameters. These percentages were regressed to the ART effect (pregnancy through ART or No ART), diet effect (WD or CD), sex effect (female or male), and concentration of the vasoactive agents (over time) as well as interaction effects between each two, three, and four factors. Mothers were included as

random effects in the model to account for the correlation between offspring of the same litter. Offspring were included as random effects to account for the correlation between two arteries extracted from the same offspring. Repeated measurements of one same artery of an offspring were taken at several time points for each vasoactive agent. Thus, to account for the correlation among the repeated measurements, a heterogeneous compound symmetry correlation structure was used to model the correlation between these time points. This correlation structure was chosen for modeling the repeated measurements based on its AIC and BIC statistics as well as its reasonable biological interpretation. Each model was constructed using backwards elimination by statistical significance. The main effects and interaction effects were selected by controlling the statistical significance level at 0.10. The studentized residual plots for each of the models suggest that the normality assumption is met in all cases. The Kenward-Roger adjustment to denominator degrees of freedom was used when fitting all three models in this study because of the unbalanced design, random effects, repeated measures, and moderate sample size. After model selection, the Tukey-Kramer HSD method was used to adjust multiple tests in comparing different levels of treatment combinations.

Data Analysis for Plasma Measurements of Diabetes Associated Biomarkers

A model was fit to each of the eight biomarkers based on ART effect (pregnancy through ART or No ART), diet effect (WD or CD), sex effect (female or male). Again, mothers were included as random effects to account for correlation of offspring from the same mother. We also examined interactions of ART, diet effect, and sex effect of the offspring. The main effects and interaction effects were selected by controlling the

significance level at 0.10. The Kenward-Roger adjustment to denominator degrees of freedom was used when fitting each protein model. Influence measure diagnostics were calculated for each observation, and highly influential mouse replicates were removed from the analysis based on their DFFITS and Cook's Distance values. Transformations were applied to maintain normality and constant variance model assumptions when necessary. Tukey-Karmer HSD model was applied to adjust multiple tests in pair wise comparisons.

2.6.2 Supporting Figures

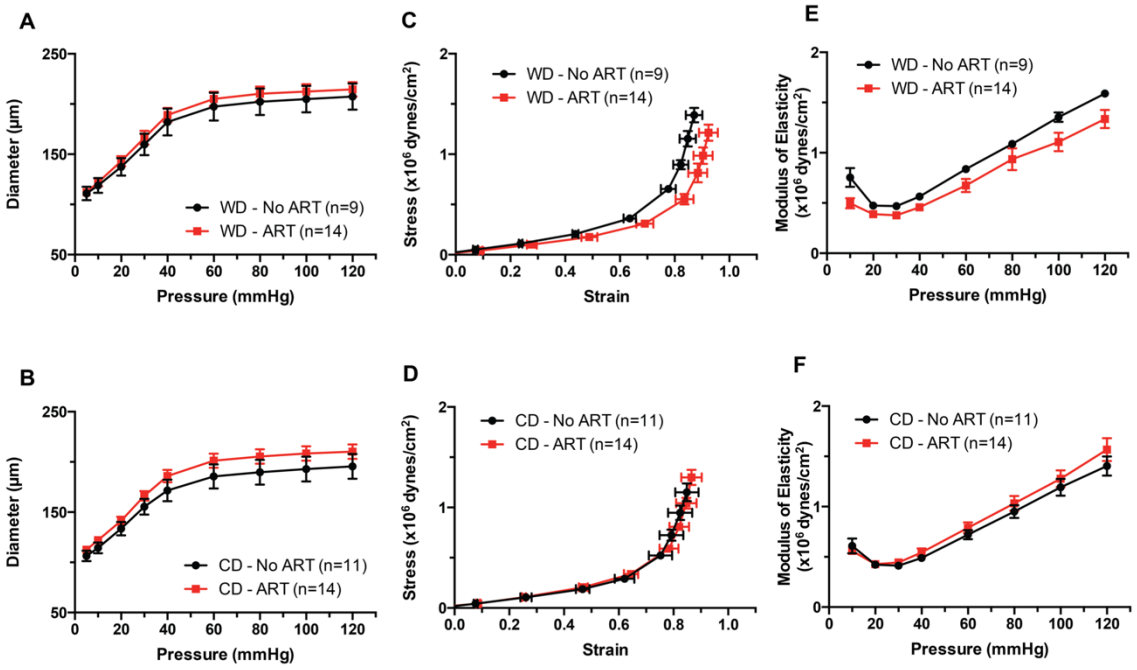

Figure S1. Effect of diet and ART on the passive diameter and elastic properties of mesenteric resistance arteries from juvenile mice. (A,B) Maximal passive diameters under calcium-free conditions at different intravascular pressures in isolated resistance arteries. (C,D) Strain-stress relationships of isolated mesenteric resistance arteries under passive conditions. (E,F) Moduli of elasticity of isolated mesenteric

resistance vessels at different intravascular pressures under passive conditions. Data are means ± SEM.

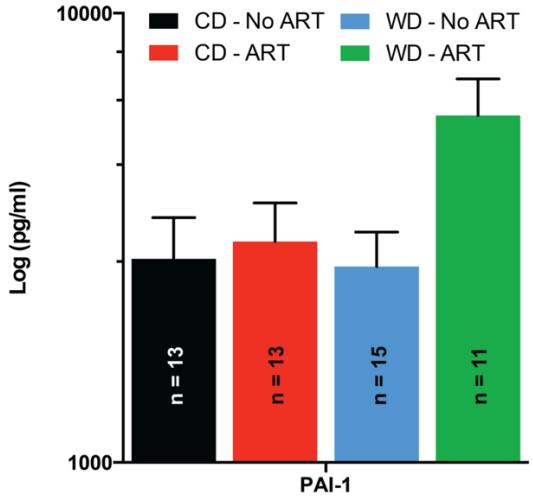

Figure S2. Effect of diet and ART on serum PAI-1 concentrations in juvenile mice. Serum PAI-1 concentrations were numerically greater (P=0.06) in WD-ART than in in WD-No ART mice. Data are means ± SEM.

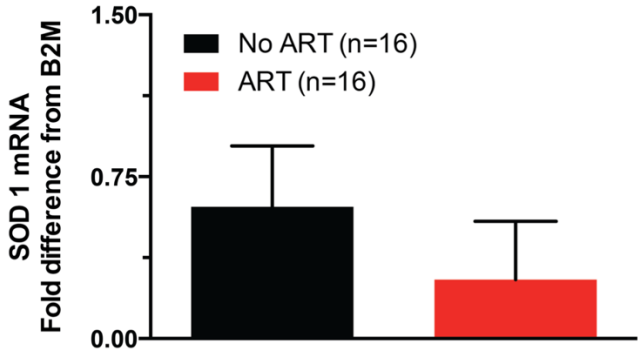

Figure S3. Effect of ART on expression of SOD-1 in juvenile mice. Quantitative RT-PCR assessment of expression of the SOD-1 gene in mice blood vessels. Gene

expression was normalized to the expression of B2m (DCT) and then averaged within the respective groups and expressed as fold difference. Data are means ± SEM. qRT-PCR procedures were as described in Schenewerk et al., and TaqMan probes for SOD1 and B2m were purchased from Applied Biosystems (Assay ID- Mm01344233_g1 and Mm00430072_m1, respectively).

2.6.3 Supporting Table

Vascular experiments

	No ART CD				No ART WD				ART CD				ART WD			
	Dam	Male offspring	Dam	Female offspring	Dam	Male offspring	Dam	Female offspring	Dam	Male offspring	Dam	Female offspring	Dam	Male offspring	Dam	Female offspring
	D1	2	D1	1	D1	1	D1	2	D1	3	D1	1	D1	4	D1	2
	D2	1	D2	1	D2	2	D2	1	D2	1	D2	3	D2	4	D2	3
	D3	1	D3	2	D3	1	D3	1	D3	1	D3	2	D3	1		
	D4	2	D4	2	D4	2	D4	2	D4	2	D4	1				
									D5	1						
	Dams	Offspring	Dams	Offspring	Dams	Offspring	Dams	Offspring	Dams	Offspring	Dams	Offspring	Dams	Offspring	Dams	Offspring
Total	4	6	4	6	4	6	4	6	5	8	4	7	3	9	2	5

Bio-Plex experiments

	No ART CD				No ART WD				ART CD				ART WD			
	Dam	Male offspring	Dam	Female offspring	Dam	Male offspring	Dam	Female offspring	Dam	Male offspring	Dam	Female offspring	Dam	Male offspring	Dam	Female offspring
	D1	2	D1	1	D1	2	D1	2	D1	1	D1	1	D1	4	D1	2
	D2	1	D2	1	D2	2	D2	1	D2	1	D2	3	D2	3	D2	3
	D3	1	D3	2	D3	2	D3	1	D3	1	D3	3	D3	1		
	D4	1	D4	1	D4	2	D4	1	D4	4	D4	1				
	D5	2	D5	1			D5	2	D5	1						
			D6	2												
	Dams	Offspring	Dams	Offspring	Dams	Offspring	Dams	Offspring	Dams	Offspring	Dams	Offspring	Dams	Offspring	Dams	Offspring
Total	5	7	6	8	4	8	5	7	5	8	4	8	3	8	2	5

Table S1. This Table describes the distribution of offspring obtained from different surrogate dams and how they were included in experiments related to vascular structure and function, and those related to the measurement of diabetes biomarkers using the Bio-Plex system.

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CHAPTER 3

MATERNAL HYPERLEPTINEMIA IS ASSOCIATED WITH MALE OFFSPRING'S ALTERED VASCULAR FUNCTION AND STRUCTURE IN MICE[2]

3.1 Introduction

Exposure to either gestational diabetes mellitus (GDM) or maternal obesity during prenatal development impacts cardiometabolic health of offspring. Programming of offspring metabolism has been particularly well studied, [1,2] and although not as abundant, there is also increasing evidence of hypertension in offspring of obese and diabetic pregnancies. For example, a meta-analysis of thirteen cohort studies found that boys born to GDM mothers have elevated systolic blood pressure [3]. This is also seen in animal models, where offspring of rats with streptozotocin-induced type I diabetes have significantly higher blood pressures than offspring of control rats [4-6]. Similarly, both male and female offspring of mice with diet-induced obesity and insulin resistance are hypertensive [7,8].

Despite the above evidence, little is known about the mechanisms by which GDM and maternal obesity lead to hypertension in offspring. One potential mechanism involves presence of elevated neonatal leptin concentrations in offspring of obese mothers with

[2] The research included in this chapter was originally published in PLoS One. Ramirez-Perez, F. I. et al. Maternal Hyperleptinemia Is Associated with Male Offspring's Altered Vascular Function and Structure in Mice, PLoS One, 2016 May 17;11(5):e0155377. doi: 10.1371/journal.pone.0155377.

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GDM. Kirk and colleagues found that in progeny of obese, insulin resistant dams, offspring's hypertension was associated with neonatal hyperleptinemia [9], and the programming effect of offspring's hypertension could be partially recapitulated by injecting offspring of control dams with leptin from postnatal days 9-15 [10]. However, it is not known whether maternal hyperleptinemia in the prenatal period also influences the development of hypertension in offspring.

Leptin is an adipokine that regulates energy homeostasis [11], and directly impacts vascular function in adulthood [12-14]. Pregnancies complicated by obesity and GDM are associated with maternal leptin resistance accompanied by hyperleptinemia [15-19]. Thus, we hypothesize that prenatal exposure to maternal hyperleptinemia may cause vascular dysfunction and hypertension in offspring. Alternatively, the problem in GDM pregnancies may be leptin resistance, i.e. the absence of leptin signaling in the mother, in which case raising maternal leptin would be predicted to protect offspring [20].

To distinguish between these possibilities (and the null hypothesis, that maternal hyperleptinemia does not affect offspring vascular function positively or negatively), we have utilized a genetic model of maternal hyperleptinemia, the $Lepr^{db/+}$ mouse. Under some conditions [20,21], but not others [22], $Lepr^{db/+}$ mice develop gestational diabetes, and their offspring become fatter and glucose intolerant [23,24]. In our laboratory conditions, on the C57Bl/6 background, $Lepr^{db/+}$ dams are profoundly hyperleptinemic, and slightly heavier than controls, but have no impairment in glucose tolerance [25]. Hyperleptinemia in this $Lepr^{db/+}$ model has the same effect on offspring metabolism as hyperleptinemia induced by delivering exogenous leptin [25,26]. Specifically, offspring of non-diabetic $Lepr^{db/+}$ dams and of leptin-infused dams weigh less, are more active, more sensitive to insulin, and have

less hepatic triglyceride accumulation than offspring of control dams [25-27], showing that prenatal exposure to maternal hyperleptinemia is beneficial to offspring's metabolism. Here, we use the same offspring of control and non-diabetic $Lepr^{db/+}$ dams to test the effects of maternal hyperleptinemia on offspring's hypertension, in the absence of maternal diabetes and obesity. Because male offspring of $Lepr^{db/+}$ dams exhibited the largest and most consistent differences in body weight and insulin sensitivity [25], these were used to examine effects on hypertension and vascular function. This model has the additional advantage of avoiding any stress caused by treatment with exogenous leptin.

To uncover the mechanisms by which maternal hyperleptinemia may alter offspring risk of hypertension, we examined resistance artery structure and function, as there is evidence of endothelial dysfunction in cells collected from newborns following delivery to a GDM pregnancy [28-31]. In addition, offspring from rodent models of maternal type I and II diabetes have impaired mesenteric artery vasodilatory responses to acetylcholine and bradykinin, but normal responses to nitric oxide (NO), indicative of endothelial dysfunction [4-7].

Resistance arteries play a major role in the regulation of blood pressure, and alterations in resistance artery vasodilation and vasoconstriction are often associated with vascular remodeling processes that modify the passive internal diameter and wall-cross sectional area (CSA) of blood vessels. In due course, these changes in vascular function and structure are important contributors to cardiovascular disease [32]. Resistance artery remodeling encompasses extensive and dynamic structural changes in cytoskeletal organization, cell-to-cell connections and extracellular matrix interactions that are controlled by a myriad of mechanical and neurohumoral stimuli [32-34]. In particular,

inward eutrophic remodeling, defined as a reduced passive luminal diameter and increased media/lumen ratio without changes in CSA of the vascular wall, is the most common structural change observed in resistance arteries of individuals suffering from essential hypertension, and its presence is highly predictive of life threatening cardiovascular events.[35-37]. Thus, to determine how maternal hyperleptinemia without insulin resistance or obesity affects the risk of hypertension in offspring, blood pressure measurements and detailed analyses of resistance artery reactivity and structural remodeling were conducted in male offspring of wild type (WT)-control and $Lepr^{db/+}$ dams, at 6 weeks of age (juvenile) and at 31 weeks of age (adult) after challenging offspring with a high fat, high sugar diet.

3.2 Materials and Methods

3.2.1 Animals and tissue collection

All animal procedures were approved by the University of Missouri–Columbia Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were housed in a 12/12 hour light/dark cycle at 20°C to 26°C with 30% to 70% humidity. $Lepr^{db/+}$ male mice (Strain: B6.BKS(D)-$Lepr^{db}$/J; stock number: 000697) obtained from Jackson Laboratory (Bar Harbor, Maine) were mated to C57Bl/6 wildtype females bred at the University of Missouri to establish the $Lepr^{db}$ colony. Wildtype females came either from this colony, or directly from the Jackson Laboratory.

An overview of the experiments involving animals is presented in Fig 1A. In order to maintain the same genetic heterogeneity within litters, WT females were mated to leptin

receptor heterozygous knockout ($Lepr^{db/+}$) males and $Lepr^{db/+}$ females were mated to WT males and only WT offspring of each cross were followed. As previously reported, on day 16.5 of pregnancy, the $Lepr^{db/+}$ dams were hyperleptinemic based on fasting leptin levels compared to the WT control mothers [25]. Litter sizes were not affected by maternal hyperleptinemia [25]. Weights, behaviors and metabolic characteristics have been reported previously for the mice used in this study [25]. For this study, juvenile (6 week old) and adult (31 week old) WT male offspring were evaluated for alterations in blood pressure as described below. Two WT male offspring from each mother were followed for 31 weeks. At 23 weeks of age, when offspring were fully adult, half were placed on a high fat, high sugar diet (HFD, 45% kcal/fat, 17% kcal/sucrose, Research Diets D12451) to identify any fetal programming effects that might interact with offspring diet. This period was chosen to permit comparison to a previous study of maternal hyperleptinemia with food restriction [38]. Following arterial blood pressure measurements at 6 or 31 weeks of age, all mice were euthanized by inducing pneumothorax followed by exsanguination, while under anesthesia and tissues were harvested for further analysis. Mesenteric arteries were studied as described below. Hearts were fixed in 4% paraformaldehyde (PFA) overnight and then embedded in optimal cutting temperature (O.C.T.) compound (Fisher Scientific, Pittsburgh, PA) for analysis.

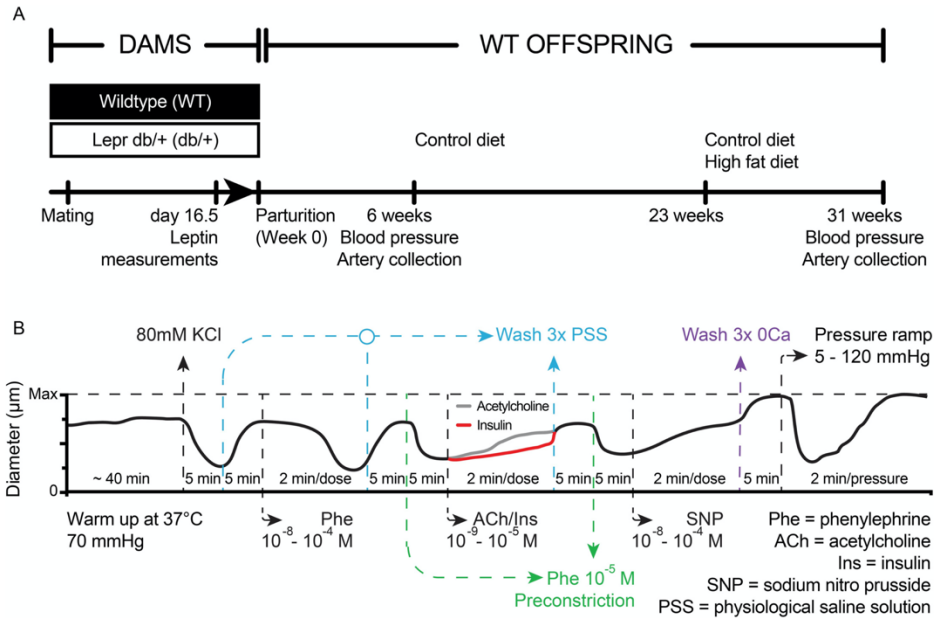

Fig 1. Experimental design. (A) Animal experiments. Blood pressure (BP) and mesenteric artery structure and function were examined in male wild type (WT) offspring of WT-control and hyperleptinemic Leprdb/+ dams. (B) Protocol to test vascular reactivity in isolated, cannulated and pressurized mesenteric resistance arteries. Two arteries were tested for each mouse. The red and green lines indicate that only one of the arteries from each mouse was exposed to either insulin or acetylcholine. At the end of each experiment all arteries were incubated in calcium-free buffer to obtain maximal passive diameters and subsequently exposed to varying levels of intraluminal pressure.

3.2.2 Blood pressure analysis

To determine if maternal hyperleptinemia altered offspring blood pressure, systolic and diastolic blood pressures were measured in juvenile (6 week old) and adult male (31 week old) offspring from WT-control and Lepr$^{db/+}$ dams using a tail cuff CODA non-invasive blood pressure system (Kent Scientific, Torrington CT, USA) as well as by carotid

catheter under anesthesia as previously described [39]. For tail-cuff measurements, individual animals were placed in a commercially acquired restraining tube and allowed to acclimatize for 10 minutes prior to initiating the blood pressure measurement protocol. For the invasive blood pressure measurements, mice were anesthetized with inhaled isoflurane and while under surgical plain anesthesia at 2% isoflurane, a carotid artery was cannulated and arterial pressure measured using a PowerLab data acquisition system and LabChart software (ADInstruments) as previously described [39].

3.2.3 Mesenteric resistance artery functional analyses

Vascular reactivity was evaluated in two second-order mesenteric resistance arteries (186-301 μm in internal diameter) harvested from offspring following blood pressure analysis as previously described [40,41]. Briefly, to evaluate vasoconstrictor responses, mesenteric artery segments from each mouse were exposed to 80 mM KCl (equimolarly substituted for NaCl) to test for viability and then to cumulative concentrations of phenylephrine to study adrenergic-dependent vasoconstriction. To evaluate endothelium-dependent vasodilation, one of the arteries was exposed to insulin, and the second to acetylcholine (ACh). Finally, both arteries were exposed to cumulative concentrations of sodium nitroprusside (SNP) to evaluate endothelium-independent vasodilatory responses. All vasodilatory responses were assessed on arteries pre-constricted with 10^{-5} M phenylephrine (Fig 1B), as there were no differences in phenylephrine-induced vasoconstriction responses between the experimental groups and all arteries exhibited similar levels of constriction in response to this concentration of phenylephrine.

3.2.4 Confocal/multiphoton microscopy imaging of mesenteric arteries

At the end of each experiment, vessels were fixed in 4% paraformaldehyde, while pressurized at 70 mmHg for 1 hour. For imaging, vessels were rinsed twice in phosphate buffered saline (PBS) and once in 0.1 M Glycine for 5 minutes each time. Cannulated vessels were flushed with 1 mL PBS to rinse their lumen and permeabilized via incubation in 0.5% Triton X-100 for 20 minutes. Vessels were washed twice in PBS and incubated for 1 hour in 0.5 µg/mL 4',6-diamidino-2-phenylindole (DAPI), 0.2 µM Alexa Fluor 633 Hydrazide (Molecular Probes) and 0.02 µM Alexa Fluor 546 phalloidin (Molecular Probes) in PBS. After being washed 3 times in PBS, vessels were imaged using a Leica SP5 confocal/multiphoton microscope with a 63x/1.2 numerical aperture water objective. Alexa Fluor 633, to image elastin, was excited with a 633 nm HeNe laser. Alexa Fluor 546 phalloidin, to image F-actin components, was excited with a 543 nm HeNe laser. DAPI, to image nuclei, was excited with a multi-photon laser at 720 nm. Collagen was imaged via second-harmonic image generation using a multi-photon laser at 850 nM. All imaging and image analyses were performed as previously described [42].

3.2.5 Elasticity measurements in mesenteric arteries

Circumferential strain, circumferential stress, Young's modulus of elasticity, and compliance were all calculated using the internal diameter and wall thickness measurements obtained during the pressure-diameter curves generated while vessels were under passive conditions. The number and area of fenestrae in the internal elastic lamina

(IEL) were determined and the modulus of elasticity specific for the IEL was calculated using images generated with Alexa 633 staining as previously described [42].

The circumferential strain was calculated as the difference between the intraluminal diameter obtained under passive conditions at each intraluminal pressure level (Dp) minus the diameter at the lowest pressure tested (5 mmHg) divided by the diameter at lowest pressure [34]:

$$\epsilon = \frac{D_P - D_{5\text{mmHg}}}{D_{5\text{mmHg}}}.$$

Circumferential stress was calculated by using the formula for thin-walled vessels Where P is the intraluminal pressure, and τ is wall thickness:

$$\sigma = \frac{P * D_P}{2\tau},$$

Compliance (C) provides information on arterial wall stiffness. It is particularly sensitive at low pressures and was calculated with the following formula [43], where ΔA is the change in the cross-sectional area of the wall relative to an increment in intraluminal pressure ΔP:

$$C = \frac{\Delta \text{A}}{\Delta \text{P}}.$$

The Young's modulus of elasticity provides information about arterial stiffness, particularly at high levels of intraluminal pressure [34]. It is the slope in the strain-stress curve at each pressure as represented in the formula below [34].

$$E = \frac{\sigma}{\epsilon}.$$

The elastic modulus can be separated in two portions depending on the level of intravascular pressure in order to separate the moduli dominated by elastin and that dominated by collagen [44-46]. We chose the first three values in the lower rage of intravascular pressures (Region I: 5, 10, 20 mmHg) to provide information on the low Young modulus of elasticity (E_{low}). At low pressures elastin dominates the elastic behavior of the artery. At higher pressures the dominant element is collagen and we chose the last three values of intraluminal pressure (Region II: 80, 100, 120 mmHg) to determine the high Young modulus of elasticity (E_{high}). E_{low} and E_{high} represent the slope of the linear regressions of Regions I and II respectively.

The modulus of elasticity specific for the internal elastic lamina (IEL) can be interpreted as an array of coupled springs, where the fenestrae in the IEL are vertices between the springs. Under this consideration, the elastic modulus can be associated to an area fraction in the elastic lamina. The area fraction is proportional to the number of holes or fenestrae (n) and their area (A, all sizes are the same) [47].

$$p = 1 - nA.$$

The number of holes per unit area is based in the symmetry used in the model. We used a honeycomb symmetry ($n = 2$) paradigm. Percolation ocurrs when the circular holes touch. For a honeycomb array the critical area fraction of material is $p_c = 1 - \pi/3\sqrt{3} = 0.395$. The normalized Young's modulus (φ) as a function of the percolation of the elastic lamina in the critical domain is:

$$\varphi_I(p) = \frac{E}{E_0} \approx \frac{2}{\pi\sqrt{3}}\left(\frac{p - p_c}{1 - p_c}\right)^{1/2},$$

While in the diluted limit is:

$$\varphi_{II}(p) = \frac{E}{E_0} = 3p - 2.$$

The total behavior of E/E_0 is described using an interpolation of the two regions φ_I and φ_{II}.

3.2.6 Heart histological analysis

To determine if cardiac lipid accumulation was affected by maternal hyperleptinemia, histological analysis was completed on adult male offspring hearts using Oil Red O staining based on published guidelines [48]. Frozen hearts were embedded and sectioned at 12 μm and 3 sections at 100 μm intervals were placed on a slide. Oil red O staining was then performed as described [48]. Quantitative analysis was performed as previously described [48] using imageJ software (NIH).

Cardiac fibrosis was also evaluated in male offspring by Picro-Sirius Red staining. Staining was performed on the heart by the histology core at IDEXX BioResearch Radil facility (Columbia, MO, USA) to assess fibrotic lesions. Tissue was visualized with a light microscope.

3.2.7 Statistical analyses

All data analyses were performed using Statistical Analysis System software version 9.4 (SAS®, SAS institute, Cary, NC). Data are presented as means ± SE. Values of $P \leq 0.05$ were considered significant.

For the data analysis of vascular functional responses, before model fitting, samples that did not constrict at least 20% relative to the maximal passive diameter with the application of high KCl were removed from analysis. For each of the applications of vasodilatory agonists (acetylcholine, insulin, and SNP), samples that did not constrict at

least 20% relative to the maximal diameter with the initial application of phenylephrine were removed from the analysis of that vasoactive agent.

The offspring arterial diameters were modeled to determine which factors were significant when testing the response of the sample with each of the vasoactive agents: phenylephrine, acetylcholine, insulin, and SNP, respectively. The diameters were normalized to represent values relative to their maximal diameter and initial constriction to account for the large variability amongst the arterial diameters. These normalized values were regressed to the maternal environment ($Lepr^{db/+}$ and WT), offspring diet (HFD-fed and standard diet (SD)-fed), and nine levels of agonist (over time) as well as interaction effects between each two and three factors. Repeated measurements of each artery of an offspring were taken at several time points for each application of acetylcholine and insulin. Mean of two repeated measurements from two arteries of the same offspring were taken at each time point for both applications of phenylephrine and SNP. Thus, to account for the correlation among the repeated measurements, a heterogeneous compound symmetry correlation structure was used. This correlation structure was chosen based on AIC (Akaike information criterion), BIC (Bayesian information criterion), and its biological interpretation. Each model was constructed using backward elimination. The main effects and interaction effects were selected by controlling the statistical significance at level 0.10. The studentized residual plots for each of the models suggest that model assumptions are met in all cases. The Kenward-Roger adjustment to denominator degrees of freedom was used when fitting all three models in this study because of the unbalanced design, random effects, repeated measures, and moderate sample size. After model

selection, the Tukey-Kramer HSD (honest significant difference) method was used to adjust multiple tests in comparing different levels of treatment combinations.

The structural and elastic properties of mesenteric arteries were modeled to determine which factors (maternal environment, Leprdb/+ or WT), offspring diet (HFD-fed or SD-fed), pressure, and interactions were significant. The same model construction and selection procedures described in the data analysis for the vascular functional responses were utilized for data analysis here. Residual plots were examined to ensure model assumptions are met. Similarly, the Kenward-Roger degree of freedom was used since experiments are of unbalanced design with correlated observations. Finally, Tukey-Karmer HSD was used to adjust multiple tests.

3.3 Results

3.3.1 Maternal hyperleptinemia did not alter offspring blood pressure

To determine the effects of maternal hyperleptinemia on offspring's hypertension, blood pressures were measured in both conscious and anesthetized animals. Blood pressure and heart rate were obtained at either 6 or at 31 weeks of age (Fig 1) in offspring fed SD or HFD. There were no significant differences in blood pressure or heart rate in juvenile or adult offspring when measured in conscious animals using the tail-cuff method (data not shown). In adult offspring, catheter measurements of diastolic blood pressure, mean arterial pressure, and heart rate were significantly increased ($P<0.05$) by consumption of HFD, regardless of prenatal exposure to hyperleptinemia (Fig 2).

The increases in blood pressure and heart rate that occurred with HFD-feeding paralleled an increase in body weight (Fig 2C). The adult male offspring from $Lepr^{db/+}$

dams weighed less at sacrifice than offspring from WT dams only when the offspring were fed SD (Fig 2C). However, these mice were a subset of those used in a previous analysis, and when the larger group was examined, offspring of $Lepr^{db/+}$ dams weighed less regardless of offspring diet [25]. These differences in offspring weight associated with maternal hyperleptinemia were not matched by differences in offspring blood pressure or heart rate (Figure 2).

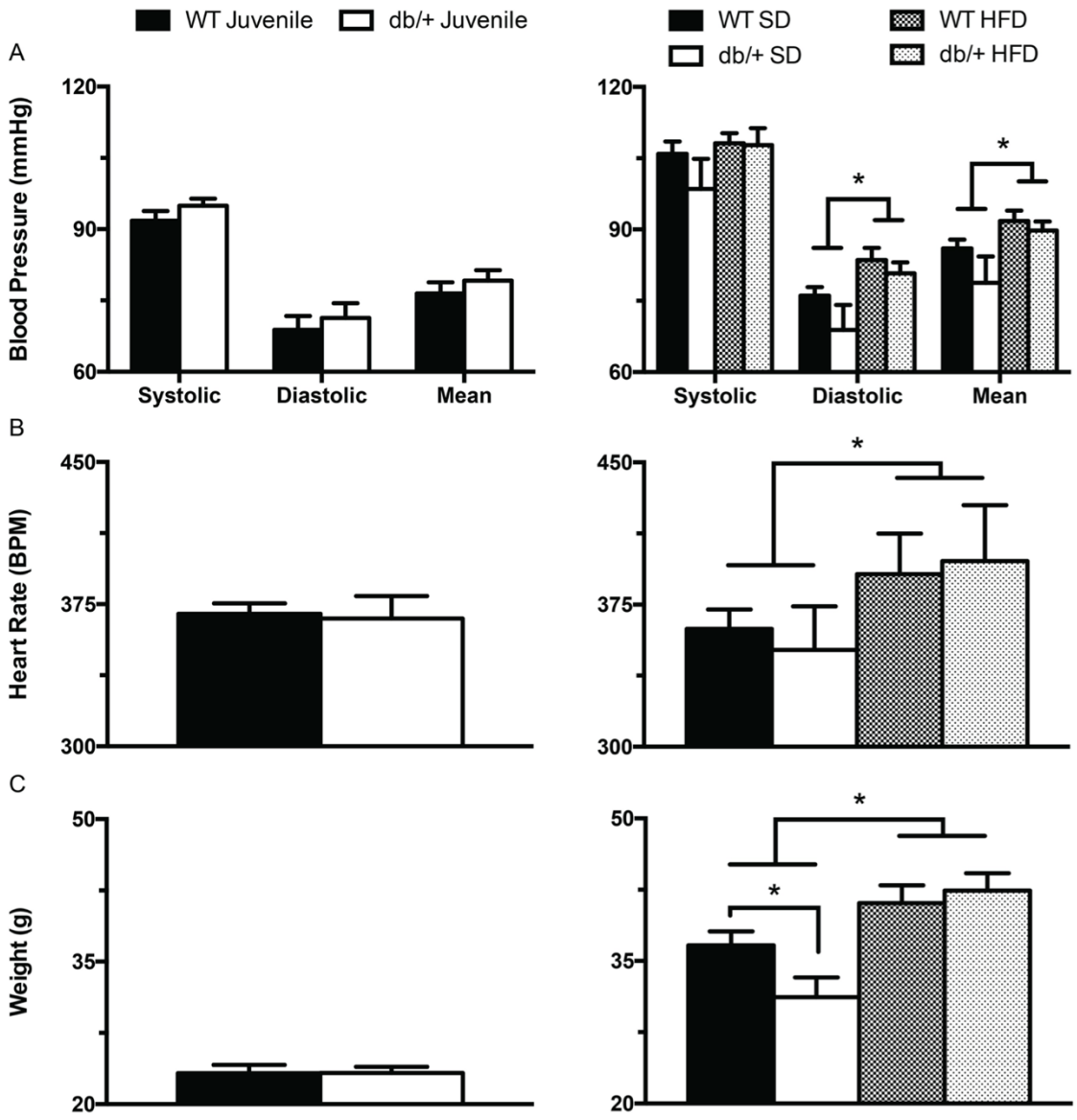

Fig 2. Effect of maternal environment and offspring diet on blood pressure (obtained via carotid catheter), heart rate and body weight of juvenile (6 week-old) and adult (31 week-old) wild type male offspring. (A) Systolic, diastolic and mean arterial pressures in 6 (left panel) and 31 (right panel) week old offspring. (B) Heart rate in 6 (left panel) and 31 (right panel) week old offspring. (C) Body weights in 6 (left panel) and 31 (right panel) week old offspring. Data are means ± SEM of n = 5–7 number of animals per treatment group combination. *P<0.05. WT, wild type; db/+, $Lepr^{db/+}$; SD, standard diet; HFD, high fat diet.

3.3.2 Responses to ACh and insulin were affected differentially by maternal hyperleptinemia, depending on offspring diet

We further examined the effect of maternal hyperleptinemia on offspring vascular function by examining mesenteric resistance artery vasoconstriction and vasodilation responses in the male WT offspring of WT-control and hyperleptinemic $Lepr^{db/+}$ dams. Mesenteric artery vasodilation and vasoconstriction responses in juvenile offspring maintained on a SD were not affected by maternal environment (Fig 3).

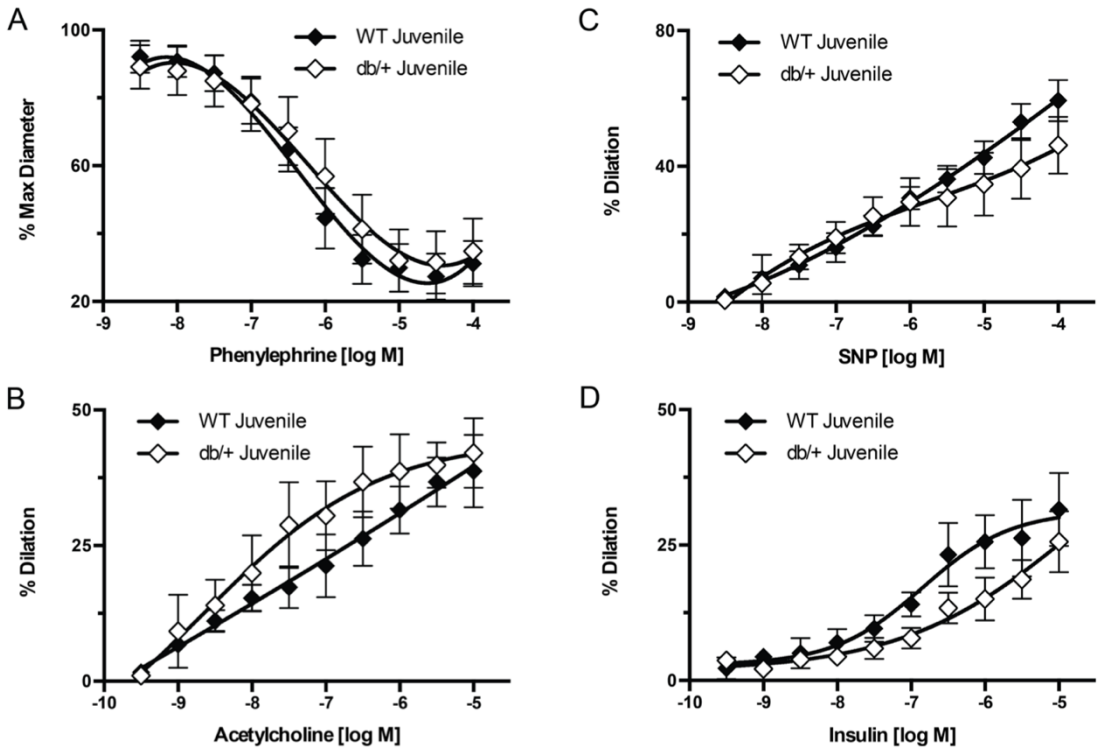

Fig 3. Effect of maternal environment on mesenteric artery responses to vasoactive agonists. Mesenteric arteries were obtained from juvenile (6 week-old) wild type (WT) male offspring of WT-control or Lepr$^{db/+}$ (db/+) dams. (A) Vascular responses to increasing concentrations of phenylephrine. (B) Vascular responses to increasing concentrations of acetylcholine. (C) Vascular responses to increasing concentrations of sodium nitroprusside (SNP). (D) Vascular responses to increasing concentrations of insulin. Data are means ± SEM of n = 4–5 number of animals (vessels) per treatment group combination.

The interacting effects of (maternal environment) x (offspring diet) on vascular function were evaluated in mesenteric arteries from adult offspring. Vasoconstriction in response to alpha-1 adrenergic stimulation was tested using phenylephrine. Arterial

vasoconstriction responses to incremental concentrations of phenylephrine did not differ between offspring from WT and $Lepr^{db/+}$ dams, regardless of offspring diet (Fig 4A–4D). Endothelial-independent vasodilation was also tested, and no significant differences were observed for SNP-induced vasodilation among any of the groups (Fig 4E–4H).

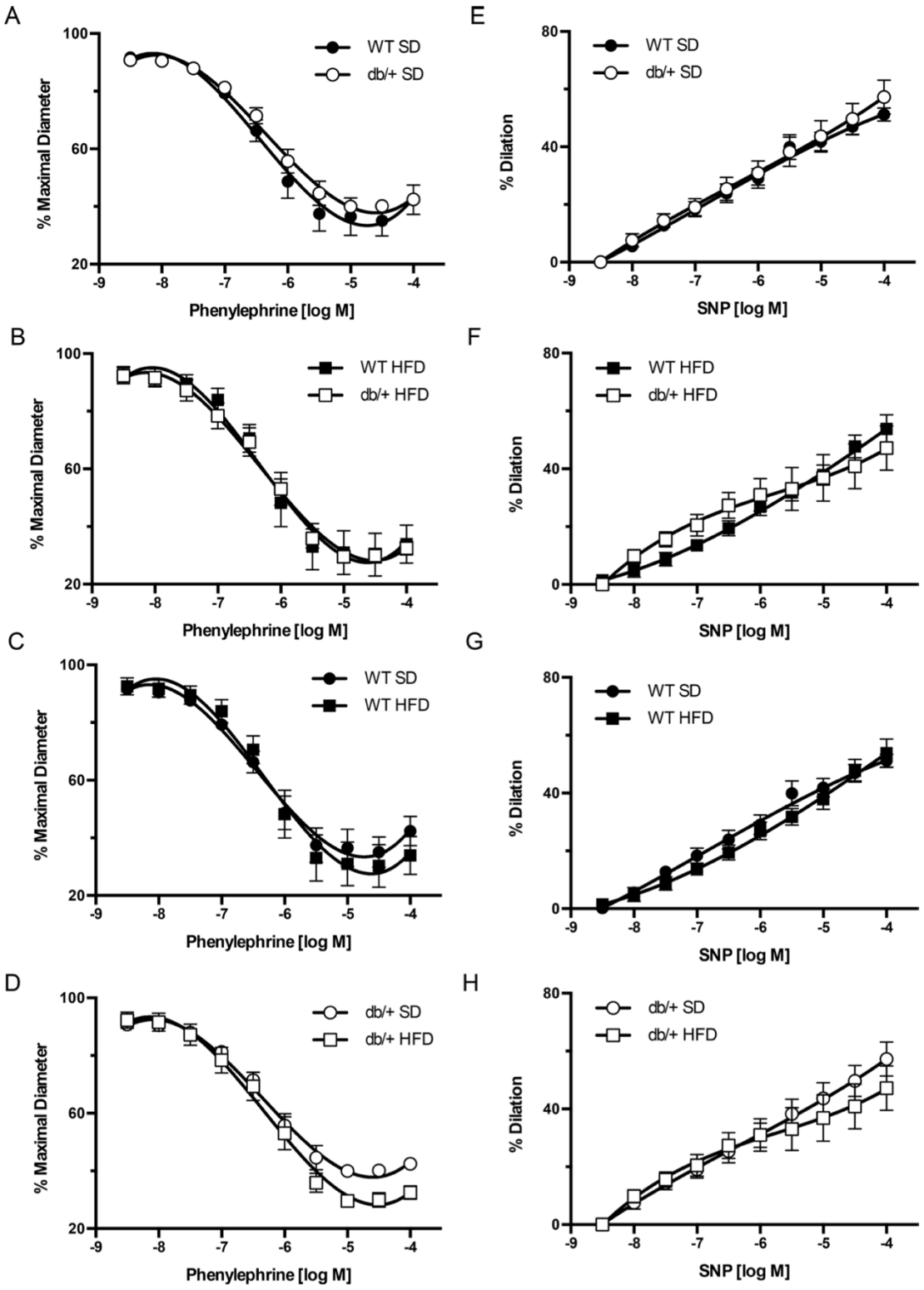

Fig 4. Effect of maternal environment and offspring diet on mesenteric resistance artery responses to phenylephrine and sodium nitroprusside (SNP). All

blood vessels were obtained from adult (31 week-old) wild type (WT) male offspring of WT-control or $Lepr^{db/+}$ (db/+) dams. (A-D) Phenylephrine-induced vasoconstriction responses. (E-H) SNP-induced vasodilatory responses. Data are means ± SEM of n = 5–7 number of animals (vessels) per treatment group combination. SD, standard diet; HFD, high fat diet.

Endothelial-dependent vasodilation responses of mesenteric arteries to ACh (Fig 5A–5D) and insulin (Fig 5E–5H) were also assessed. When offspring were fed a SD, those born to WT and Leprdb/+ dams exhibited no differences in ACh-induced vasodilation (Fig 5A). However, offspring of WT dams had greater ($P<0.05$) vasodilatory responses to ACh when fed a HFD than a SD (Fig 5C). No differences in ACh vasodilation responses were observed between arteries of offspring fed the HFD from WT and Leprdb/+ dams (Fig 5B) or between SD- and HFD-fed offspring from Leprdb/+ dams (Fig 5D).

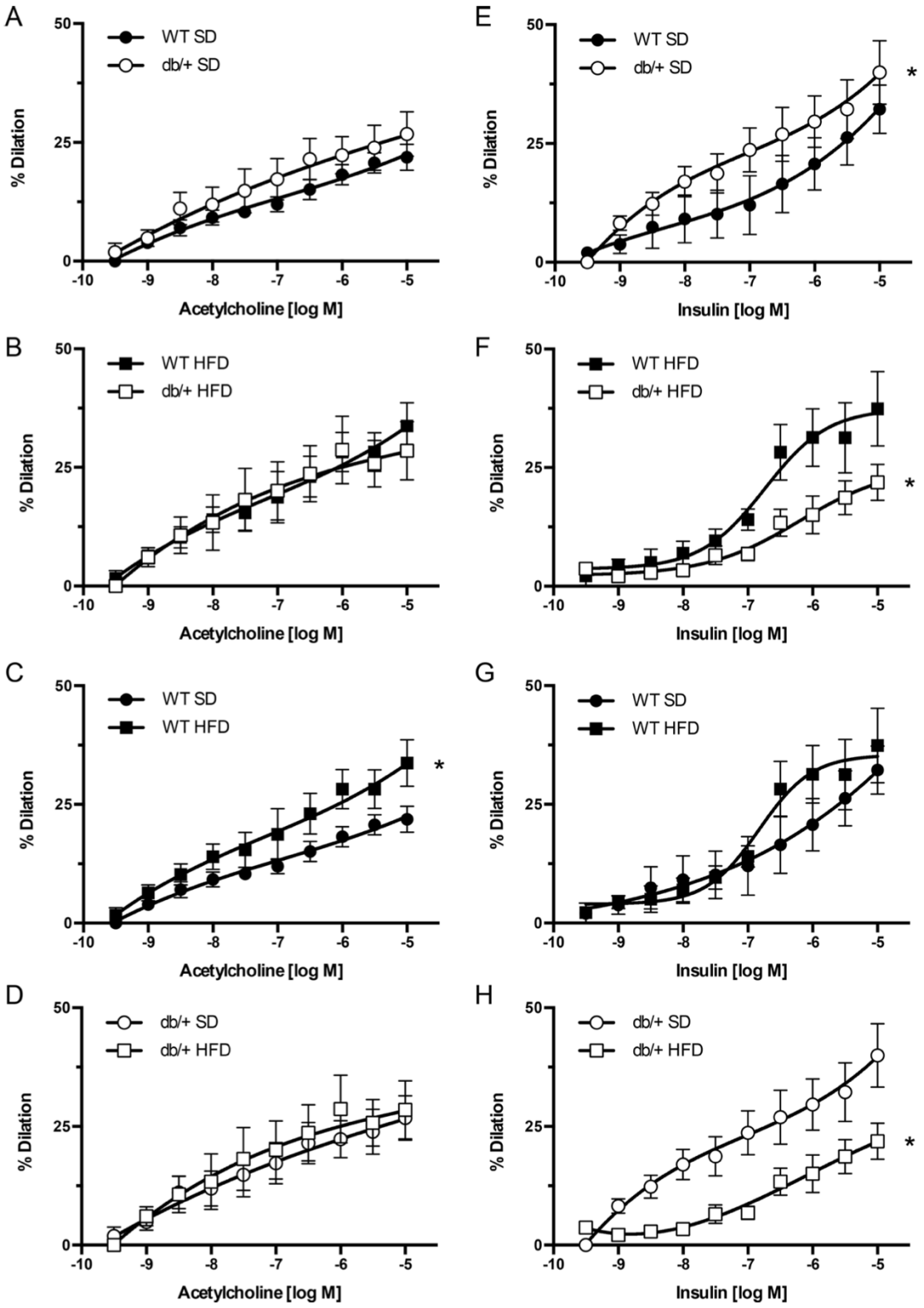
A
WT SD
db/+ SD
% Dilation
Acetylcholine [log M]
B
WT HFD
db/+ HFD
% Dilation
Acetylcholine [log M]
C
WT SD
WT HFD
*
% Dilation
Acetylcholine [log M]
D
db/+ SD
db/+ HFD
% Dilation
Acetylcholine [log M]
E
WT SD
db/+ SD
*
% Dilation
Insulin [log M]
F
WT HFD
db/+ HFD
*
% Dilation
Insulin [log M]
G
WT SD
WT HFD
% Dilation
Insulin [log M]
H
db/+ SD
db/+ HFD
*
% Dilation
Insulin [log M]

Fig 5. Effect of maternal environment and offspring diet on mesenteric resistance artery responses to acetylcholine and insulin. All blood vessels were obtained from adult (31 week-old) wild type (WT) male offspring of WT-control or $Lepr^{db/+}$ (db/+) dams. (A-D) Acetylcholine-induced vasodilatory responses. (E-H) Insulin-induced vasodilatory responses. Data are means ± SEM of n = 5–6 number of animals (vessels) per treatment group combination. *P<0.05. SD, standard diet; HFD, high fat diet.

In offspring fed a SD, the vasodilation response to insulin was significantly greater (P<0.05) in arteries from offspring of $Lepr^{db/+}$ dams than in offspring of WT dams (Fig 5E). In contrast, insulin dependent vasodilation was significantly blunted (P<0.05) in HFD-fed offspring of Leprdb/+ dams compared to HFD-fed offspring of WT-control dams (Fig 5F) and SD-fed offspring of Leprdb/+ dams (Fig 5H). There were no differences in insulin-induced vasodilation between SD- and HFD-fed offspring from WT-control dams (Fig 5G).

3.3.3 The elastic properties of mesenteric arteries were affected by a (maternal environment) x (offspring diet) interaction

We further examined the structural properties of mesenteric arteries to determine if the observed differences in vasodilation were associated with differences in vascular remodeling. No differences in vascular structural characteristics were observed in juvenile offspring (Fig 6).

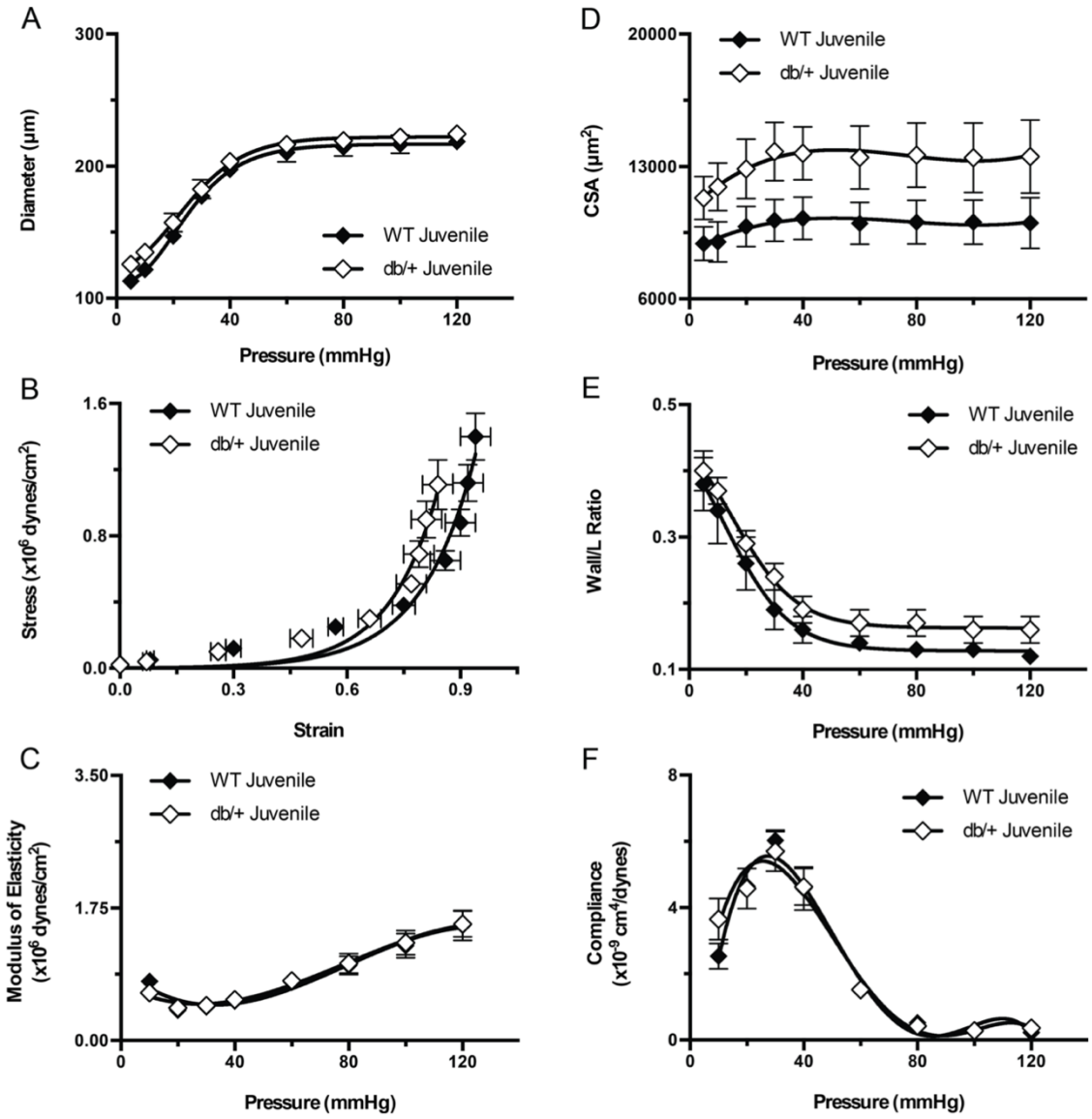

Fig 6. Effect of maternal environment on the structure and mechanical properties of mesenteric resistance arteries from juvenile (6 week-old) wild type (WT) male offspring of WT-control or Lepr$^{db/+}$ (db/+) dams. (A) Pressure-diameter curves of blood vessels kept under passive conditions. (B) Strain-stress relationship curves of mesenteric arteries kept under passive conditions at different intravascular pressures. (C) Elastic moduli of mesenteric arteries kept under passive conditions at different intravascular pressures. (D) Cross sectional area (CSA) of the vascular wall in mesenteric

arteries kept under passive conditions at different intravascular pressures. (E) Vascular wall to intravascular lumen ratio of mesenteric arteries kept under passive conditions at different intravascular pressures. (F) Compliance of mesenteric arteries kept under passive conditions at different intravascular pressures. Data are means ± SEM of n = 5 number of animals (vessels) per treatment group combination.

In adult mice, mesenteric vascular remodeling in response to HFD differed between WT male offspring from $Lepr^{db/+}$ and WT-control dams. In offspring fed a SD, the passive luminal diameter of arteries was significantly greater ($P<0.05$) in offspring of $Lepr^{db/+}$ dams than in offspring from WT dams (Fig 7A). HFD feeding significantly increased passive luminal diameters and CSAs in offspring from WT dams (Fig 7C and 7G), but not in offspring from $Lepr^{db/+}$ dams (Fig 7D and 7H). As a result, on HFD, the CSA was significantly reduced ($P<0.05$) in offspring of Leprdb/+ dams versus that from WT dams (Fig 7F). No differences were observed in the arterial wall-to-lumen ratios among any of the groups (data not shown).

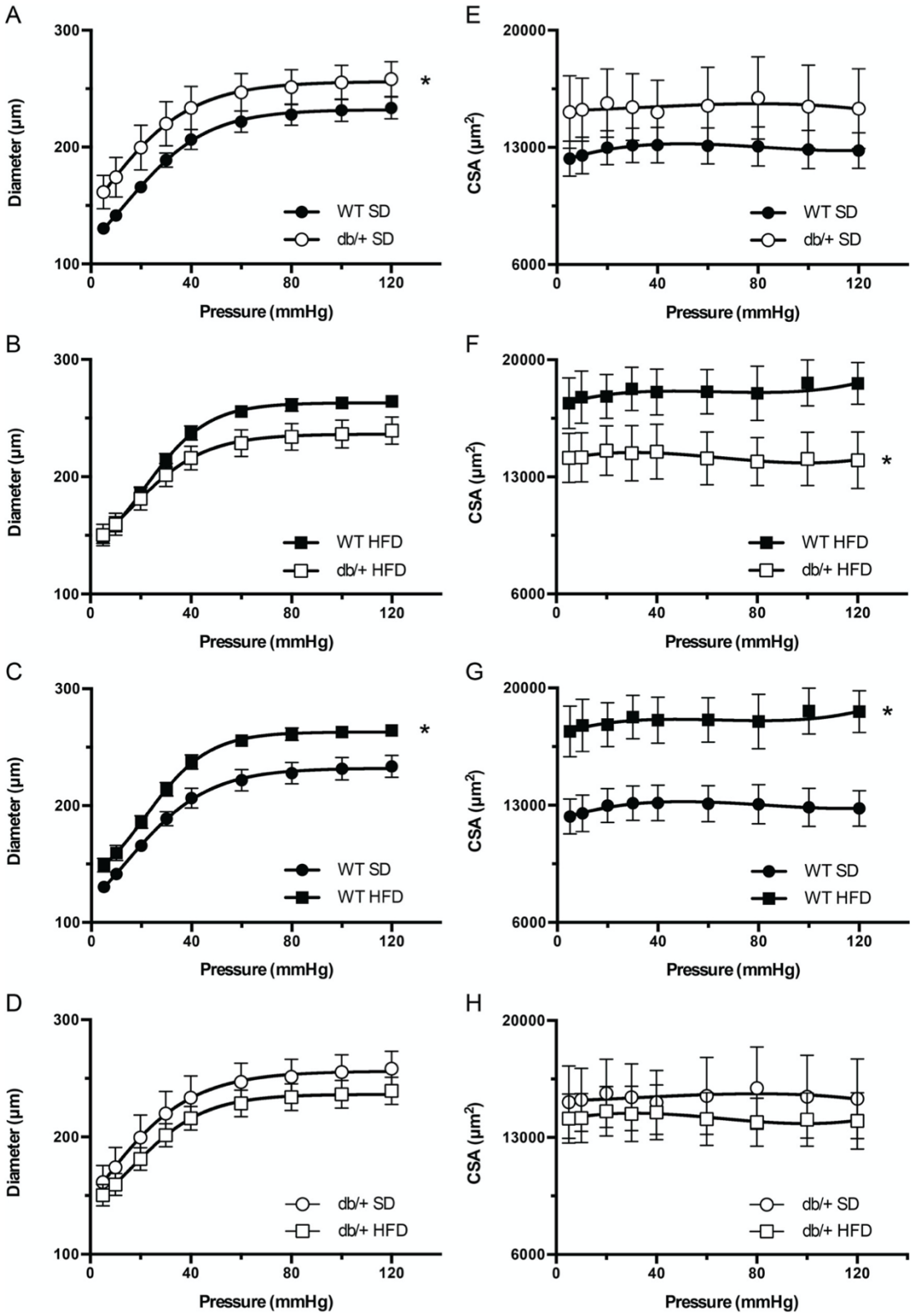
A
B
C
D
E
F
G
H
Diameter (μm)
CSA (μm²)
Pressure (mmHg)
WT SD
db/+ SD
WT HFD
db/+ HFD
*
100
200
300
6000
13000
20000
0
40
80
120

Fig 7. Effect of maternal environment and offspring diet on the structural properties of mesenteric resistance arteries from adult (31 week-old) wild type (WT) male offspring of WT-control or Lepr$^{db/+}$ (db/+) dams. (A-D) Pressure-diameter curves of blood vessels kept under passive conditions. (E-H) Cross sectional area (CSA) of the vascular wall in mesenteric arteries kept under passive conditions at different intravascular pressures. Data are means ± SEM of n = 5–7 number of animals (vessels) per treatment group combination. *P<0.05. SD, standard diet; HFD, high fat diet.

Arterial stiffness was affected by maternal environment, and diet had an effect depending on maternal environment. Offspring of Lepr$^{db/+}$ dams had significantly reduced (P<0.05) vascular wall strain values (Figure 8A-B) and higher moduli of elasticity (Figure 8E-F), indicative of arterial stiffness compared to offspring of WT dams. There were no differences in vascular wall stress (Figure 8C-D) or elastic moduli (Figure 8G-H) between diets. To investigate arterial stiffness at low pressures, we analyzed arterial compliance (Figure 8I-L) and the low-modulus of elasticity (Figure 8I-L, inset). The low-modulus of elasticity in mesenteric arteries from offspring of Lepr$^{db/+}$ dams was increased (P<0.05) compared to offspring of WT dams, when offspring were fed a HFD (Figure 8J, inset). This effect of HFD was correlated with a tendency for an increased vascular compliance at low pressures (Figure 8J); however, the difference in compliance did not reach statistical significance. No other significant effects of maternal environment or diet on arterial compliance or the low-modulus of elasticity were observed. Thus, maternal hyperleptinemia increased arterial stiffness at high pressures, independent of offspring diet, whereas at low pressures, there was a (maternal environment) x (offspring diet) interaction,

such that offspring of WT, but not Lepr$^{db/+}$ dams, had reduced arterial stiffness (increased compliance) in response to HFD.

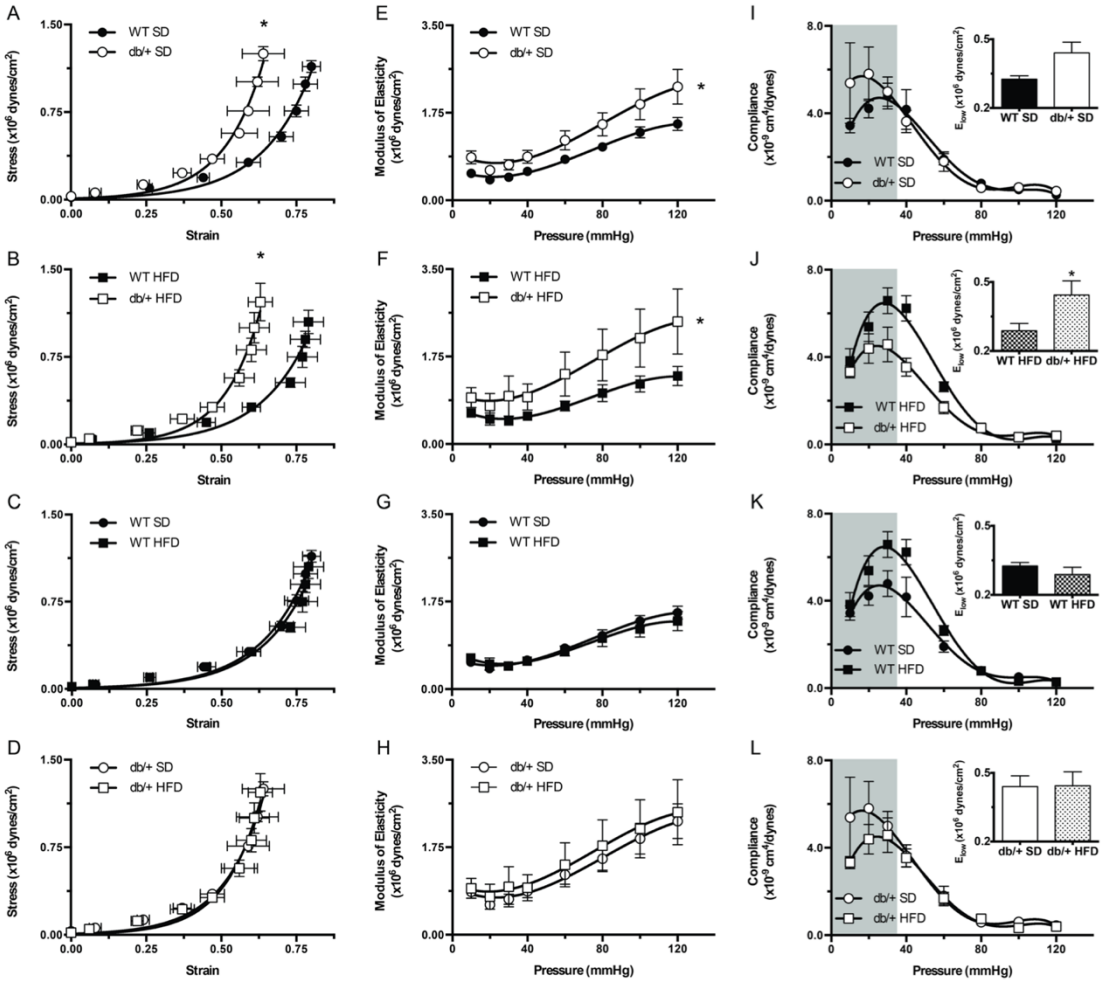

Fig 8. Effect of maternal environment and offspring diet on the mechanical properties of mesenteric resistance arteries from adult (31 week-old) wild type (WT) male offspring of WT-control or Lepr$^{db/+}$ (db/+) dams. (A-D) Strain-stress relationship curves of mesenteric arteries kept under passive conditions at different intravascular pressures. (E-H) Elastic moduli of mesenteric arteries kept under passive conditions at different intravascular pressures. (I-L) Compliance of mesenteric arteries kept under passive conditions at different intravascular pressures. The shaded areas represent the data

used to calculate the elastic moduli at low pressures, which are shown in the insets. Data are means ± SEM of n = 5–7 number of animals (vessels) per treatment group combination. *P<0.05. SD, standard diet; HFD, high fat diet.

3.3.4 The structural composition of mesenteric resistance arteries was affected by a (maternal environment) x (offspring diet) interaction

The cytoskeletal and extracellular matrix composition of blood vessels directly impacts vascular structure and stiffness. Therefore, we examined F-actin, elastin and collagen contents of mesenteric arteries from adult offspring of WT-control and $Lepr^{db/+}$ dams by confocal/multiphoton microscopy (Fig 9). Maternal environment did not affect the F-actin cytoskeletal composition of these arteries (Figure 9F). However, offspring fed a HFD, regardless of maternal genotype, had significantly reduced (P<0.05) F-actin cytoskeletal volume in the medial layer compared to vessels from offspring on a SD (Fig 9F-H). The elastin content (Fig 9I-K) was also significantly reduced (P<0.05) in HFD-fed offspring compared to SD, regardless of maternal environment. The number of vascular smooth muscle cells and collagen content of the arteries was not different among any of the groups (Fig 9L and 9M).

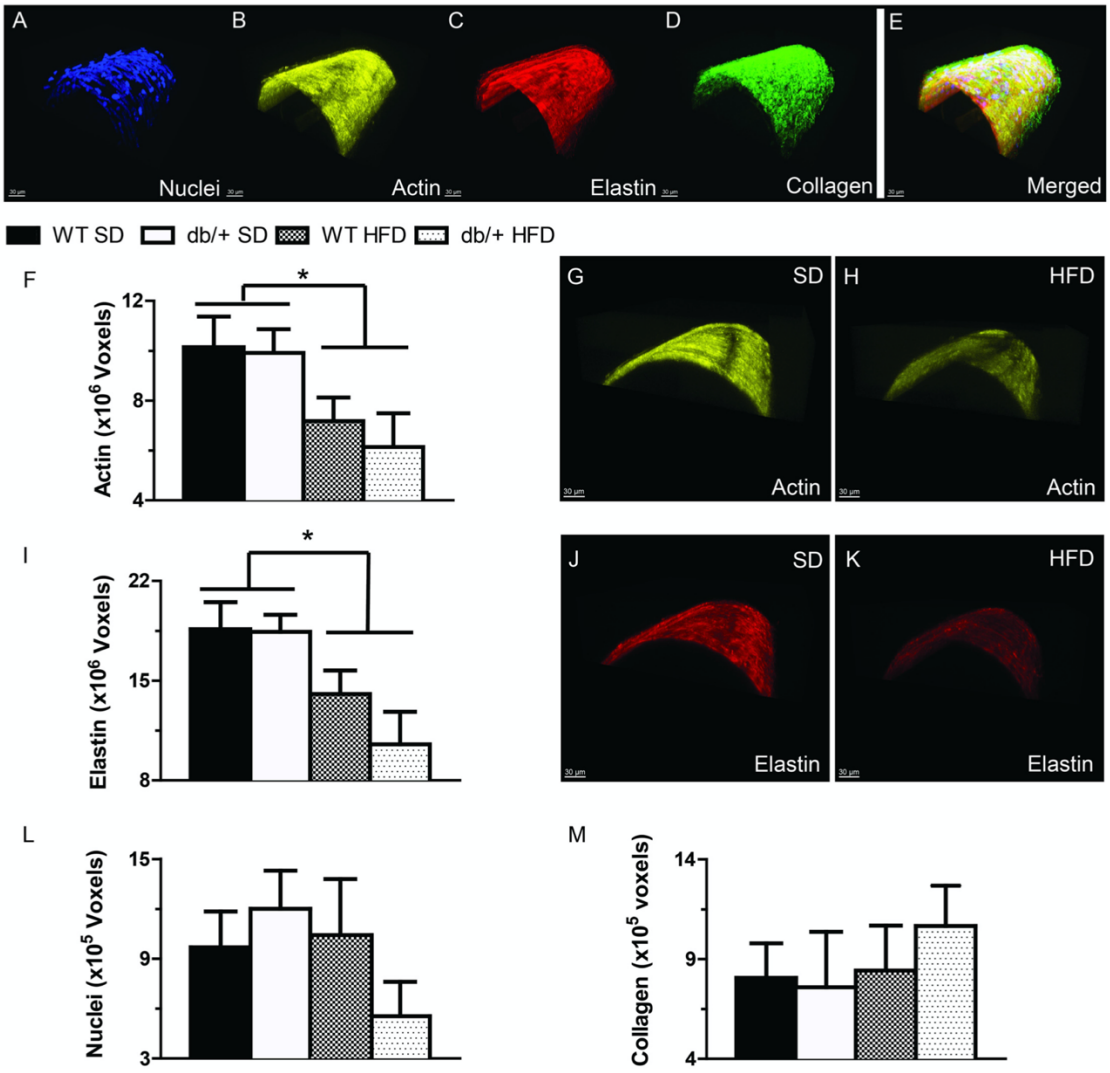

Fig 9. Effect of maternal environment and offspring diet on the morphological characteristics of mesenteric resistance arteries from adult (31 week-old) wild type (WT) male offspring of WT-control or Lepr$^{db/+}$ (db/+) dams. (A-E) Representative confocal images of mesenteric resistance arteries showing (A) nuclei; (B) F-actin; (C) elastin; (D) collagen; and (E) merged image. (F-H) Group data and representative images showing that feeding a high-fat diet was associated with a significant reduction in arterial F-actin content. (I-K) Group data and representative images showing that feeding a high-fat diet was associated with a significant reduction in arterial elastin content. (L) Vascular

smooth muscle cell number, represented by nuclei contained within the medial layer of mesenteric arteries. (M) The amount of collagen contained in the wall of mesenteric arteries. Data are means ± SEM of n = 5–7 number of animals (vessels) per treatment group combination. *P<0.05. SD, standard diet; HFD, high fat diet.

The number and area of fenestrae in the internal elastic lamina (Fig 10) were also evaluated in the mesenteric arteries of the adult offspring. Overall, offspring from Lepr$^{db/+}$ dams had fewer (P<0.05) fenestrae compared to offspring from WT dams, regardless of diet, although in pairwise comparisons, the difference was only significant in HFD-fed offspring (Fig 10E). The mean area occupied by each fenestra was also significantly reduced by HFD (Fig 10F). The calculation of the internal elastic lamina elastic moduli normalized by the fenestrae critical value (E/E0) was significantly affected by maternal environment and diet. The E/E0 was significantly greater (P<0.05) in vessels from mice fed a HFD and in vessels from offspring of Lepr$^{db/+}$ dams (Fig 10G).

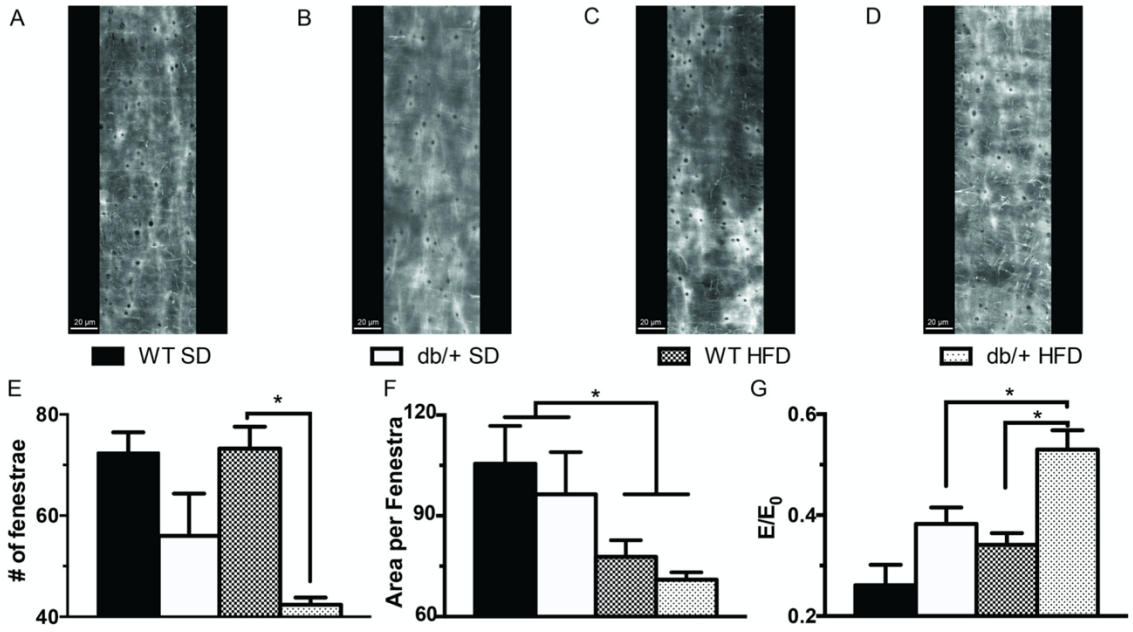

Fig 10. Effect of maternal environment and offspring diet on the internal elastic lamina characteristics of mesenteric resistance arteries from adult (31 week-old) wild type (WT) male offspring of WT-control or Lepr$^{db/+}$ (db/+) dams. (A-D) Representative confocal images of the internal elastic lamina in mesenteric resistance arteries from each of the treatment group combinations. (E-G) Group data showing the number and area of fenestrae within the internal elastic lamina and the elastic modulus of elasticity normalized as a function of the percolation of the internal elastic lamina and its fenestrae. Data are means ± SEM of n = 4–5 number of animals (vessels) per treatment group combination. *P<0.05. SD, standard diet; HFD, high fat diet.

3.3.5 Cardiac lipid accumulation and fibrosis were not affected by maternal environment or offspring diet

Lipid accumulation and fibrosis (data not shown) were assessed in hearts of WT male offspring from WT-control and Lepr$^{db/+}$ dams on either the SD or HFD. No differences were found in either parameter among any of the offspring groups.

3.4 Discussion

The adverse maternal environments of GDM and maternal obesity are characterized by maternal leptin resistance and hyperleptinemia [15-19]. As a consequence, there is both reduced leptin signaling in the mother, and exposure of the mother and placenta to high leptin concentrations. Here we evaluated the effect of high maternal leptin, in the absence

of maternal hyperglycemia or obesity, on offspring cardiovascular health, with specific emphasis on blood pressure and resistance artery function and structure. There was no difference in blood pressure in offspring of control and $Lepr^{db/+}$ dams, showing that maternal hyperleptinemia is not responsible for the hypertension observed in offspring of diabetic or obese mothers. However, maternal hyperleptinemia significantly impacted mesenteric artery function and structure in offspring, particularly the arterial response to high fat, high sugar diet consumption. These data suggest that maternal leptin interacts in complex ways with other factors in the maternal and postnatal environments to influence vascular health in offspring.

Alterations to resistance artery function and structure have profound effects on the development of hypertension and CVD [32,36,37]. Exposure to an adverse maternal environment also can lead to the development of hypertension and CVD [49-51]. However, there is limited information on the role that alterations in resistance artery function and structure play in programming of hypertension by the maternal environment [4-7,39,52]. In the offspring of hyperleptinemic dams, differences in resistance artery function were present without hypertension or obesity in the offspring suggesting first, that differences in resistance artery function and structure were directly programmed *in utero*, rather than resulting secondarily from differences in blood pressure or metabolism in the offspring. The absence of significant changes in arterial function or structure in juvenile mice and their presence in adult mice also suggest that the *in utero* effects of maternal hyperleptinemia on the offspring vasculature are mostly programming effects that are expressed only in the mature individual. Moreover, contrary to our initial expectations, maternal hyperleptinemia resulted in beneficial rather than detrimental effects in the

offspring vasculature. It increased vasodilatory responses to insulin and increased the passive diameter (outward remodeling) of mesenteric resistance arteries. These beneficial effects, however, occurred only in mice fed a SD. Adverse effects of maternal hyperleptinemia on the offspring vasculature included a specific detrimental response to insulin-induced vasodilation observed only when mice were fed a HFD, and an increase in arterial stiffness that was independent of diet effects.

The observation that changes in arterial function and structure were not associated with significant changes in blood pressure when mice were fed a SD supports the notion that alterations in vascular function and mechanics precede clinical alterations in cardiovascular function [42,53]. In addition, at least in the SD-fed offspring, programmed alterations in vasomotor responses, vascular remodeling and arterial stiffness may have offset each other, or been offset by other factors that were not measured, like cardiac output or fluid volume, resulting in no net change in blood pressure. Alternatively, it is possible that subtle changes in blood pressure were not detectable by tail cuff because of restraint stress, or by catheter, because of anesthesia.

With regard to vascular function, the observation that there were no significant changes in vascular responses to phenylephrine or SNP suggest that maternal hyperleptinemia had no programing effects on vascular smooth muscle responsiveness to vasoconstrictor or vasodilator agonists. Programing effects of maternal hyperleptinemia on vascular function were particular to the endothelium. Moreover the fact that vessels from SD-fed offspring of $Lepr^{db/+}$ dams had enhanced responses to insulin, but not to ACh, suggest that the beneficial effects of maternal hyperleptinemia on vascular function are associated with improvements in insulin signaling upstream of NO production by

endothelial NO synthase (eNOS). This is further supported by the observation that the detrimental effect on vascular function seen in HFD-fed offspring of hyperleptinemic dams consisted of a reduced vasodilatory responsiveness to insulin, but not to ACh. This becomes particularly interesting when once considers that HFD-feeding was associated with an augmented vasodilatory response to ACh in the offspring of WT-control damns, but not in the offspring of hyperleptinemic dams. Enhanced ACh responses following HFD have been shown in offspring of HFD-fed dams before [54] and in obese, diabetic db/db mice, [43] although others have reported reduced ACh response following extended exposure to HFDs [55]. Previous studies have also shown diminished insulin-induced vasodilation following HFD, as occurred in the offspring of hyperleptinemic dams, but only after a longer diet period (10 weeks) [56]. Taken together, these data suggest that maternal hyperleptinemia programs the vascular endothelium in mesenteric resistance vessels not to respond to overnutrition with an enhanced capacity for eNOS-dependent vasodilation and to reduce its responsiveness to insulin. The mechanisms associated with these responses are likely highly complex and remain to be determined.

Alterations in vasomotor responses are often associated with vascular remodeling processes and changes in the physical structure and mechanical properties of the vascular wall [32]. Remodeling is an intricately controlled process that encompasses changes in cytoskeletal organization, cell-to-cell connections and extracellular matrix composition and structure [32-34]. Previously, Souza-Smith et al. [43] showed that over-nutrition in the type 2 diabetic db/db mouse is associated with outward remodeling of the mesenteric resistance circulation. The increase in passive luminal diameter (outward remodeling) was attributed to hemodynamic changes caused by the increased blood flow associated with the

characteristic hyperphagia of this animal model. In our current study, mesenteric vessels obtained from offspring of hyperleptinemic dams remodeled outwardly as did those obtained from WT-control dams fed a HFD. It is possible that in the HFD-fed mice outward remodeling was caused by hemodynamic changes attributable to presence of this diet in the gut. However, for the outward remodeling observed in the arteries of offspring from hyperleptinemic dams fed a SD, the only observation that provides a potential mechanism for this phenomenon is the increased vasodilatory responsiveness to insulin seen in the same arteries. The plausibility of this mechanism is supported by the observation that feeding a HFD to offspring of hyperleptinemic dams did not induce outward remodeling in their mesenteric arteries and that this was associated with a reduced vasodilatory response to insulin. As in the study by Souza-Smith et al. [43] outward remodeling of the mesenteric arteries was associated with an increased CSA of the vascular wall, indicating that the remodeling was hypertrophic according to the characterization of remodeling introduced by Mulvany et al. [57].

As in previous studies, HFD consumption increased mean arterial blood pressure, due primarily to an increased diastolic blood pressure [58-60]. However, this increase in blood pressure was observed only in catheter measurements made in anesthetized animals and not in blood pressure measurements obtained using tail-cuff plethysmography. Others have shown that diet-induced obesity increases blood pressure using telemetry [53,59]. Therefore, it is likely that feeding of a HFD for 8 weeks had started changes in blood pressure regulation that induce hypertension in the present study. However, we cannot discard the possibility that the changes in blood pressure we observed were caused by changes in the sensitivity of the HFD-fed animals to isoflurane.

Mechanically, the arteries from offspring of hyperleptinemic dams had reduced strain levels and were stiffer than arteries from offspring of WT-control dams. This occurred without significant changes in arterial compliance al low pressures. Consumption of a HFD exacerbated the stiffening of arteries in offspring of hyperleptinemic dams, making the elastic modulus of their vessels at low pressures significantly greater than that in vessels from offspring of WT-control dams. This programming effect of maternal hyperleptinemia was not associated with any significant changes in the amount of vascular smooth muscle cells, F-actin stress fibers, elastin or fibrillar collagen contained within the vascular wall. Structurally, the outward hypertrophic remodeling associated with consumption of a HFD was associated with an overall reduction in F-actin and elastin content within the vascular wall. Paradoxically the reduction in elastin content was also associated with a significant reduction in the area occupied by fenestrae in the IEL and a specific reduction in the number of fenestra in the IEL of arteries from offspring fed a HFD that were obtained from hyperleptinemic dams. Consumption of a HFD has been previously shown to be associated with significant reduction in the fenestrae of vessels [42,61]. Calculation of the elastic modulus normalized as a function of the percolation of the internal elastic lamina and its fenestrae suggests that a reduction in the number and size of fenestrae may participate in augmenting the stiffness of mesenteric arteries in animals fed a HFD [62]. In comparison, the mechanism responsible for the diet-induced vascular hypertrophy in offspring of WT-control dams is not clear, because there were no significant changes in the amount of vascular smooth muscle nuclei, F-actin, fibrillar collagen or elastin contained in the wall of those vessels. It remains to be determined if the change in CSA was caused by presence of bigger cells with less actin stress fibers or by presence of

extracellular matrix proteins other than fibrillar collagen. In a previous study of C57Bl6 fed a 45% fat diet for 32 weeks, outward remodeling of mesenteric arteries occurred in association with adventitial and smooth muscle cell hyperplasia [61].

Further study is needed to untangle the complex underlying mechanisms responsible for functional and structural differences in the vasculature from mice exposed to prenatal maternal hyperleptinemia. Maternal leptin does not cross the placenta to reach the fetal circulation [63,64]; therefore the observed differences are not due to maternal leptin acting directly on developing fetal vasculature. Rather, maternal hyperleptinemia likely alters maternal metabolism and changes placental function to alter the delivery of nutrients and growth factors to the growing fetus [65-75]. Our other studies indicate that offspring born to hyperleptinemic mothers have better metabolic health overall, with lower body weights, increased spontaneous activity [25], and improved insulin and leptin sensitivity, which may in turn affect vascular function, as exemplified by the increased insulin-dependent vasodilation observed in offspring of hyperleptinemic dams. One possibility is that enhanced leptin sensitivity could affect expression of matrix metalloproteinases [75,76], which play a key role in artery remodeling [77]. Physical activity is known to slow the progression of CVD and improve vascular homeostasis by decreasing reactive oxygen species and increasing NO bioavailability in the endothelium [78].

Overall, this study indicates that exposure to high leptin levels *in-utero* affects vascular function in a manner dependent on the vasoactive stimulus and postnatal diet. Maternal hyperleptinemia was largely beneficial to vascular function when offspring were fed a SD, and deleterious when they were fed a HFD. This is supportive of the hypothesis

that alterations in maternal serum leptin may contribute to the changes in cardiovascular health observed in offspring of obese or diabetic pregnancies. This study also strengthens the idea that programming of arterial function may precede changes in blood pressure, and thus, may be a key mechanism by which maternal environment can alter cardiovascular health [39,52]. While alterations to vascular function are linked to the development of hypertension, it is important to note that these changes appear to occur prior to the onset of hypertension and that multiple vascular changes are observed in multiple vascular beds. Thus, studies over a longer time course, and in other vascular beds, may be necessary to fully understand whether vascular changes induced by maternal hyperleptinemia persist and lead to overt beneficial or adverse changes in blood pressure and CVD.

3.5 Acknowledgments

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3.6 References

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CHAPTER 4

ABSTRACTS

4.1 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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Maternal obesity and the use of assisted reproductive technologies (ART) are two suboptimal developmental 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 (BW) 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 were measured intracellularly and extracellularly in mesenteric resistance arteries. ART resulted in increased BW at weaning. This effect decreased over time and diet was the predominant determinant of BW 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 pre- and/or postnatally affects weight, MAP, and gene expression while ART increases oxidative stress in mesenteric resistance arteries of juvenile offspring, no synergistic effects were observed.

4.2 Lysophosphatidic acid induces integrin activation in vascular smooth muscle and alters arteriolar myogenic vasoconstriction

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In vascular smooth muscle cells (VSMC) increased integrin adhesion to extracellular matrix (ECM) proteins, as well as the production of reactive oxygen species (ROS) are strongly stimulated by lysophosphatidic acid (LPA). We hypothesized that LPA-induced generation of ROS increases integrin adhesion to the ECM. Using atomic force microscopy (AFM) we determined the effects of LPA on integrin adhesion to fibronectin (FN) in VSMC isolated from rat (Sprague–Dawley) skeletal muscle arterioles. In VSMC, exposure to LPA (2 μM) doubled integrin-FN adhesion compared to control cells ($P < 0.05$). LPA-induced integrin-FN adhesion was reduced by pre-incubation with antibodies against β1 and β3 integrins (50 μg/ml) by 66% ($P < 0.05$). Inhibition of LPA signaling via blockade of the LPA G-protein coupled receptors LPAR1 and LPAR3 with 10 μM Ki16425 reduced the LPA-enhanced adhesion of VSCM to FN by 40% ($P < 0.05$). Suppression of ROS with tempol (250 μM) or apocynin (300 μM) also reduced the LPA-induced FN adhesion by 47% ($P < 0.05$) and 59% ($P < 0.05$), respectively. Using confocal microscopy, we observed that blockade of LPA signaling, with Ki16425, reduced ROS by 45% ($P < 0.05$), to levels similar to control VSMC unexposed to LPA. In intact isolated arterioles, LPA (2 μM) exposure augmented the myogenic constriction response to step increases in intraluminal pressure (between 40 and 100 mm Hg) by 71% ($P < 0.05$). The blockade of

LPA signaling, with Ki16425, decreased the LPA-enhanced myogenic constriction by 58% ($P < 0.05$). Similarly, blockade of LPA-induced ROS release with tempol or gp91 ds-tat decreased the LPA-enhanced myogenic constriction by 56% ($P < 0.05$) and 55% ($P < 0.05$), respectively. These results indicate that, in VSMC, LPA-induced integrin activation involves the G-protein coupled receptors LPAR1 and LPAR3, and the production of ROS, and that LPA may play an important role in the control of myogenic behavior in resistance vessels through ROS modulation of integrin activity.

4.3 Low-Dose Mineralocorticoid Receptor Blockade Prevents Western Diet–Induced Arterial Stiffening in Female Mice

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Women are especially predisposed to development of arterial stiffening secondary to obesity because of consumption of excessive calories. Enhanced activation of vascular mineralocorticoid receptors impairs insulin signaling, induces oxidative stress, inflammation, and maladaptive immune responses. We tested whether a subpressor dose of mineralocorticoid receptor antagonist, spironolactone (1 mg/kg per day) prevents aortic and femoral artery stiffening in female C57BL/6J mice fed a high-fat/high-sugar western diet (WD) for 4 months (ie, from 4–20 weeks of age). Aortic and femoral artery stiffness were assessed using ultrasound, pressurized vessel preparations, and atomic force microscopy. WD induced weight gain and insulin resistance compared with control diet–fed mice and these abnormalities were unaffected by spironolactone. Blood pressures and heart rates were normal and unaffected by diet or spironolactone. Spironolactone prevented WD-induced stiffening of aorta and femoral artery, as well as endothelial and vascular smooth muscle cells, within aortic explants. Spironolactone prevented WD-induced impaired aortic protein kinase B/endothelial nitric oxide synthase signaling, as well as impaired endothelium-dependent and endothelium-independent vasodilation.

Spironolactone ameliorated WD-induced aortic medial thickening and fibrosis and the associated activation of the progrowth extracellular receptor kinase 1/2 pathway. Finally, preservation of normal arterial stiffness with spironolactone in WD-fed mice was associated with attenuated systemic and vascular inflammation and an anti-inflammatory shift in vascular immune cell marker genes. Low-dose spironolactone may represent a novel prevention strategy to attenuate vascular inflammation, oxidative stress, and growth pathway signaling and remodeling to prevent development of arterial stiffening secondary to consumption of a WD.

4.4 Endothelial Mineralocorticoid Receptor Mediates Diet-Induced Aortic Stiffness in Females

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Rationale: Enhanced activation of the mineralocorticoid receptors (MRs) in cardiovascular tissues increases oxidative stress, maladaptive immune responses, and inflammation with associated functional vascular abnormalities. We previously demonstrated that consumption of a Western diet (WD) for 16 weeks results in aortic stiffening, and that these abnormalities were prevented by systemic MR blockade in female mice. However, the cell-specific role of endothelial cell MR (ECMR) in these maladaptive vascular effects has not been explored.

Objective: We hypothesized that specific deletion of the ECMR would prevent WD-induced increases in endothelial sodium channel activation, reductions in bioavailable nitric oxide, increased vascular remodeling, and associated increases in vascular stiffness in females.

Methods and Results: Four-week-old female ECMR knockout and wild-type mice were fed either mouse chow or WD for 16 weeks. WD feeding resulted in aortic stiffness and endothelial dysfunction as determined in vivo by pulse wave velocity and ex vivo by atomic force microscopy, and wire and pressure myography. The WD-induced aortic stiffness was associated with enhanced endothelial sodium channel activation, attenuated

endothelial nitric oxide synthase activation, increased oxidative stress, a proinflammatory immune response and fibrosis. Conversely, cell-specific ECMR deficiency prevented WD-induced aortic fibrosis and stiffness in conjunction with reductions in endothelial sodium channel activation, oxidative stress and macrophage proinflammatory polarization, restoration of endothelial nitric oxide synthase activation.

4.5 Endothelial Estrogen Receptor-α Does Not Protect Against Vascular Stiffness Induced by Western Diet in Female Mice

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Consumption of a diet high in fat and refined carbohydrates (Western diet [WD]) is associated with obesity and insulin resistance, both major risk factors for cardiovascular disease (CVD). In women, obesity and insulin resistance abrogate the protection against CVD likely afforded by estrogen signaling through estrogen receptor (ER)α. Indeed, WD in females results in increased vascular stiffness, which is independently associated with CVD. We tested the hypothesis that loss of ERα signaling in the endothelium exacerbates WD-induced vascular stiffening in female mice. We used a novel model of endothelial cell (EC)-specific ERα knockout (EC-ERαKO), obtained after sequential crossing of the ERα double floxed mice and VE-Cadherin Cre-recombinase mice. Ten-week-old females, EC-ERαKO and aged-matched genopairs were fed either a regular chow diet (control diet) or WD for 8 weeks. Vascular stiffness was measured in vivo by pulse wave velocity and ex vivo in aortic explants by atomic force microscopy. In addition, vascular reactivity was assessed in isolated aortic rings. Initial characterization of the model fed a control diet did not reveal changes in whole-body insulin sensitivity, aortic vasoreactivity, or vascular stiffness in the EC-ERαKO mice. Interestingly, ablation of ERα in ECs reduced WD-induced vascular stiffness and improved endothelial-dependent dilation. In the setting of a

WD, endothelial ERα signaling contributes to vascular stiffening in females. The precise mechanisms underlying the detrimental effects of endothelial ERα in the setting of a WD remain to be elucidated.

4.6 Arterial Stiffening in Western Diet-Fed Mice Is Associated with Increased Vascular Elastin, Transforming Growth Factor-β, and Plasma Neuraminidase

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Consumption of excess fat and carbohydrate (Western diet, WD) is associated with alterations in the structural characteristics of blood vessels. This vascular remodeling contributes to the development of cardiovascular disease, particularly as it affects conduit and resistance arteries. Vascular remodeling is often associated with changes in the elastin-rich internal elastic lamina (IEL) and the activation of transforming growth factor (TGF)-β. In addition, obesity and type II diabetes have been associated with increased serum neuraminidase, an enzyme known to increase TGF-β cellular output. Therefore, we hypothesized that WD-feeding would induce structural modifications to the IEL of mesenteric resistance arteries in mice, and that these changes would be associated with increased levels of circulating neuraminidase and the up-regulation of elastin and TGF-β in the arterial wall. To test this hypothesis, a WD, high in fat and sugar, was used to induce obesity in mice, and the effect of this diet on the structure of mesenteric resistance arteries was investigated. 4-week old, Post-weaning mice were fed either a normal diet (ND) or WD for 16 weeks. Mechanically, arteries from WD-fed mice were stiffer and less distensible, with marginally increased wall stress for a given strain, and a significantly

increased Young's modulus of elasticity. Structurally, the wall cross-sectional area and the number of fenestrae found in the internal elastic lamina (IEL) of mesenteric arteries from mice fed a WD were significantly smaller than those of arteries from the ND-fed mice. There was also a significant increase in the volume of elastin, but not collagen in arteries from the WD cohort. Plasma levels of neuraminidase and the amount of TGF-β in mesenteric arteries were elevated in mice fed a WD, while ex vivo, cultured vascular smooth muscle cells exposed to neuraminidase secreted greater amounts of tropoelastin and TGF-β than those exposed to vehicle. These data suggest that consumption of a diet high in fat and sugar causes stiffening of the vascular wall in resistance arteries through a process that may involve increased neuraminidase and TGF-β activity, elevated production of elastin, and a reduction in the size and number of fenestrae in the arterial IEL.

4.7 Dipeptidyl peptidase-4 inhibition with linagliptin prevents western diet-induced vascular abnormalities in female mice

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Background: Vascular stiffening, a risk factor for cardiovascular disease, is accelerated, particularly in women with obesity and type 2 diabetes. Preclinical evidence suggests that dipeptidylpeptidase-4 (DPP-4) inhibitors may have cardiovascular benefits independent of glycemic lowering effects. Recent studies show that consumption of a western diet (WD) high in fat and simple sugars induces aortic stiffening in female C57BL/6J mice in advance of increasing blood pressure. The aims of this study were to determine whether administration of the DPP-4 inhibitor, linagliptin (LGT), prevents the development of aortic and endothelial stiffness induced by a WD in female mice.

Methods: C56Bl6/J female mice were fed a WD for 4 months. Aortic stiffness and ex vivo endothelial stiffness were evaluated by Doppler pulse wave velocity (PWV) and atomic force microscopy (AFM), respectively. In addition, we examined aortic vasomotor responses and remodeling markers via immunohistochemistry. Results were analyzed via 2-way ANOVA, $p < 0.05$ was considered as statistically significant.

Results: Compared to mice fed a control diet (CD), WD-fed mice exhibited a 24 % increase in aortic PWV, a five-fold increase in aortic endothelial stiffness, and impaired endothelium-dependent vasodilation. In aorta, these findings were accompanied by medial

wall thickening, adventitial fibrosis, increased fibroblast growth factor 23 (FGF-23), decreased Klotho, enhanced oxidative stress, and endothelial cell ultrastructural changes, all of which were prevented with administration of LGT.

Conclusions: The present findings support the notion that DPP-4 plays a role in development of WD-induced aortic stiffening, vascular oxidative stress, endothelial dysfunction, and vascular remodeling. Whether, DPP-4 inhibition could be a therapeutic tool used to prevent the development of aortic stiffening and the associated cardiovascular complications in obese and diabetic females remains to be elucidated.

4.8 Regular Exercise Reduces Endothelial Cortical Stiffness in Western Diet–Fed Female Mice

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We recently showed that Western diet–induced obesity and insulin resistance promotes endothelial cortical stiffness in young female mice. Herein, we tested the hypothesis that regular aerobic exercise would attenuate the development of endothelial and whole artery stiffness in female Western diet–fed mice. Four-week-old C57BL/6 mice were randomized into sedentary (ie, caged confined, n=6) or regular exercise (ie, access to running wheels, n=7) conditions for 16 weeks. Exercise training improved glucose tolerance in the absence of changes in body weight and body composition. Compared with sedentary mice, exercise-trained mice exhibited reduced endothelial cortical stiffness in aortic explants (sedentary 11.9±1.7 kPa versus exercise 5.5±1.0 kPa; $P<0.05$), as assessed by atomic force microscopy. This effect of exercise was not accompanied by changes in aortic pulse wave velocity ($P>0.05$), an in vivo measure of aortic stiffness. In comparison, exercise reduced femoral artery stiffness in isolated pressurized arteries and led to an increase in femoral internal artery diameter and wall cross-sectional area ($P<0.05$), indicative of outward hypertrophic remodeling. These effects of exercise were associated with an increase in femoral artery elastin content and increased number of fenestrae in the internal elastic lamina ($P<0.05$). Collectively, these data demonstrate for the first time that

the aortic endothelium is highly plastic and, thus, amenable to reductions in stiffness with regular aerobic exercise in the absence of changes in in vivo whole aortic stiffness. Comparatively, the same level of exercise caused destiffening effects in peripheral muscular arteries, such as the femoral artery, that perfuse the working limbs.

4.9 Absence of Endothelial ERα Results in Arterial Remodeling and Decreased Stiffness in Western Diet–Fed Male Mice

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The role of estrogen receptor-α (ERα) signaling in the vasculature of females has been described under different experimental conditions and our group recently reported that lack of endothelial cell (EC) ERα in female mice fed a Western diet (WD) results in amelioration of vascular stiffness. Conversely, the role of ERα in the male vasculature in this setting has not been explored. In conditions of overnutrition and insulin resistance, augmented arterial stiffness, endothelial dysfunction, and arterial remodeling contribute to the development of cardiovascular disease. Here, we used a rodent model of decreased ERα expression in ECs [endothelial cell estrogen receptor-α knockout (EC-ERαKO)] to test the hypothesis that, similar to our findings in females, loss of ERα signaling in the endothelium of insulin-resistant males would result in decreased arterial stiffness. EC-ERαKO male mice and same-sex littermates were fed a WD (high in fructose and fat) for 20 weeks and then assessed for vascular function and stiffness. EC-ERαKO mice were heavier than littermates but exhibited decreased vascular stiffness without differences in endothelial-dependent vasodilatory responses. Mesenteric arteries from EC-ERαKO mice had significantly increased diameters, wall cross-sectional areas, and mean wall thicknesses, indicative of outward hypertrophic remodeling. This remodeling paralleled an increased

vessel wall content of collagen and elastin, inhibition of matrix metalloproteinase activation and a decrease of the incremental modulus of elasticity. In addition, internal elastic lamina fenestrae were more abundant in the EC-ERαKO mice. In conclusion, loss of endothelial ERα reduces vascular stiffness in male mice fed a WD with an associated outward hypertrophic remodeling of resistance arteries.

4.10 Amiloride Improves Endothelial Function and Reduces Vascular Stiffness in Female Mice Fed a Western Diet

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Obese premenopausal women lose their sex related cardiovascular disease protection and develop greater arterial stiffening than age matched men. In female mice, we have shown that consumption of a Western diet (WD), high in fat and refined sugars, is associated with endothelial dysfunction and vascular stiffening, which occur via activation of mineralocorticoid receptors and associated increases in epithelial Na+ channel (ENaC) activity on endothelial cells (EnNaC). Herein our aim was to determine the effect that reducing EnNaC activity with a very-low-dose of amiloride would have on decreasing endothelial and arterial stiffness in young female mice consuming a WD. To this end, we fed female mice either a WD or control diet and treated them with or without a very-low-dose of the ENaC-inhibitor amiloride (1 mg/kg/day) in the drinking water for 20 weeks beginning at 4 weeks of age. Mice consuming a WD were heavier and had greater percent body fat, proteinuria, and aortic stiffness as assessed by pulse-wave velocity than those fed control diet. Treatment with amiloride did not affect body weight, body composition, blood pressure, urinary sodium excretion, or insulin sensitivity, but significantly reduced the development of endothelial and aortic stiffness, aortic fibrosis, aortic oxidative stress, and mesenteric resistance artery EnNaC abundance and proteinuria in WD-fed mice. Amiloride

also improved endothelial-dependent vasodilatory responses in the resistance arteries of WD-fed mice. These results indicate that a very-low-dose of amiloride, not affecting blood pressure, is sufficient to improve endothelial function and reduce aortic stiffness in female mice fed a WD, and suggest that EnNaC-inhibition may be sufficient to ameliorate the pathological vascular stiffening effects of WD-induced obesity in females.

4.11 Uric acid promotes vascular stiffness, maladaptive inflammatory responses and proteinuria in western diet fed mice

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Objective: Aortic vascular stiffness has been implicated in the development of cardiovascular disease (CVD) and chronic kidney disease (CKD) in obese individuals. However, the mechanism promoting these adverse effects are unclear. In this context, promotion of obesity through consumption of a western diet (WD) high in fat and fructose leads to excess circulating uric acid. There is accumulating data implicating elevated uric acid in the promotion of CVD and CKD. Accordingly, we hypothesized that xanthine oxidase(XO) inhibition with allopurinol would prevent a rise in vascular stiffness and proteinuria in a translationally relevant model of WD-induced obesity.

Materials/Methods: Four-week-old C57BL6/J male mice were fed a WD with excess fat (46%) and fructose (17.5%) with or without allopurinol (125 mg/L in drinking water) for 16 weeks. Aortic endothelial and extracellular matrix/vascular smooth muscle stiffness was evaluated by atomic force microscopy. Aortic XO activity, 3-nitrotyrosine (3-NT) and aortic endothelial sodium channel (EnNaC) expression were evaluated along with aortic expression of inflammatory markers. In the kidney, expression of toll like receptor 4 (TLR4) and fibronectin were assessed along with evaluation of proteinuria.

Results: XO inhibition significantly attenuated WD-induced increases in plasma uric acid, vascular XO activity and oxidative stress, in concert with reductions in proteinuria. Further, XO inhibition prevented WD-induced increases in aortic EnNaC expression and associated endothelial and subendothelial stiffness. XO inhibition also reduced vascular pro-inflammatory and maladaptive immune responses induced by consumption of a WD. XO inhibition also decreased WD-induced increases in renal TLR4 and fibronectin that associated proteinuria.

Conclusions: Consumption of a WD leads to elevations in plasma uric acid, increased vascular XO activity, oxidative stress, vascular stiffness, and proteinuria all of which are attenuated with allopurinol administration.

4.12 Glycemic control by the SGLT2 inhibitor empagliflozin decreases aortic stiffness, renal resistivity index and kidney injury

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Background: Arterial stiffness is emerging as an independent risk factor for the development of chronic kidney disease. The sodium glucose co-transporter 2 (SGLT2) inhibitors, which lower serum glucose by inhibiting SGLT2-mediated glucose reabsorption in renal proximal tubules, have shown promise in reducing arterial stiffness and the risk of cardiovascular and kidney disease in individuals with type 2 diabetes mellitus. Since hyperglycemia contributes to arterial stiffness, we hypothesized that the SGLT2 inhibitor empagliflozin (EMPA) would improve endothelial function, reduce aortic stiffness, and attenuate kidney disease by lowering hyperglycemia in type 2 diabetic female mice (db/db).

Materials/methods: Ten-week-old female wild-type control (C57BLKS/J) and db/db (BKS.Cg-Dock7m+/+Leprdb/J) mice were divided into three groups: lean untreated controls (CkC, n = 17), untreated db/db (DbC, n = 19) and EMPA-treated db/db mice (DbE, n = 19). EMPA was mixed with normal mouse chow at a concentration to deliver 10 mg kg−1 day−1, and fed for 5 weeks, initiated at 11 weeks of age.

Results: Compared to CkC, DbC showed increased glucose levels, blood pressure, aortic and endothelial cell stiffness, and impaired endothelium-dependent vasorelaxation.

Furthermore, DbC exhibited impaired activation of endothelial nitric oxide synthase, increased renal resistivity and pulsatility indexes, enhanced renal expression of advanced glycation end products, and periarterial and tubulointerstitial fibrosis. EMPA promoted glycosuria and blunted these vascular and renal impairments, without affecting increases in blood pressure. In addition, expression of "reversion inducing cysteine rich protein with Kazal motifs" (RECK), an anti-fibrotic mediator, was significantly suppressed in DbC kidneys and partially restored by EMPA. Confirming the in vivo data, EMPA reversed high glucose-induced RECK suppression in human proximal tubule cells.

Conclusions: Empagliflozin ameliorates kidney injury in type 2 diabetic female mice by promoting glycosuria, and possibly by reducing systemic and renal artery stiffness, and reversing RECK suppression.

4.13 Diet-Induced Obesity Promotes Kidney Endothelial Stiffening and Fibrosis Dependent on the Endothelial Mineralocorticoid Receptor

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Obesity is characterized by enhanced MR (mineralocorticoid receptor) activation, vascular stiffness, and associated cardiovascular and kidney disease. Consumption of a Western-style diet (WD), high in saturated fat and refined carbohydrates, by female mice, leads to obesity and vascular stiffening. Use of ECMR (endothelial cell–specific MR) knockout mice supports that ECMR activation is critical for development of vascular and cardiac fibrosis and stiffening. However, the role of ECMR activation in kidney inflammation and fibrosis remains unknown. We hypothesized that cell-specific deletion of ECMR would prevent WD-induced central aortic stiffness and protect the kidney from endothelial dysfunction and vascular stiffening. Four-week-old female ECMR KO and wild-type mice were fed either mouse chow or WD for 16 weeks. WD feeding increased body weight and fat mass, proteinuria, as well as vascular stiffness indices (pulse wave velocity and kidney artery stiffening) and impaired endothelial-dependent vasodilatation without blood pressure changes. The WD-induced kidney arterial stiffening was associated with attenuated eNOS (endothelial NO synthase) activation, increased oxidative stress, proinflammatory immune responses, alterations in extracellular matrix degradation pathways, and fibrosis. ECMR deletion prevented these abnormalities by improving eNOS activation and reducing macrophage proinflammatory M1 polarization, expression of TG2

(transglutaminase 2), and MMP (matrix metalloproteinase)-9. Our data support the concept that ECMR activation contributes to endothelial dysfunction, increased kidney artery fibrosis/stiffening, and impaired NOS (NO synthase) activation, processes associated with macrophage infiltration and polarization, inflammation, and oxidative stress, collectively resulting in tubulointerstitial fibrosis in females consuming a WD.

4.14 IGF-1 Deficiency Promotes Pathological Remodeling of Cerebral Arteries: A Potential Mechanism Contributing to the Pathogenesis of Intracerebral Hemorrhages in Aging

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Clinical and experimental studies show that age-related decline in circulating insulin-like growth factor-1 (IGF-1) levels promotes the pathogenesis of intracerebral hemorrhages, which critically contribute to the development of vascular cognitive impairment and disability in older adults. Yet, the mechanisms by which IGF-1 deficiency compromises structural integrity of the cerebral vasculature are not completely understood. To determine the role of IGF-1 deficiency in pathological remodeling of middle cerebral arteries (MCAs), we compared alterations in vascular mechanics, morphology, and remodeling-related gene expression profile in mice with liver-specific knockdown of IGF-1 (Igf1f/f + TBG-Cre-AAV8) and control mice with or without hypertension induced by angiotensin-II treatment. We found that IGF-1 deficiency resulted in thinning of the media and decreased wall-to-lumen ratio in MCAs. MCAs of control mice exhibited structural adaptation to hypertension, manifested as a significant increase in wall thickness, vascular smooth muscle cell (VSMC) hypertrophy, decreased internal diameter and up-regulation of extracellular matrix (ECM)-related genes. IGF-1 deficiency impaired hypertension-induced adaptive media hypertrophy and dysregulated ECM remodeling, decreasing elastin

content and attenuating adaptive changes in ECM-related gene expression. Thus, circulating IGF-1 plays a critical role in maintenance of the structural integrity of cerebral arteries. Alterations of VSMC phenotype and pathological remodeling of the arterial wall associated with age-related IGF-1 deficiency have important translational relevance for the pathogenesis of intracerebral hemorrhages and vascular cognitive impairment in elderly hypertensive patients.

4.15 Sexual Dimorphism in Obesity-Associated Endothelial ENaC Activity and Stiffening in Mice

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Obesity and insulin resistance stiffen the vasculature, with females appearing to be more adversely affected. As augmented arterial stiffness is an independent predictor of cardiovascular disease (CVD), the increased predisposition of women with obesity and insulin resistance to arterial stiffening may explain their heightened risk for CVD. However, the cellular mechanisms by which females are more vulnerable to arterial stiffening associated with obesity and insulin resistance remain largely unknown. In this study, we provide evidence that female mice are more susceptible to Western diet–induced endothelial cell stiffening compared with age-matched males. Mechanistically, we show that the increased stiffening of the vascular intima in Western diet–fed female mice is accompanied by enhanced epithelial sodium channel (ENaC) activity in endothelial cells (EnNaC). Our data further indicate that: (i) estrogen signaling through estrogen receptor α (ERα) increases EnNaC activity to a larger extent in females compared with males, (ii) estrogen-induced activation of EnNaC is mediated by the serum/glucocorticoid inducible kinase 1 (SGK-1), and (iii) estrogen signaling stiffens endothelial cells when nitric oxide is lacking and this stiffening effect can be reduced with amiloride, an ENaC inhibitor. In

aggregate, we demonstrate a sexual dimorphism in obesity-associated endothelial stiffening, whereby females are more vulnerable than males. In females, endothelial stiffening with obesity may be attributed to estrogen signaling through the ERα–SGK-1–EnNaC axis, thus establishing a putative therapeutic target for female obesity-related vascular stiffening.

4.16 Chronic Elevation of Endothelin-1 Alone May Not Be Sufficient to Impair Endothelium-Dependent Relaxation

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Endothelin-1 (ET-1) is a powerful vasoconstrictor peptide considered to be causally implicated in hypertension and the development of cardiovascular disease. Increased ET-1 is commonly associated with reduced NO bioavailability and impaired vascular function; however, whether chronic elevation of ET-1 directly impairs endothelium-dependent relaxation (EDR) remains elusive. Herein, we report that (1) prolonged ET-1 exposure (ie, 48 hours) of naive mouse aortas or cultured endothelial cells did not impair EDR or reduce eNOS (endothelial NO synthase) activity, respectively ($P>0.05$); (2) mice with endothelial cell–specific ET-1 overexpression did not exhibit impaired EDR or reduced eNOS activity ($P>0.05$); (3) chronic (8 weeks) pharmacological blockade of ET-1 receptors in obese/hyperlipidemic mice did not improve aortic EDR or increase eNOS activity ($P>0.05$); and (4) vascular and plasma ET-1 did not inversely correlate with EDR in resistance arteries isolated from human subjects with a wide range of ET-1 levels ($r=0.0037$ and $r=-0.1258$, respectively). Furthermore, we report that prolonged ET-1 exposure downregulated vascular UCP-1 (uncoupling protein-1; $P<0.05$), which may contribute to

the preservation of EDR in conditions characterized by hyperendothelinemia. Collectively, our findings demonstrate that chronic elevation of ET-1 alone may not be sufficient to impair EDR.

4.17 Western diet induces renal artery endothelial stiffening that is dependent on the epithelial Na^+ channel

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Consumption of a Western diet (WD) induces central aortic stiffening that contributes to the transmittance of pulsatile blood flow to end organs, including the kidney. Our recent work supports that endothelial epithelial Na+ channel (EnNaC) expression and activation enhances aortic endothelial cell stiffening through reductions in endothelial nitric oxide (NO) synthase (eNOS) and bioavailable NO that result in inflammatory and oxidant responses and perivascular fibrosis. However, the role that EnNaC activation has on endothelial responses in the renal circulation remains unknown. We hypothesized that cell-specific deletion of the α-subunit of EnNaC would prevent WD-induced central aortic stiffness and protect the kidney from endothelial dysfunction and vascular stiffening. Twenty-eight-week-old female αEnNaC knockout and wild-type mice were fed either mouse chow or WD containing excess fat (46%), sucrose, and fructose (17.5% each). WD feeding increased fat mass, indexes of vascular stiffening in the aorta and renal artery (in vivo pulse wave velocity and ultrasound), and renal endothelial cell stiffening (ex vivo atomic force microscopy). WD further impaired aortic endothelium-dependent relaxation and renal artery compliance (pressure myography) without changes in blood pressure. WD-

induced renal arterial stiffening occurred in parallel to attenuated eNOS activation, increased oxidative stress, and aortic and renal perivascular fibrosis. αEnNaC deletion prevented these abnormalities and support a novel mechanism by which WD contributes to renal arterial stiffening that is endothelium and Na+ channel dependent. These results demonstrate that cell-specific EnNaC is important in propagating pulsatility into the renal circulation, generating oxidant stress, reduced bioavailable NO, and renal vessel wall fibrosis and stiffening.

4.18 LIMK (LIM Kinase) Inhibition Prevents Vasoconstriction- and Hypertension-Induced Arterial Stiffening and Remodeling

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Increased arterial stiffness and vascular remodeling precede and are consequences of hypertension. They also contribute to the development and progression of life-threatening cardiovascular diseases. Yet, there are currently no agents specifically aimed at preventing or treating arterial stiffening and remodeling. Previous research indicates that vascular smooth muscle actin polymerization participates in the initial stages of arterial stiffening and remodeling and that LIMK (LIM kinase) promotes F-actin formation and stabilization via cofilin phosphorylation and consequent inactivation. Herein, we hypothesize that LIMK inhibition is able to prevent vasoconstriction- and hypertension-associated arterial stiffening and inward remodeling. We found that small visceral arteries isolated from hypertensive subjects are stiffer and have greater cofilin phosphorylation than those from nonhypertensives. We also show that LIMK inhibition prevents arterial stiffening and inward remodeling in isolated human small visceral arteries exposed to prolonged vasoconstriction. Using cultured vascular smooth muscle cells, we determined that LIMK inhibition prevents vasoconstrictor agonists from increasing cofilin phosphorylation, F-actin volume, and cell cortex stiffness. We further show that localized LIMK inhibition prevents arteriolar inward remodeling in hypertensive mice. This

indicates that hypertension is associated with increased vascular smooth muscle cofilin phosphorylation, cytoskeletal stress fiber formation, and heightened arterial stiffness. Our data further suggest that pharmacological inhibition of LIMK prevents vasoconstriction-induced arterial stiffening, in part, via reductions in vascular smooth muscle F-actin content and cellular stiffness. Accordingly, LIMK inhibition should represent a promising therapeutic means to stop the progression of arterial stiffening and remodeling in hypertension.

4.19 TRAF3IP2 (TRAF3 Interacting Protein 2) Mediates Obesity-Associated Vascular Insulin Resistance and Dysfunction in Male Mice

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Insulin resistance in the vasculature is a characteristic feature of obesity and contributes to the pathogenesis of vascular dysfunction and disease. However, the molecular mechanisms underlying obesity-associated vascular insulin resistance and dysfunction remain poorly understood. We hypothesized that TRAF3IP2 (TRAF3 interacting protein 2), a proinflammatory adaptor molecule known to activate pathological stress pathways and implicated in cardiovascular diseases, plays a causal role in obesity-associated vascular insulin resistance and dysfunction. We tested this hypothesis by employing genetic-manipulation in endothelial cells in vitro, in isolated arteries ex vivo, and diet-induced obesity in a mouse model of TRAF3IP2 ablation in vivo. We show that ectopic expression of TRAF3IP2 blunts insulin signaling in endothelial cells and diminishes endothelium-dependent vasorelaxation in isolated aortic rings. Further, 16 weeks of high fat/high sucrose feeding impaired glucose tolerance, aortic insulin-induced vasorelaxation, and hindlimb postocclusive reactive hyperemia, while increasing blood pressure and arterial stiffness in wild-type male mice. Notably, TRAF3IP2 ablation protected mice from such high fat/high sucrose feeding-induced metabolic and vascular defects. Interestingly, wild-type female mice expressed markedly reduced levels of

TRAF3IP2 mRNA independent of diet and were protected against high fat/high sucrose diet-induced vascular dysfunction. These data indicate that TRAF3IP2 plays a causal role in vascular insulin resistance and dysfunction. Specifically, the present findings highlight a sexual dimorphic role of TRAF3IP2 in vascular control and identify it as a promising therapeutic target in vasculometabolic derangements associated with obesity, particularly in males.

VITA

Francisco I. Ramirez-Perez was born in México City, México, in January 26th 1981. After graduating high school in 2002 and the same year was accepted at the *Universidad de Guanajuato*, where he joined the Engineering Physics Department and obtained his Bachelor's degree in Engineering Physics in 2009. His thesis research was on high energy particle physics, titled: "Modelos Extendidos con Dimensiones Extras, Split Fermion" (Extended Models with Extra Dimensions, Split Fermion). While in college, Francisco volunteered training high school students to participate at national physics competitions. Francisco joined the Master's program at the same university in the Physics department, in the Biophysics program. While working on his thesis Francisco was invited to be part of a collaboration working on non-invasive protocol to detect cancer at Dalton Cardiovascular Research Center at University of Missouri in Columbia, MO, USA under the supervision of Dr. Luis Polo-Parada. He graduated with a Master's degree in Physics in 2011. His thesis research summarized the work he performed while at University of Missouri, titled: "Estudio de la viabilidad celular por medio de espectroscopia fotoacústica de la línea celular HS936 al ser expuesta a radiación láser: teoría y experimento" (Study of cell viability using photoacoustic spectroscopy on the HS936 cell line when exposed to laser radiation: theory and experiment). He was invited to apply at the Ph.D. program at University of Missouri and in 2012 he started his Ph.D. working under the supervision of Dr. Luis A. Martinez-Lemus and Dr. Luis Polo-Parada at Dalton Cardiovascular Research Center in the Biological Engineering Department of the University of Missouri. Francisco research and interest is focused on vascular remodeling, hypertension, obesity and diabetes.