THE EFFECT OF POSTNATAL GROWTH RESTRICTION ON CARDIOVASCULAR DEVELOPMENT IN FEMALE MICE

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PUBLIC ABSTRACT

According to the World Health Organization (WHO) and the United Nations Children's Fund (UNICEF), growth restriction in early life affects ~15% of newborns and 23% of children under the age of 5. Growth restriction is a reduction or delay in physical development during gestation or early postnatal life that increases the risk for chronic diseases in adulthood. Data from the Neonatal Nutrition and Exercise Research Laboratory (NNERL) indicate females are more severely affected by postnatal growth restriction and have an increased risk for cardiovascular disease (CVD) but it is unclear how these changes occur.

As estradiol and testosterone levels are one of the largest biological differences between males and females and influence cardiac function, it is hypothesized they influence the development of cardiac dysfunction in postnatally growth restricted females (PNGR) through calcium (Ca^{2+}) handling proteins and cardiac fibrosis. Thus, this dissertation comprises three objectives that have been designed to fill this gap, 1) determine the effect of postnatal growth restriction on estradiol and testosterone levels, 2) determine the effect of postnatal growth restriction on Ca^{2+} handling proteins in the heart, and 3) determine the effect of postnatal growth restriction on cardiac remodeling and fibrosis.

All three objectives were achieved with a validated nutritive mouse model to induce postnatal growth restriction. To achieve this model, mouse dams were started on a low protein (8%) or normal protein (20%) diet prior to mating. The dams on the low protein diet produce less milk for their offspring which reduces nutrient intake and growth. After the mouse pups were born a validated cross-fostering method isolates growth restriction to the postnatal period by placing normal protein pups with a low protein fed mom. On postnatal day 21, all mice were weaned and placed on the same 20% normal protein diet into adulthood.

Study one collected blood samples at two time points that corelate with human ages: postnatal day 70 is equal to middle adulthood (~45 years) and postnatal day 130 equates to later adulthood (~70 years). Once all samples were collected, 18 different hormones were compared between PNGR and non-growth restricted female mice. There were no differences in testosterone between the groups at postnatal day 70 or 130 and estradiol was not detectable in 75% of the PNGR samples. However, 18-hydroxycortisol was elevated in the PNGR mice which suggests hypertension in the PNGR females. For study two cardiac samples were collected at three ages, postnatal day 21, 70, and 130 to measure Ca^{2+} handling protein abundance. PNGR female mice had lower sarcoendoplasmic reticulum calcium ATPase (Serca2) proteins as compared to control mice but none of the upstream regulators of Serca2 were altered. It is possible there is a disruption of SERCA2 gene expression or an interruption in translation of the Serca2 that reduces adulthood protein expression in the PNGR female mice. For the final study cardiac samples were collected to assess endothelin-1 protein abundance. A second cardiac sample was collected at postnatal day 130 to determine collagen abundance as a marker of cardiac fibrosis. Endothelin-1 was not increased in PNGR mice as compared to control mice at any time point and histology staining did not reveal excess collagen in the PNGR mice.

Together the results from these three studies help to fill a gap in the literature by revealing changes in cardiac function from postnatal growth restriction in female mice. The female PNGR mice have increased likelihood of hypertension as suggested by an increase in 18-hydroxycortisol and a reduction in Serca2 which leads to impairments in Ca^{2+} reuptake and increases their risk for cardiac dysfunction. Interventions should center on alleviating impaired relaxation in the heart through improving Ca^{2+} reuptake and mitigating hypertension.

ABSTRACT

In 2020, UNICEF and WHO approximated 15% of babies born annually are considered small for gestational age and roughly 23% of children up to the age of 5 suffer from early life growth restriction worldwide. Alarmingly, early life growth restriction is linked with chronic disease development in adulthood. The NNERL has established PNGR causes smaller and less binucleated cardiomyocytes, disrupted collagen proteins, impaired Ca^{2+} signaling, widened QRS complexes and elongated isovolumic relaxation time, all suggestive of cardiac dysfunction that disproportionately affects females. The increased severity in females suggests a possible sex steroid phenomenon, as sex steroids present one of the largest biological differences between males and females. Therefore, the purpose of this dissertation was to determine the influence of postnatal growth restriction on cardiovascular development with a focus on sex steroids, in a mouse model. Study one hypothesized PNGR would increase testosterone but decrease estradiol in adulthood; study two hypothesized PNGR would reduce Ca^{2+} regulating protein abundance disrupting Ca^{2+} kinetics and reducing cardiac function; and finally study three hypothesized that PNGR female mice have increased collagen deposition leading to cardiac fibrosis and stiffness of the heart.

All three studies were conducted with a validated nutritive cross-fostering model to isolate growth restriction to the postnatal period. In brief, mouse dams were placed on either a normal protein diet (20%) or a low protein diet (8%) two weeks prior to mating. The dams on the low protein diet produce ~20% less milk which reduces overall nutrient intake and stunts organ growth in the offspring. On postnatal day (PN) 1 cross-fostering was accomplished by placing normal protein born pups with a low protein fed dam or a different normal protein fed dam (CON). On PN 21, all mice were weaned and placed on the same 20% normal protein diet into adulthood.

In study one blood samples were collected from female mice at two time points PN 70 and 130. Serum was then separated from plasma and steroid levels were assessed with mass spectrometry. Testosterone levels were not different between PNGR and CON mice at either timepoint, while estradiol was only detected in 25% of PNGR and 66% of CON. However, a precursor steroid, 18-hydroxycortisol, was elevated in PNGR female mice and suggests the PNGR mice suffer from hypertension. For study two, cardiac samples were collected at PN 21, 70, and 130 to assess Ca^{2+} handling protein abundance. Serca2 was reduced in PNGR female mice indicating a disruption in Ca^{2+} reuptake. However, there were no differences between CON and PNGR for the regulators of Serca2 [phospholamban (PLN), phosphorylated PLN (P-PLN), protein kinase A (PKA) and phosphorylated PKA (P-PKA)]. Finally, in study three the female mice were euthanized at PN 21, 70, and 130 and hearts were excised for protein quantification of endothelin-1 (ET-1) and a second heart was collected at PN 130 for histology staining of collagen. ET-1 is a potent stimulator protein of cardiac remodeling and fibrosis while Masson's Trichrome stain detects collagen fibers. At PN 21, 70, and 130, ET-1 protein in the heart was not different between CON and PNGR female mice and at PN 130 the PNGR mice did not have more collagen in the myocardium than CON mice.

Together, the three studies of this dissertation filled a gap in the literature and confirm impaired relaxation in the PNGR female hearts is linked to changes in Ca^{2+} modulation via a reduction in Serca2 but without cardiac fibrosis. Despite not being able to determine estradiol levels in 75% of PNGR samples from study one, the elevated 18-hydroxycortisol in PNGR females suggest hypertension may be increasing their risk of CVD. Future research is recommended to focus on increasing Serca2 regulated Ca^{2+} reuptake and reducing hypertension to mitigate impaired relaxation in the PNGR females.

This dissertation is dedicated to many people whom without their support, this PhD journey would not be possible, and to three students who will never get to finish their degree at MSU, Brian, Arielle, and Alexandria. To my husband Mike, through the ups and down, you never once doubted my success and made many sacrifices for me to pursue this dream. To our dog Otis who provided me snuggles at all the right times and reminded me to stay active midday. Lastly, to my family, from whom I learned at an early age to always believe in myself and to know that I can do anything I put my mind too. This dissertation does not exist without all of you.

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CHAPTER 1:

INTRODUCTION

In 2020, the most recent worldwide report from UNICEF and WHO identified approximately 15% of babies born annually are considered small and roughly 21% of children under the age of 5 suffer from early life growth restriction (1,2). Malnutrition, placental insufficiency, the inability to absorb nutrients, and a lack of nutrients are major contributors to growth restriction (3–7). Through the analysis of epidemiological studies, Barker championed the Developmental Origins of Health and Disease (DOHaD) hypothesis and suggested that early life growth restriction is linked with increased risk of chronic diseases such as CVD, diabetes, hypertension, sarcopenia, cancer, lung disease and metabolic diseases (8–16). Recent research has confirmed the DOHaD hypothesis, highlighted by the Cohen group's 2016 review, which suggested any interruption in nutritional availability during gestational growth and lactation periods will result in *adapted* cardiovascular development to ensure immediate short-term survival (17). However, this modified phenotype for short term survival will ultimately lead to cardiac dysfunction in adulthood (8,18–22).

Currently, there is an abundance of research investigating the intrauterine growth restricted field, but less emphasis has been placed on the postnatal environment (8,14,22–28). In human and animal models, the postnatal window is also susceptible to insults that influence organ growth and development, but this is organ dependent. However, due to the logistical constraints in conducting studies on human hearts, animal models have helped understand underlying mechanisms leading to increased CVD in postnatal growth restricted populations.

Rodents in particular, have been a valuable model to study cardiovascular development because of their accelerated lifespans and similarities of genes to humans (29–32). Although, a mouse heart's cellular maturity and functionality develop in the postnatal period, this period correlates to the 3rd trimester in humans, which is where a majority of growth restriction occurs in

humans (33,34). Importantly, the postnatal period in a mouse also includes the first year of life in an infant. With this knowledge, the NNERL established postnatal growth restriction causes smaller and less binucleated cardiomyocytes, disrupts collagen proteins, impairs Ca^{2+} signaling, widens QRS complexes and elongates isovolumic relaxation time (IVRT) (35–38). The physiological data reported from the NNERL show negative defects on the cardiovascular system disproportionately affects females as compared to male mice (35,39). The PNGR females have altered Ca^{2+} signaling, increased ventricular stiffness, and reduced cardiac function, while the males are not compromised. The cardiac pathology in PNGR female mice is suggestive of diastolic dysfunction as marked by increased cardiac stiffness and impaired relaxation which increases their risk of developing heart failure.

Since cardiac pathology due to postnatal growth restriction is more severe in females, it suggests a possible sex steroid phenomenon, as sex steroids present one of the largest biological differences between males and females (40). There is evidence to suggest abnormally low estrogen is a risk factor for CVDs, particularly evident in females with heart failure. Many of the studies investigating low estrogen focused on post-menopausal females as this is the most common period of reduced estrogen and CVD (41–44). However, a restricted diet or an inadequate diet will disrupt the production, metabolism, and detoxification of hormones leading to a disruption in growth and maturation (45,46). Adequate nutrition is essential during all phases of maturation (i.e. infancy, childhood, puberty) and helps to develop and maintain a healthy body system throughout life (47). A study by Teixeira et al., reported reduced estrogen and increased testosterone in postnatally undernourished male rats as compared to normally nourished male rats, highlighting the importance of early life nutrition for endocrine development in males (48). Further evidence for the link between postnatal malnutrition and hormone disruption is a study by Ramos et al., (49)

which reported reduced thyroid hormones in PNGR rat offspring, without mention of rodent sex. Importantly, a reduction in thyroid hormones is correlated with a reduction in plasma estrogen and testosterone bioavailability through sex-hormone binding globulin levels (50). With the established link between reduced hormones and cardiac dysfunction, Ramos' results suggest a correlation between PNGR and reduced sex hormones which could ultimately increase the females' risk of cardiac dysfunction (42–44,51–53).

Despite not clear mechanisms, the connection exists between lower estrogen and CVD (54–56). Estrogen is one of the major female hormones and regulates endothelial function, vascular health, and Ca^{2+} handling proteins to promote cardiovascular health (51,52,57–60). Current postnatal growth restriction research suggests diastolic dysfunction in females is likely through changes in Ca^{2+} flux, cardiac remodeling, and myocardial stiffness (35,37,39). Figure 1.1 demonstrates this dissertation's hypothesis for a link between diastolic dysfunction and reduced estrogen. Support for this hypothesis comes from intrauterine growth restricted rodent studies that have revealed earlier senescence and loss of hormones in female offspring and the changes in hormone levels in different studies investigating PNGR rodents (23,24,48,49,53). However, the evidence remains minimal for the influence of postnatal growth restriction on hormonal status directly in PNGR female mice. In spite of the evidence for diastolic dysfunction and the hypothesized changes in hormone synthesis in PNGR populations, biological sex differences in CVD pathogenesis are still not elucidated and the need to understand the underlying mechanisms is imperative (43). Therefore, the purpose of this dissertation is to determine the influence of postnatal growth restriction (in a mouse model) on cardiovascular development and function with a focus on sex steroids.

As seem in figure 1.1, postnatal growth restriction is hypothesized to decrease serum estradiol (the most abundant form of estrogen, E2) in female mice with a subsequent increase in serum testosterone (T). Changes in sex steroids are correlated with a reduction in thyroid hormones which were previously reported to be reduced by growth restriction (49,53). Both E2 and T influence signaling pathways that augment key enzymes and proteins to influence cardiac function (51,55,61,62). Specifically, E2 has a direct effect on Ca^{2+} handling proteins, including ryanodine receptors (RYR), SERCA2, PLN, P-PLN and PKA (51,63). These Ca^{2+} handling proteins regulate Ca^{2+} flux between the cytosol and sarcolemma which influences cardiac contractility and relaxation. Thus, any changes in the coordinated movements of Ca^{2+} in and out of the cell via defects in the regulating proteins will reduce cardiac contractility and prolong relaxation (64).

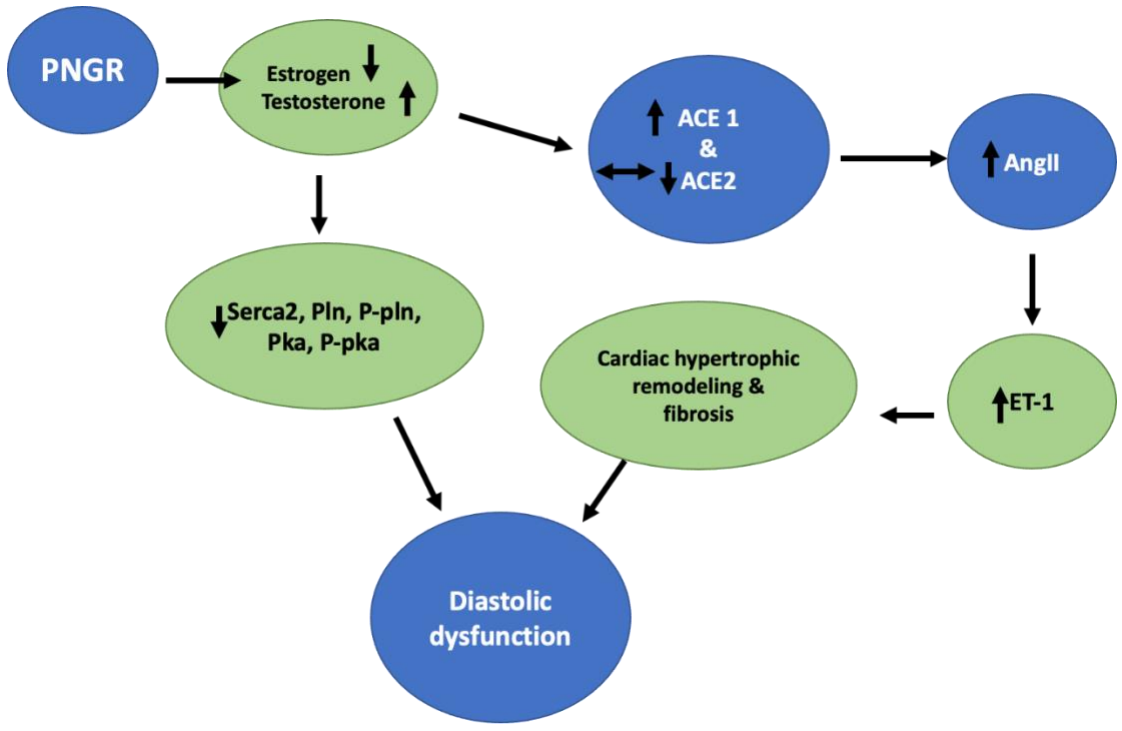

Figure 1.1 *Hypothesized pathways for PNGR induced cardiac dysfunction*
Through altered sex hormones in females, the PNGR mice experience diastolic cardiac dysfunction. Blue bubbles are documented effects of growth restriction, (43,51,395,396), whereas green bubbles are hypotheses to be investigated in this dissertation. PNGR, postnatal growth restriction

Changes in Ca^{2+} homeostasis are accompanied by changes in cardiac morphology, reduced cardiac output and increased risk of arrythmias (65–69). The multiple defects in cardiac energy supply and demand, ultimately leads to cardiac dysfunction and failure (66). Additionally, decreases in E2 levels can increase ET-1 expression, which acts downstream of Angiotensin II (AngII) to induce cardiac remodeling (52,70–72). Although not fully understood, the interaction of AngII and ET-1 generate ROS and induce inflammation contributing to hypertrophy/cardiac fibrosis leading to stiffness and diastolic dysfunction evident by increased LV mass, reduced LV end-diastolic dimension, impaired contractility, and prolonged relaxation rates (73–75). Preliminary evidence has suggested cardiac fibrotic tissue accumulates in the PNGR female mice hearts, but no studies exist to confirm this hypothesis yet (37,39). Therefore, the overall objective of this dissertation is to determine the relationship between sex steroids and cardiac structure and function in PNGR female mice which research has shown leads to diastolic dysfunction and an increased risk of CVD (35,36,39). To accomplish the objective the following specific aims were developed.

Specific Aim 1: To determine if postnatal growth restriction alters the sex steroids estradiol and testosterone, in adulthood in female mice.

Hypothesis 1.1: PNGR female mice will have decreased estradiol in serum at PN 70 and PN 130 as compared to the CON female mice.

Hypothesis 1.2: PNGR female mice will have increased testosterone in serum at PN 70 and 130 as compared to the CON female mice.

Specific Aim 2*:* To determine the influence of postnatal growth restriction on Ca^{2+} handling proteins that regulate cardiac function in the PNGR female mouse hearts.

Hypothesis 2.1: PNGR female mice will have reduced expression of SERCA2 as compared to CON female mice across the lifespan (PN 21, 70 & 130).

Hypothesis 2.2: PNGR female mice will have unchanged expression of PLN but decreased expression of P-PLN as compared to CON female mice across the lifespan (PN 21, 70 & 130).

Hypothesis 2.3: PNGR female mice will have unchanged expression of PKA proteins but decreased expression of P-PKA as compared to CON female mice across the lifespan (PN 21, 70 & 130).

Specific Aim 3: To determine the effect of postnatal growth restriction on endothelin-1 and cardiac fibrosis to indicate cardiac hypertrophy and stiffness in adulthood.

Hypothesis 3.1: PNGR female mice will have increased endothelin-1 protein expression as compared to CON female mice across the lifespan (PN 21, 70 & 130).

Hypothesis 3.2: PNGR female mice will have increased cardiac fibrotic tissue as compared to CON female mice in adulthood (PN 130).

In conclusion, the previous epidemiological evidence and physiological research has highlighted a link between postnatal growth restriction and cardiac dysfunction in females, without compromise in males. However, cardiac development in the PNGR population is under-researched and deserves more attention through a targeted female-specific approach. With the successful completion of these objectives, this dissertation will fill significant knowledge gaps in the DOHaD literature concerning female-specific cardiac dysfunction and lay the foundation for evidence-based practices to be developed to ultimately reduce CVD and mortality rates from postnatal growth restriction in females.

CHAPTER 2:

LITERATURE REVIEW

The following review will summarize the literature encompassing the postnatal development of the female cardiovascular system in a mouse model. Included after the review is a justification of the methodology to be used in this dissertation and the importance of animal models in laying the foundation for evidence-based practices for humans. The review provides rationale for the aims and hypotheses examined in this dissertation.

2.1 Epidemiological Evidence for Growth Restriction and Disease Risk

In 1975, Dörner demonstrated that the hormone levels, metabolites, and neurotransmitters during early development could "program" brain development, and a disruption in these physiological factors would lead to increased disease susceptibility (76–78). The term "programming", as Dörner first used it, highlights the idea that an insult during critical periods of early life causes a lifetime of consequences (79). Although Dörner initially commented on the brain's susceptibility, further research examined the disruptive programming of other organs. Barker championed the DOHaD hypothesis by analyzing large epidemiological cohorts and hypothesizing a critical window of plasticity in fetal development (10,11,20,80–82). However, when the developmental window closes, the fetus' ability to adapt to new environments or stressors ends (83). The fetus's newly adapted phenotype may not be suited to the environment in which they will continue to grow in and increases their susceptibility for chronic diseases in adulthood.

The Hertfordshire Cohort, Helsinki Birth Cohort, and the Dutch Famine Cohort were the foundational epidemiological studies that demonstrated infants born during a famine (exemplifying early life undernutrition) had increased risks of developing cardiovascular and renal diseases, metabolic disorders, and type 2 diabetes in adulthood (10,80,81,84–86). Further analysis of these growth restricted cohorts accumulated evidence on different risk severity associated with the timing of the insult: early versus late in utero versus postnatal (87). For example, the Dutch

Cohort found infants exposed during earlier gestation had higher risks of cancer, cognitive dysfunction, atherosclerosis, and coronary heart disease (88,89). While work from the Crispi Lab group reported more severity in late-onset IUGR and was characterized by globular remodeling of the heart as result of pressure overload (90,91). More recent analysis of a cohort from the Great Chinese Famine suggests the difference in timing of nutritional insults affects males and females differently as well (92,93). Although, the mechanisms are not fully understood yet, Huang et al., reported females exposed to famine were more likely to develop ascending aortic dilatation than males (92). The shift in research towards two developmental windows allowed researchers to determine the risk from adverse early environments is different for individual organs, as organs develop on individual timelines.

As this dissertation focuses on the cardiovascular system, it is important to examine the cardiovascular developmental windows. Figure 2.1, taken from Tan et al., provides an overview of the 3 phases of cardiovascular development in humans (94). Figure 2.1 shows how nutritional insults in early life alter specifics of cardiovascular development, including the shifts from cardiomyocyte hyperplasia to hypertrophy and glycolytic metabolism to oxidative metabolism. For example, intrauterine insults could result in reduced hyperplasia due to early terminal differentiation of cardiomyocytes and alterations in gene expression related to energy production and oxygen homeostasis (95–97). In contrast, insults during postnatal life could lead to excess ventricular hypertrophic growth through increases in sarcomeric proteins and profibrotic markers in response to greater cardiac output demands and cardiac load of extrauterine life (94,98,99).

In summary, the epidemiological evidence highlights the importance of not just in-utero famine exposure and low birth weight on disease risk in adulthood but also how important the early postnatal environment is in influencing developmental programming, particularly for

females. The new evidence shapes the current research focus to better understand postnatal development and disease risk. Despite the confirmation of postnatal nutrition and increased CVD risk, the mechanism by which this relationship occurs is still not fully understood.

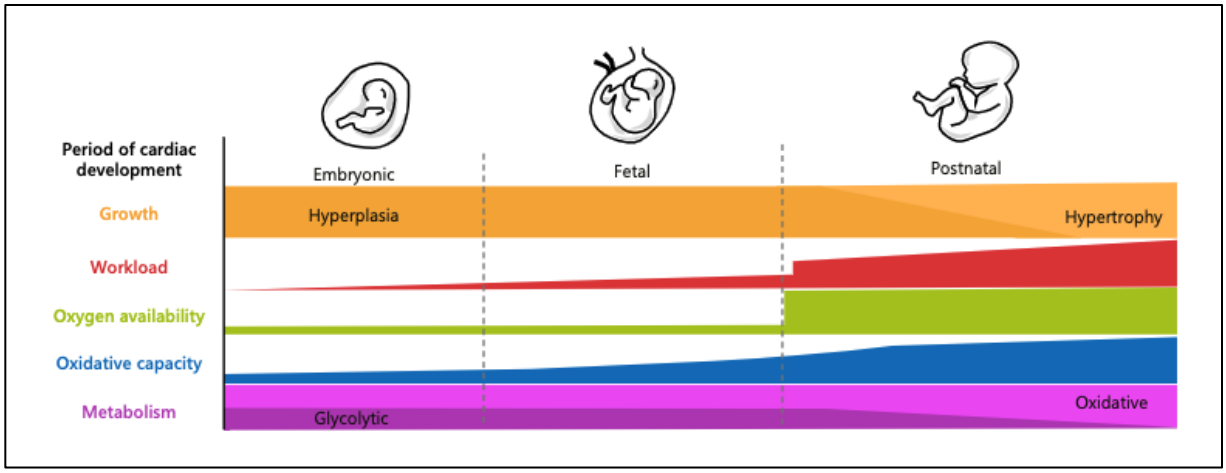

Figure 2.1 *Periods of human cardiac development*
Tan et al., 2020 (94)

2.2 Early Life Growth Restriction

Early life growth restriction is defined by pediatric literature and the DOHaD hypothesis as a condition in which a fetus or infant has features of malnutrition (larger head, hands, and feet compared to body, loss of buccal fat, scaphoid abdomen, and Doppler abnormalities) and is below the 10th percentile of gestational age or two standard deviations from population norms caused by delayed growth and maturation (4,27,100–103). However, recent evidence suggests that early life growth restriction should be broken into two windows of development: intrauterine (in the womb) and postnatal (after birth). Each window has its own effect on growth and development. Intrauterine growth restriction (IUGR) is well documented in the medical field, (8,14,21,22,25–27,104,105) and occurs due to an abnormal intrauterine environment from a lack of oxygen or nutrients and/or maternal diseases (gestational diabetes, hypertension etc.), which results in increased chronic disease susceptibility in the offspring (8,14,21,22,25–28,105–107). The focus of

this dissertation is on the postnatal window in a mouse model and as such, the term IUGR will only be used when discussing growth-restriction before birth. Postnatal growth restriction will be used to define the postnatal window immediately following birth through the first 21 days (of a mouse's life), and early life growth-restriction will be used to define the combined pre- and postnatal window of development in humans. As will be discussed in detail below, postnatal growth in a mouse model equates to roughly the third trimester and early postnatal development in a human, but the development is tissue specific (108–111).

In contrast to the negative effects of IUGR, the effects of postnatal growth restriction are not well documented. The postnatal period (in humans) encompasses the first days after birth up to the first 5 years of life where rapid hypertrophic growth occurs (0-12 months = infancy, 12 months-5 years = childhood) (112,113). Following birth, the infants are exposed to their new external environment but are not fully developed, leaving them vulnerable to growth insults during this window, like the intrauterine window (113,114). Similar to IUGR, postnatal growth restriction metrics have not been unanimously agreed upon in the literature, but the most common categorization is through height and weight percentiles. Failure to grow in postnatal life is defined as below the 10^{th} percentile (z-score <-2) of expected height or weight for age (115–117). Vulnerability during postnatal development can be particularly true if an infant is born premature, where various organ development will take place outside of the womb (118). In almost all species, the cardiovascular system continues to mature in early life, however in different capacities. In humans and sheep, cardiomyocytes begin to mature and differentiate in late gestation and continue through the first couple weeks of postnatal life, while in mice and rats, cardiomyocytes do not begin to proliferate and differentiate until after birth lasting until about 21 days post birth (97). Research has proposed select organs, including the brain, are "spared" by compensatory nutrient

redistribution and develop normally (119). In this case, the infants can compensate in ways to sustain life outside the womb and grow into adulthood. The compensation of other organs like the heart, however, become detrimental with age and increase the infant's risk for chronic diseases (18,76,119,120). Unfortunately, the mechanisms by which the heart adapts to early life growth restriction is not fully understood and therefore interventions and/or treatments are lacking.

2.3 The Heart and Growth Restriction

The cardiovascular system's development is a delicate and complex interaction of pathways that are influenced by genetics, the external environment, and nutrient availability (121). Although the heart is the first organ to develop, the heart continues to grow in the postnatal period. The pattern of cardiac growth is relatively consistent across different species, but the key time points of proliferation and differentiation vary. There are 3 types of cardiomyocytes that go through the maturational process as the heart develops: mononucleated, binucleated, and polynucleated. After cardio-genesis but early in gestation, cardiomyocyte endowment is determined by hormonal pathways, including paracrine factors and endocrine growth hormones that trigger proliferation of mononucleated cardiomyocytes. Later in gestation, cardiomyocytes become terminally differentiated bi- and multinucleated cells (34,97).

Humans are primarily considered a precocial species, meaning offspring are born with eyes open and organs mostly developed. Cardiomyocytes progress in humans from mononucleated to binucleated in the later part of gestation, roughly around the 3rd trimester (97), followed by hypertrophy, or an increase in cardiomyocyte size. The process of maturation begins in late gestation but continues through the first weeks/months of postnatal life, in response to extrauterine life demands (97,122,123). In contrast, mice are an altricial species, meaning offspring are born immature, and some organ development, including the cardiovascular system, continues in early

postnatal life (108). Specifically, PN 1-10 in a mouse are considered the proliferative phase with endowment ending between PN 7-10, which is when the differentiation and hypertrophic phase begin lasting until around PN 21 (97,123,124). Hypertrophy is the last phase of development that prepares the heart for efficiency and pumping throughout life. Thus, any insult during the postnatal window will manipulate cardiomyocyte proliferation and differentiation, leading to permanent cardiac impairments. Despite the difference in developmental timelines, mouse and human cardiomyocytes go through the same phases (**Figure 2.2**) with both species susceptible to environmental insults (22,35–38,125). The period of PN 1-21 in a mouse has been equated to the third trimester and first few months of postnatal life in humans (97,108,126). More importantly, research has found pathological conditions from fetal growth restriction primarily occur (70-80% of incidences) in late gestation (33), thus the window of postnatal growth restriction in the mouse model is clinically relevant to human research.

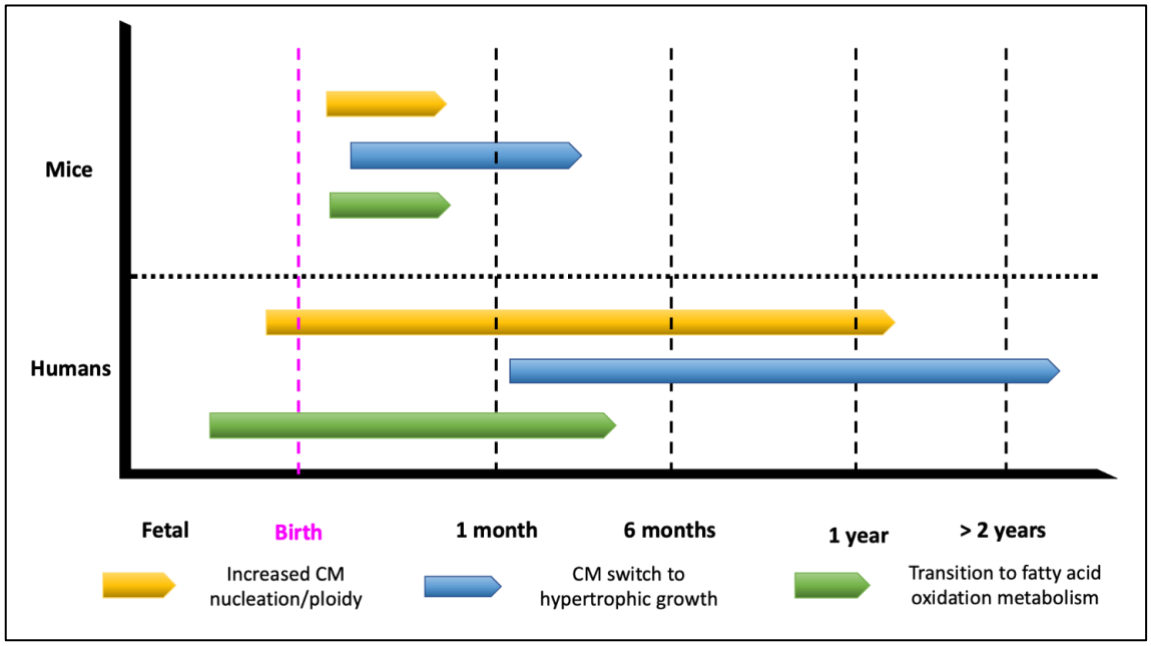

Figure 2.2 *Timelines of cardiac development between mouse and human*
Recreated from Velayutham et al., 2019 (99)

Postnatal growth restriction is an emergent risk factor for CVD in adulthood, as Barker demonstrated the heart was one of the first organs distressed by growth restriction and is related to a 47% increase in CVD (82,127). Scientists have continually confirmed the DOHaD hypothesis with epidemiological examinations of other cohorts (128–131), but primarily focused on IUGR. Most IUGR models suffer from alterations in Ca^{2+} signaling, arterial remodeling, increased hypertension and atherosclerosis increasing their risk of CVD (96,132). Whereas recent evidence from animal models suggests PNGR models suffer from diastolic dysfunction marked by increases in Ca^{2+} transients, ventricular stiffness, reduced ventricular chamber volume, and increased wall thickness to sustain cardiac function, but the adaptations eventually lead to heart failure and mortality (35,133,134). Since it is becoming clear CVD develops differently in intrauterine versus postnatal growth restriction and in males versus females (135,136) it is highly likely male and female offspring respond differently to an early life growth insult. However, the plethora of epidemiological evidence has not been able to distinguish a difference between growth restricted male and female CVD risk (104,137–140).

In 1994, Spinillo et al. examined ~1500 singleton pregnancies, running a logistic regression on incidence of sex on fetal growth restriction (an alternative term for IUGR) risk and found an increased odds ratio that demonstrated females were at a greater risk of growth restriction than males (141). Then in 2009, Melamed et al. expanded Spinillo's work and performed a retrospective study on 78,000 singleton pregnancies (fetal sex: 51% male, 49% female) from one facility to assess the effect of fetal sex on short term neonatal outcomes (142). Melamed's study controlled for maternal age, parity, birthweight, head circumference, gestational age, diabetes, preeclampsia, epidural analgesia, and abnormal amounts of amniotic fluid. The study demonstrated that female fetuses were more likely to experience IUGR than males when defined as below the 10^{th} percentile

in birthweight (142). Meanwhile, a retrospective study by Quiñones et al. reported male and female IUGR fetuses had similar health outcomes regarding respiratory distress syndrome, intraventricular hemorrhages, necrotizing enterocolitis, and periventricular leukomalacia (143), with no mention of CVD outcomes.

There are inconsistencies in how scientists' control for additional risk factors of CVD in human data sets and the reliability of birth weight recall. A meta-regression analysis by Lawlor et al. did not detect a sex difference in the inverse relationship between birth weight and blood pressure (144), while Gamborg et al. found a stronger association between birth weight and increased blood pressure in females weighing <4kg in Nordic cohorts (145). However, contrary to Lawlor and Gamborg, Vos et al. calculated a young adulthood 10-year risk score greater in low-birth-weight males than low-birth-weight females (146). The contradictive results in the literature are due to study design differences in estimating risk and inherent limitations of retrospective analyses (147,148). Furthermore, due to ethical concerns and logistical constraints of human aging, very few long-term effect investigations into human growth restriction exist. The limitations in human research have led to ambiguity in results and reinforce the need for animal models.

2.4 Postnatal Growth Restriction in the Mouse Model

Postnatal growth restriction pertains to the window of development immediately following birth up to PN day 21 in a mouse model. The three common methods used to induce postnatal growth restriction are a protein/nutrient-restricted diet during postnatal life via the mother (35,37–39,149–151), litter size manipulation (152,153), or a timed separation of pups from suckling dams prior to weaning age (154). Research has found that the latter two methods may confound the data due to a stressful environment that can increase cortisol release and manipulate physiological growth and mask cardiometabolic defects (153,155–157). Therefore, protein/nutrient-restrictive

models, especially isocaloric models, are lower-stress alternatives to induce growth-restricted offspring (12,154).

Evidence has accumulated on the negative effects of postnatal growth restriction. The PNGR offspring have reduced lean mass and fat mass (representative of their reduced body size) (36–39,149), increased sympathetic and decreased parasympathetic activity (158,159), reduced total and binucleated cardiomyocytes (35), reduced protein synthesis (150), reduced glucose tolerance in adulthood (160), loss of GH/IGF-1 signaling (12), low nephron endowment (161), right ventricular and pulmonary vascular remodeling (162), increased left ventricular end-diastolic diameter and left ventricular hypertrophy (152,158), decreased leptin concentrations (163), reduced locomotor activity (149,163,164), and increased anxious behavior (163), highlighting the importance and sensitivity of the postnatal developmental window. Bell & Slotkin reported reduced postnatal nutrition influences cardiac development and found reduced cardiac membrane binding receptors, which lead to reduced adrenergic responsiveness (disregarding sex differences) (165). Similarly, Visker et al. documented prolonged QRS and QT duration with electrocardiogram suggesting an increased risk of cardiac arrythmias and supporting Bell & Slotkin's work (36,37). In a second study by Visker et al., the PNGR mice presented with increased IVRT and isovolumic contraction time (IVCT) via echocardiogram clearly highlighting cardiac stiffness and suggesting increased cardiac collagen deposition in the extracellular matrix (37). Although many studies of growth restriction have previously investigated males and females combined or solely the male sex due to difficulties examining female biology, differences exist between how males and females regulate cardiovascular function. Therefore, a gap in the literature exists and more research is needed to investigate female-centric risks to reduce mortality.

The NNERL has been at the forefront of postnatal growth restriction and cardiovascular dysfunction research and most recently discovered that the PNGR female mice were more severely affected than males (35,37,39,149). The PNGR female mice have diastolic cardiovascular impairments and an overall reduced exercise capacity (35,36,38,149). Specifically, Ferguson et al. demonstrated reduced maximal exercise capacity due to blunted HR response, the thinning of LV posterior wall and a reduction in the binucleation and cross-sectional area (CSA) of cardiomyocytes (35). The PNGR females had smaller volumetric load, indicated by reduced stroke volume (SV) and end diastolic volume (35). Despite not looking at the right ventricle specifically, Ferguson's hypothesis for diastolic impairment in females is supported by Wedgwood et al., who reported exacerbated right ventricular remodeling in a rat model of postnatal growth restriction (162). Most recently, Ferguson et al. published a study that confirms reduced physical activity in female PNGR mice but also suggests an adverse response to exercise (39). PNGR female mice presented with reduced aerobic capacity from an adverse shift towards glycolytic fibers in skeletal muscle and concentric remodeling in the heart (39). Overall, the evidence from PNGR models suggests a sex bias where females are more severely impaired than males. To develop therapeutic countermeasures for female PNGR populations, more research is needed to uncover the mechanisms underlying cardiac impairment.

2.5 Cardiac Dysfunction in Postnatal Growth Restriction

The female PNGR mice suffer from cardiac dysfunction indicated by increases in left ventricular (LV) wall thickness, IVRT and deceleration time (DT), and reductions in LV volume and E-wave deceleration rate (EWDR) defined as deceleration rate of early transmitral diastolic velocity (E/DT) (manuscript currently in review) (166). Cardiac dysfunction marked by impaired relaxation, increased stiffness and normal ejection fraction is often termed diastolic dysfunction

(DD). However, 2+ criteria must be present to diagnose DD from the following: E/A ratio, e', E-wave velocity, left atrial volume indexed (LAVI) and tricuspid regurgitation peak velocity (TRpV) (167). After diagnosis, the severity of DD is graded on a 3-point scale by changes in E/A ratio, IVRT, and LV end-diastolic filling pressure (LVEDP). Mild DD is categorized as E/A ratio <0.8, DT >200ms and IVRT >100ms, moderate DD is categorized by E/A ratio >0.8 but <2 with a normal DT and LVEDP, and severe DD is categorized by E/A ratio >2.0, DT <160 ms, IVRT >60ms and elevated LVEDP (167). Furthermore, the PNGR female mice demonstrate reduced cardiomyocyte nucleation and cardiac protein composition and increased cardiac Ca^{2+} transients and vascular stiffness, further supporting cardiac dysfunction (35–37,39). Together these findings can be seen as adaptations to an adverse environment in early life but one that results in impaired relaxation in adulthood. Although the mechanisms underlying impaired relaxation in PNGR female mice are not completely clear, key mechanisms hypothesized include alterations in cardiac Ca^{2+} handling proteins and extracellular matrix (ECM) remodeling.

2.5.1 Cardiac Dysfunction via Ca^{2+} Handling Proteins

Ca^{2+} is integral in regulating the excitation-contraction coupling (ECC) mechanism for cardiac muscle contractions (168). The heart uses Ca^{2+} in a delicate, positive feedback loop to trigger each muscle cell contraction. In short, the sarcoplasmic reticulum (SR) releases stored Ca^{2+} to bind to the tropomyosin complex and reveal actin-myosin binding sites. Followed by the release of ADP and Pi, the newly bound actin and myosin complex is responsible for each muscle contraction and is repeated thousands of times a day. Since Ca^{2+} regulates cardiac contractility and relaxation, any disruption in Ca^{2+} flux will cause dysfunction. The main proteins that influence the influx of Ca^{2+} to control the contractility of the heart are SERCA2, PLN, P-PLN, PKA, and P-PKA.

SERCA2 is vital to the recycling/reuptake of Ca^{2+} which facilitates effective muscle contractility and relaxation. With reduced or dysfunctional SERCA2 proteins, there is a decrease in the rate of Ca^{2+} reuptake that can inhibit actin-myosin unbinding and hinders cardiac contractile dynamics (169,170). Dysfunctional SERCA2 has been linked with ischemic heart disease, cardiomyopathies, and congestive heart failure due to its role in regulating Ca^{2+} uptake (171). The most abundant isoform in the heart is SERCA2a, which is present in both adult and neonatal stages of heart development (172). SERCA2a activity is tightly regulated by the phosphorylation of another endogenous protein, PLN (173,174). When cytosolic Ca^{2+} levels are decreased, PLN will inhibit SERCA2a activity by reducing the pumps affinity to sequester Ca^{2+} back in the SR (170,175). Opposingly, when cytosolic Ca^{2+} levels are higher, PLN is phosphorylated by the activation of the Ca^{2+}/calmodulin kinase (CaMKII), allowing SERCA2a to sequester Ca^{2+} back into the SR (176). Thus, the ratio of phosphorylated to un-phosphorylated PLN will determine the increase or decrease of SERCA2a activity, altering Ca^{2+} flux and the rate of contractile dynamics in the heart. Importantly, PLN does not auto-phosphorylate, but rather PKA phosphorylates PLN at serine^{16} to increase SERCA2 activity and increase relaxation rates (175,177,178). Any decrease in PKA expression or phosphorylation activity will reduce PLN and SERCA2 activity and cause a deregulation of Ca^{2+} kinetics and a disruption in cardiac relaxation (179,180). However, the effect of PNGR on key Ca^{2+} handling proteins is still unknown, leaving a gap in the literature.

2.5.2 Cardiac Remodeling via AngII and ET-1

Left ventricular hypertrophy (LVH) caused by concentric remodeling of the myocardium is a response to stabilize LV function in the face of increased afterload, protein mutations and/or ischemic events (181,182). LVH can cause elevated ventricular pressures, cardiac stiffness, and impaired myocardial relaxation that provokes the development of diastolic dysfunction while

maintaining systolic function (183–185). Initially, LVH is considered compensatory, even beneficial, as it enhances LV volume and contraction kinetics/strength to keep up with hearts demand. Eventually the continued hypertrophy reduces LV volume, weakens contractions, and transitions to decompensated diastolic failure marked by fibrosis (75). The transition to decompensation is not fully understood, however the transition is made evident through increases in ECM deposition. Proper formation and organization of the ECM is essential for homeostasis, and in pathophysiology any defects in or excess deposition of ECM components characterizes fibrosis (186,187).

In pressure overloaded hearts LVH and fibrosis are caused by cardiac wall stress and/or humoral and paracrine factors including AngII, calcineurin, aldosterone, and/or catecholamines (73,188). AngII has been recognized as a hypertrophic and fibrotic stimulus via multiple pathways including ET-1 and transforming growth factor-beta (TGF-B) (73,75,189). Previous research has reported elevated AngII and ET-1 in IUGR populations highlighting these molecules as potential targets of cardiac remodeling in PNGR females (190–192). ET-1 is one of the most potent vasoconstrictors, produced by endothelial cells to stimulate vascular smooth muscle contraction, cell proliferation, angiogenesis, and migration, as well as proinflammatory effects in response to hypoxia, oxidized LDL, AngII, proinflammatory cytokines, and bacterial toxins (193–195). ET-1 acts in an autocrine and paracrine manner through G protein-coupled endothelin receptor ET_A, responsible for mediating vasoconstrictive and proliferative actions and ET_B, responsible for mediating NO release and ET-1 clearance (196). More specifically, ET-1 is linked with fibrosis through its ability to stimulate cardiomyocyte hypertrophy as well as to promote matrix protein synthesis and cardiac fibroblast proliferation through ET_A receptors which leads to fibrosis (186,197). In a model of pressure overload, the transition from endothelial cell to mesenchymal is

a direct contributing factor to cardiac fibrosis (198,199). Further evidence for ET-1 induced fibrosis comes from studies that reported increased secretion of ET-1 in human failing hearts and upregulation in experimental models of cardiac fibrosis (75).

In support of the ET-1 hypothesized pathway, studies have reported IUGR elicits an increase in ET-1. An increase in ET-1 coincides with a decrease in endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) bioavailability, which together elicits an inflammatory response to increase cardiac remodeling/fibrosis and increases risk of CVDs (186,192,195). Despite differences in the IUGR and PNGR development windows, the two groups rely on similar mechanisms for growth and their developmental timelines overlap, suggesting ET-1 as a likely candidate for PNGR cardiac remodeling. Furthermore, the current literature on PNGR rodents suggests concentric remodeling and increased stiffness of the cardiovascular system is indicative of changes in the extracellular matrix (35,37,39). However, no evidence exists to confirm this hypothesis, leaving a gap in the literature. This dissertation aims to close that gap by using Masson's Trichrome staining to confirm fibrosis and collagen accumulation to better understand LVH and impaired relaxation in the PNGR females.

2.6 Sex Differences in Cardiovascular Structure, Function, and Disease

Only recently have scientists begun to understand that specific organ adaptations exist between males and females (200–203). Females have smaller LV chambers than males, even when indexed to body size, and smaller stroke volumes but higher resting heart rates to maintain adequate cardiac output (204). Females also have higher levels of LV stiffness as compared to males, that is accentuated with aging and likely driven by differences in endothelium inflammation and NO signaling (205–207). Females have different electrical systems, driven by greater Ca^{2+} sensitivity, smaller Ca^{2+} peak transients, higher ATPase activity, and lower excitation-contraction

(EC) coupling gain than males, leading to weaker contractions in females (59,208,209). Furthermore, females have a longer repolarization period than males, due to differences in potassium channel proteins and estrogen's lowering effect on SR Ca^{2+} content, which could increase risk of arrythmias (59,208,210). Females also have differences in cardiac metabolism, including lower glucose uptake and utilization but greater fatty acid uptake and metabolic inefficiency than males (211–213).

A recent review article by Vaccarezza et al. highlighted that females are more likely than males to experience acute myocardial infarctions without obstructed arteries, stress-induced cardiomyopathies, peripheral artery disease, abdominal aortic aneurysms, and most often, heart failure with preserved ejection fraction (HFpEF), citing the anatomical and functional differences between males and females (136,204,214). The differences in CVD pathophysiology of males and females are likely due to differences in cardiovascular structure and function which are governed by estrogen, gene expression, and inflammation responses (204,212). Furthermore, females have been reported to have a higher sensitivity to stressful events/environments than males which also increases their risk of developing CVD (215–217). Despite the recognition of sex differences and increasing knowledge, the different mechanisms through which females develop CVDs remain unexplained, highlighting a gap to be filled (41,214,218–220).

One of the defining factors of CVD risk in females is the loss of estrogen through menopause. Estrogen has been targeted as treatment for women because of its cardioprotective effects in pre-menopausal females. The Women's Health Initiative (WHI) examined the effect of estrogen supplementation as female-specific CVD treatment in postmenopausal women. Unfortunately, the WHI study was stopped early due to adverse effects on cardiac function and increased cancer risk in females, triggering controversy over estrogen's role in cardiac protection.

Since the WHI study, research on sex hormone treatments has been stagnant (54,221). However, recent analysis of the WHI data found the timing of estrogen supplementation was key in determining risk and that early intervention with hormones was paramount (54,222,223). The new timing hypothesis warrants a resurgence of hormonal research in female CVD pathology and is supported by a recent commission by the Lancet to reduce the global burden of CVD by 2030. The commission aims to identify sex-specific biological mechanisms of CVD in women and to understand how estrogens affect the cardiovascular system, closing the knowledge gap and improving prevention and care for females (135). Although the Lancet does not directly comment on growth-restricted females, this population is included in the global burden. Therefore, a revival in research concerning female-specific disease pathology is in line with this dissertation's aim to fill a gap in female CVD pathology by investigating the effect of postnatal growth restriction on sex steroids in female mice.

2.7 The Role of Sex Steroids on Cardiovascular Function

Endocrine disorders can manifest disruptions in several organ systems. The cardiovascular system is particularly vulnerable to dysfunction from endocrine disorders due to its reliance on endocrine hormones for growth and function and its need to maintain homeostasis (224). Thyroid hormones exert chronotropic and inotropic effects on the heart, and if left untreated, hypothyroidism can have a serious impact by reducing cardiac contractility and cardiac output and increasing diastolic pressure and systemic vascular resistance (225). In another tightly orchestrated feedback loop, thyroid hormones, specifically Triiodothyronine (T_3), upregulate the expression of SERCA2 and downregulate the expression of PLN, playing a role in Ca^{2+} flux (53). T_3 also stimulates the synthesis and secretion of NO to influence vascular resistance responses. Thus,

reduced T_3 levels in PNGR female mice would lead to reduced cardiovascular function through several pathways.

Disruptions in the hypothalamic-pituitary-thyroid axis function (i.e. reduced levels of T_3) are established in the PNGR population and play a role in cardiovascular dysfunction (49,53). Although the hypothalamic-pituitary-thyroid axis primarily centers on hypo- and hyperthyroidism and thyroid hormone levels, sex steroid bioavailability is regulated by circulating thyroid levels, highlighting the complex interplay between hormones and cardiac function (226). Research has determined sex steroids influence cellular metabolism in males and females, in addition to cellular Ca^{2+} homeostasis and mitochondrial metabolism in females (51,227,228). Estrogen and testosterone are known to influence cardiac function through enzymes and proteins that control contractility and vessel resistance. Therefore, the reduction in thyroid hormones (49) and hypothesized reduction in sex steroids are likely responsible for the cardiac dysfunction in PNGR females.

2.7.1 Estrogen & the Heart

Estrogen is the most abundant sex hormone in the female body system and is primarily produced in the ovaries and adrenal glands. Evidence has reported estrogen's effect on the cardiovascular system, particularly in modulating Ca^{2+} handling proteins and mitochondrial metabolism (51,227). There are 3 main forms of estrogen: Estrone (E1), Estradiol (E2) and Estriol (E3) (229). E1 is responsible for sexual development and function and can be converted into E2 if needed, while E3 plays a minor role in stimulating blood flow but is the predominant hormone during pregnancy (227,230). E2 is considered the most common form of estrogen (227) and is often associated with physiological responses and will be referred to throughout the remainder of this dissertation.

E2 acts as a system regulator in several organs and systems. Acting through 3 different receptors—estrogen receptor alpha (ERα), estrogen receptor beta (ERβ) and G-protein coupled receptor (GPER)—E2 influences cascades of enzymes that alter vascular resistance, contractility, and protein mobilization in the cardiovascular system. E2 works through both genomic and non-genomic pathways. Genomic pathways are dependent on tissue specific transcriptional regulation and take hours or days for a response or modification, while non-genomic pathways occur more quickly (5-20 minutes) and do not require any changes in gene expression (231,232). In the heart, genomic and non-genomic pathways will be dependent on where ERs are located. ERα and ERβ initiate classical genomic signaling and are found in cardiomyocytes, endothelial cells, smooth muscle cells, and cardiac fibroblasts (233–239). The third estrogen receptor, GPER, is a cytoplasmic or membrane receptor, primarily involved in non-genomic signaling (240).

Most predominately, E2 modulates endothelium function by increasing eNOS activity and protein expression and inhibiting ET-1, which increases NO availability and regulates stiffness/pressure of the vascular system (43,241). eNOS plays a crucial role in vasorelaxation/vasoconstriction and, during prolonged eNOS dysregulation, blood pressure responses increase, highlighting a direct involvement in estrogen-mediated protection (43,241,242). In addition to eNOS regulation, E2 regulates ET-1 production. ET-1 is a potent vasoconstrictor and a profibrotic mediator on its own, but ET-1 also acts downstream of AngII, a major mediator in the renin angiotensin system (RAS) (194). E2 decreases ET-1 transcription through inhibition of AngII-erk activation via nuclear signaling disruption (71). Importantly, preliminary results from the NNERL have reported increased ACE1 and reduced ACE2, suggesting an increase in AngII and increased RAS activity in PNGR female mice. The alterations in ACE1/2 add evidence to support the AngII-ET-1 induced cardiac remodeling hypothesis. In

addition to the AngII-erk pathway, E2 inhibits ET-1 activity via Ca^{2+} influx control and ERα and GPER signaling (196,243). The activation of ERα and GPER by E2 reduces free circulating ET-1 but also reduces ET-1 expression in cells (196,243). Therefore, the inhibition of ET-1 provides a novel mechanism for the cardioprotective nature of E2, although its regulation by E2 is not fully understood (71,72).

Through E2's interactions with ERα and ERβ, E2 regulates cardiac contractility through Ca^{2+} homeostasis (244). Reduced E2 levels have been linked with reduced stability and activity of Ryr2 channels, reduced SERCA2 proteins, and phosphorylation of PLN, altering Ca^{2+} flux (51). Ryr2 is highly expressed in cardiomyocytes and integral for Ca^{2+} induced Ca^{2+} release (CICR). A decrease in E2 will cause a leaky Ryr2 channel, creating higher open probability and increased Ca^{2+} release leading to cardiac dysfunction (57). Further evidence from ovariectomized (OVX) animal studies, to mimic reduced estrogen, found increased resting diastolic Ca^{2+} levels, mediated by SERCA2 dysfunction, that lead to impaired relaxation and diastolic dysfunction (59,60). SERCA2 is regulated by Pln phosphorylation and Pka, highlighting additional proteins of interest. Studies have reported E2 deficiency reduces phosphorylation of Pln, eliciting a reduction in SERCA2 activity and inhibiting Ca^{2+} reuptake (51,245). In support of impaired Ca^{2+} flux and cardiac dysfunction through reduced estrogen, Yang et al. found ovariectomized guinea pigs had reduced Ca^{2+} handling mechanisms, which led to larger SR Ca^{2+} stores and higher frequency Ca^{2+} sparks (246). Yang's results in guinea pigs provides support for research from Ferguson et al. that found increased Ca^{2+} sparks in PNGR female mouse hearts under stress indicative of cardiac dysfunction (35), and both provide support for this dissertation's hypothesis.

2.7.2 Testosterone & the heart

From the androgen steroid family, T is the most abundant sex hormone in the male body and is a precursor to E2 in males and females. The principal source of T is the testes and, to a lesser extent, the adrenal glands. T is synthesized through a release of gonadotropin-releasing hormone (GnRH), which binds to receptors on anterior pituitary glands and stimulates synthesis of luteinizing hormone (LH) into circulation. Once LH is circulating, it binds to LH receptors on Leydig cells and stimulates G_s to activate cAMP/PKA pathway, promoting the translocation of cholesterol into mitochondria and increasing gene transcription for T synthesis (247). T synthesis is under tight regulation via a negative feedback loop. The feedback loop will prevent GnRH and LH release when T levels are too high, halting the synthesis of more T (248). Through the activation of the aromatase enzyme, T gets converted into E2, which is the primary process for males to develop E2 in their bodies (61). Albeit at a much lower concentration, T is also produced in the ovaries, providing females with a low level of T in circulation which can also be converted to E2 through aromatase enzymes, creating an additional E2 source for females.

Most androgenic effects are genomic and mediated by androgen receptors (AR) located in bodily tissues, including the atria and ventricles (249). Androgens, including T, bound to ARs will have direct and indirect effects on the vasculature, suggesting a role for them in mediating CVD. Once T binds to an AR, it dissociates from the heat shock protein (HSP), and this new complex migrates into the nucleus to trigger gene transcription (250). Through gene transcription, T can modulate the metabolism, differentiation, and growth of cardiac myocytes (251). For example, T increases phosphorylation of CaMKII and Pln, leading to hypertrophic growth (251). Cardiac Ca^{2+} handling is also regulated by T, through SERCA2-PLN modulation. As SERCA2 regulates Ca^{2+}

reuptake, any alteration to T synthesis or circulating levels will increase PLN phosphorylation and increase SERCA2 affinity for Ca^{2+}, disrupting Ca^{2+} reuptake (61).

Males and females not only differ in CVD pathophysiology, but they also differ in hormone levels. Reduced T level is considered a risk factor for CVD in males, whereas an increase of T can be detrimental to cardiac function in females (252–254). Thus, diminished E2 and increased T in females, as hypothesized in figure 1.1, will impair NO synthesis and its associated effects, increase AngII and cardiac remodeling through ET-1, reduce Ca^{2+} handling mechanisms which alters cardiovascular contractility and relaxation, all leading to cardiac dysfunction. Therefore, the overall objective of this dissertation is to determine the influence of PNGR on cardiac development via a sex steroid pathway in female mice. To accomplish the objective the following specific aims were developed.

Specific Aim 1: To determine if postnatal growth restriction alters the sex steroids estradiol and testosterone, in adulthood in female mice.

Hypothesis 1.1: PNGR female mice will have decreased estradiol in serum at PN 70 and PN 130 as compared to the CON female mice.

Hypothesis 1.2: PNGR female mice will have increased testosterone in serum at PN 70 and 130 as compared to the CON female mice.

Specific Aim 2*:* To determine the influence of postnatal growth restriction on Ca^{2+} handling proteins that regulate cardiac function in the PNGR female mouse hearts.

Hypothesis 2.1: PNGR female mice will have reduced expression of SERCA2 as compared to CON female mice across the lifespan (PN 21, 70 & 130).

Hypothesis 2.2: PNGR female mice will have unchanged expression of PLN but decreased expression of P-PLN as compared to CON female mice across the lifespan (PN 21, 70 & 130).

Hypothesis 2.3: PNGR female mice will have unchanged expression of PKA proteins but decreased expression of P-PKA as compared to CON female mice across the lifespan (PN 21, 70 & 130).

Specific Aim 3: To determine the effect of postnatal growth restriction on endothelin-1 and cardiac fibrosis to indicate cardiac hypertrophy and stiffness in adulthood.

Hypothesis 3.1: PNGR female mice will have increased endothelin-1 protein expression as compared to CON female mice across the lifespan (PN 21, 70 & 130).

Hypothesis 3.2: PNGR female mice will have increased cardiac fibrotic tissue as compared to CON female mice in adulthood (PN 130).

2.8 Animal Models and Method Justification

Animal models are an invaluable tool to study genetic, molecular, and cellular incidents that influence early life growth and development. Early life growth restriction is a major health concern for human and experimental models, requiring a greater understanding. However, it cannot be ethically justified to design an experimental study to cause growth restriction in humans. Therefore, animal models, especially mice, help provide novel insight into developmental programming of CVD across the lifespan and ensure safety and efficacy for future human experiments (108,255). For this dissertation, the inbred Friend leukemia virus B mouse strain (FVB/N) from Charles River Laboratories (Wilmington, MA, USA) will be used. There is a large reproducible reference database on growth, body composition, skeletal muscle phenotype, cardiac function, and metabolism for these mice (35–39,149,150,256–258). Additionally, dams' birth large litters and do not reject newborn pups after being handled, which allows for success of a cross-fostering model to induce growth restriction during the postnatal window. Mice have similar embryology, anatomy, and physiology to humans, share 99% of the same DNA, and are genetically

homologous within each litter allowing us to use siblings at different time points (30,108,259). Mice are favored over rats in this dissertation, as mice have a much larger reproducible genotype and phenotype database available and have an accelerated life span to study aging and disease development (mouse life expectancy is 1.5-2 years vs. rat life expectancy is 2.5-3 years) (255). Although the timeline for cardiac development is different (as discussed above, Figure 2.2) between mice and humans, mice share similarities in development and growth patterns and similar molecular mechanisms in basic cellular processes that allow us to evaluate physiological mechanisms underlying PNGR cardiac dysfunction (259).

2.8.1 Postnatal Growth Restriction Nutrient Model

Evidence in humans and animals reports early life growth restriction leads to increased CVD risk in adulthood (15,16,85,140,260–263). To elicit growth restriction during different developmental windows, several nutrient models have been developed and used, including protein or energy-restriction during gestation and/or postnatal life (35,150,164,264), manipulation of litter size (153,262), timed separation of offspring from mothers prior to weaning (265,266), placental insufficiency (17,267) and intrauterine tubal ligation (268). However, it is thought that with the last four methods, there are confounding factors that lead to increased stress on the pups (handling of animals, hypoxic in utero environments, etc.) that can alter biological results (269,270). Therefore, protein restricted diets, particularly isocaloric diets such as the one to be used in this dissertation, are a low-stress option achieving the same growth restricted result. Furthermore, the protein restricted diet allows us to isolate growth restriction to the postnatal window of development. The postnatal window has been most neglected in developmental programming research, despite epidemiological literature emphasizing the importance of not only birth weight but also weight and development at one year of age.

For this dissertation, a validated nutritive model is used to induce growth restriction isolated to the postnatal window, postnatal days (PN) 1-21. The postnatal period in mice correlates to the 3rd trimester of fetuses into their first year of postnatal life, but development is tissue specific (97,110,111,271). The cross-fostering model can be seen in Figure 2.3. FVB (Charles River Laboratories, Wilmington, MA, USA) mouse dams were fed either a normal protein diet (20% protein; Research Diets, New Brunswick, NJ, USA) or a low protein isocaloric diet (8% protein) beginning 2 weeks prior to mating. One male was introduced into each cage with two female mice for 24 hours to ensure all pups were born the same age. On PN day 1, all pups born from control fed dams were weighed, pooled, and redistributed to one of two experimental groups: (1) Control (CON), pups born from a normal protein fed dam and cross-fostered to a different normal protein fed dam, or (2) postnatal growth restricted (PNGR), pups born from a normal protein fed dam but cross-fostered to a dam fed the low protein diet, isolating growth restriction to the early postnatal window. In this model, the low protein diet reduced the milk production of the dam by roughly 20%, which restricts the amount of milk available to the pups (264,272,273). The different protein content diets were isocaloric (3853.9 kcal vs 3867.5 kcal) and contained the same amount of fat (Table 2.1) (36). Of particular importance in this study was the similar content between diets of soybean oil (70g each), as phytoestrogens in diets can have effects on sex steroids and development in females (274). Energy restriction through this model results in overall reduced body mass, underdeveloped organs, and reduced skeletal muscle—symptoms of early life growth restriction while minimizing external stressors on the pups (154,266,272,273).

Although differences exist between mouse and human models, ages have been correlated between the two species to allow for analysis of age-related diseases. PN 21 is weaning in a mouse and is matched with adolescence in humans at the beginning of puberty, while PN 70 is considered

middle adulthood (~40-50 years old) where signs of CVD begin to develop (108). Lastly, PN 130 is a timepoint in older adulthood (~70 years) to assess disease progression (108,259). The three time points chosen for this dissertation are appropriate as they pertain to growth and disease development in PNGR as published in the literature. The PNGR mice have lower body mass at PN 21, 70, and 130; increased mitochondrial leak and Ca^{2+} transients at PN 70; and echocardiogram changes and reduced exercise capacity at PN 70 and 130 (35,36,38,39,256). Finally, this dissertation is only investigating the female mice as the NNER Laboratory has determined the

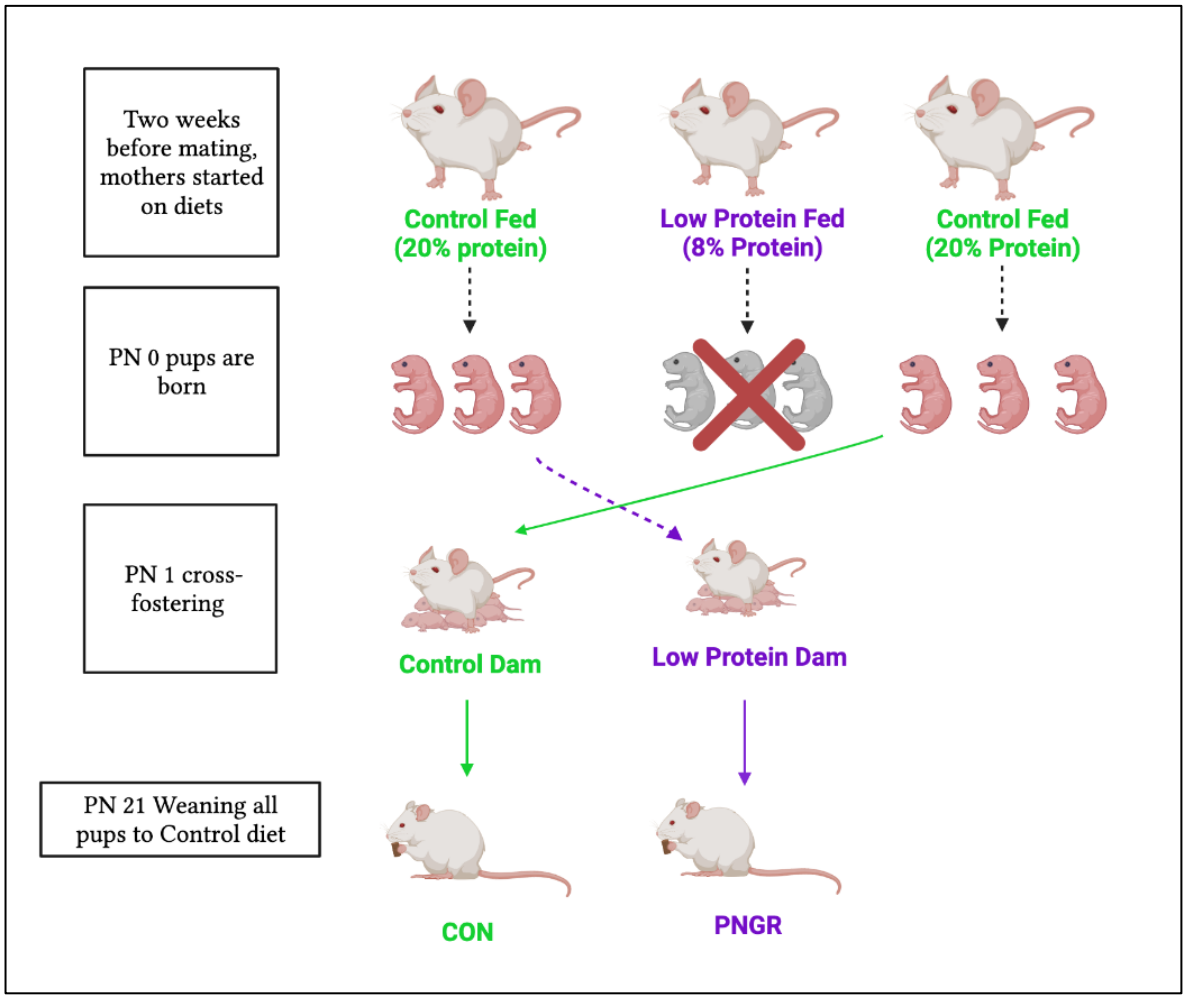

Figure 2.3 *Validated nutritive cross-fostering model*
Growth restriction is isolated to the postnatal period through cross-fostering one day post birth (36–39,149). PN, postnatal day; CON, control; PNGR, postnatal growth restriction.

female mice and not the males have reduced exercise capacity and impaired cardiac function. As the evidence suggests a female-specific cardiac pathology, our goal was to determine the female-specific mechanisms underlying their cardiac dysfunction and increased CVD risk. Male mice exhibited detriments in other organs and were allocated to additional studies of skeletal muscle function.

Table 2.1 *Macronutrient composition of diets*

Diet	Protein (g)	Fat (g)	Carbohydrate (g)	Fiber (g)
CON	196.6	70	609.4	50
LP	80.6	70	728.8	60
Diet	**Protein (%)**	**Fat (%)**	**Carbohydrate (%)**	**Fiber (%)**
CON	19.7	7	60.9	5
LP	8.1	7	73.1	6

Diets were a casein and cornstarch base with fat content consisting solely of soybean oil. CON, control diet; LP, low-protein diet.

2.8.2 Immunoblotting- Infrared Western Blot (IRWB)

The immunoblotting technique has been used for many years. Originally introduced by W. Neal Burnette, the initial intent of an immunoblot was to determine the presence or absence of a protein in a specific tissue (275). As technology progressed, the immunoblot technique was enhanced to not only determine presence or absence but to also determine the abundance of a specific protein. Most recently the immunoblot technique has been enhanced further using near infrared technology to better analyze protein abundance against total protein in the sample, rather than a control (276). Infrared techniques combined with digital imaging have improved the scientific rigor of immunoblots, allowing scientists a simple and fast way to quantify proteins. The infra-red western blot (IRWB) was used in this dissertation to determine the effect of PNGR on

the abundance of Ca2+ handling proteins, SERCA2, PLN and P-PLN, PKA and P-PKA, (Cell Signaling, Kit #8575, MA) and ET-1 (Abcam, ab117757) in cardiac tissue. These specific proteins are the key regulators of Ca^{2+} cycling in the heart, while ET-1 plays a central role in cardiac/vascular stiffness and remodeling.

In short, proteins were extracted from sample tissue and an assay was performed to measure protein concentrations. Once the concentrations are known, all samples were standardized allowing comparison between samples. Gel electrophoresis was then used to separate the sample's proteins by molecular weight followed by an electrical transfer out of the gel to a membrane, producing a series of bands for different proteins (277). Once the proteins were bound to the membrane, primary and secondary antibody incubation labeled the specific protein of interest and allowed the detection and measurement via infra-red imaging. Quantification of the specific proteins was performed with densitometry software, Empiria Studio v.1.3 (LI-COR).

2.8.3 Mass Spectrometry

According to a study by Nilsson in 2015 mass spectrometry assays had the best detectability reported for sex steroids, particularly estradiol (278). Before and since Nilsson's report, other mass spectrometry techniques have been optimized to use smaller volumes of sample but still maintain sensitivity and specificity. One optimization was the switch from gas chromatography to liquid chromatography which uses liquid to move the sample through with high-pressure pumps that separate the components of the sample, followed by mass spectrometry which measures the mass-to-charge ratio of the ions to identify the components (279–281).

For this dissertation I used liquid chromatography with tandem mass spectrometry (LC-MS/MS) protocol with electrospray ionization process (ESI) and multiple reaction monitoring (MRM) methods on an ultra-high performance liquid chromatography that was coupled with a

triple-quadruple (UHPLC-QQQ) mass spectrometer. ESI ionization is a steroid dependent positive or negative polarity technique that helps increase sensitivity of low value measurements. The MRM portion of the protocol is an advanced version of single reaction monitoring that allows the identification of multiple product ions from more than one precursor ion which is helpful when investigating sex steroids in serum (282). Finally, the purpose of the triple-quadrupole mass spectrometer used in this dissertation is a 2x filtering system, ensuring specificity of the results.

In short, the protocol consists of chromatographically separating target steroids in a ~30-minute cycle. Samples are first combined with internal standards and diluted with deionized water and loaded into individual extraction cartridges. After loading, samples are eluted 2x with 0.7 mL methyl-*tert*-butyl ether, dried under a vacuum (Savant, Thermo Fisher), and ultimately reconstituted in 0.1 mL 40:60 methanol to water ratio to prepare for mass spectrometry measurement. Agilent 1260 and 1290 dual front-end HPLC/UPLC pumps are then used to filter out everything but the steroids of interest into column one, followed by pumping through the secondary column filter to reach the mass spectrometer for measurement recording. Agilent 6495 triple quadrupole mass spectrometer was used to measure sex steroids with MRM. All values were reported as total pG/mL. The limit of quantitation is defined as the lowest level that can be detected with a CV of < 10% and is specific for each steroid of interest (283).

2.8.4 Masson's Trichrome Staining

Fibrosis in the heart is associated with aging and several cardiac diseases in response to physiological or pathological cues. Cardiac fibrosis is pathological remodeling of the ECM that leads to fibrotic scars and impaired cardiac muscle function (284). To assess the presence and quantity of fibrosis, histological analysis of heart tissue is an accepted and sensitive technique (284). Histology is a routine lab technique that uses microscope examination of tissue appearance,

organization, and/or function. Staining is the most common method used in histology as this allows the visualization of tissue morphology and structure. The histology method has six key stages for analysis: fixation, processing, embedding, sectioning, staining, and imaging (285). Depending on the staining protocol, the fixation, processing, and embedding may differ between kits.

For the purpose of this dissertation, Masson's Trichrome (Cat#25088-1, Polysciences, PA) staining for collagen fiber identification was used, as it is one of the most commonly used staining methods for cardiovascular fibrosis quantification. Masson's trichrome is a fast, reliable, multi-step staining protocol that allows the differentiation between smooth muscle and type I collagen fibers in tissue sections to indicate a fibrotic phenotype (286). The multi-color kit stains collagen fibers bright blue, muscle tissue appears red, nuclei appear dark brown, and the cytoplasm appear pink. For Masson's Trichrome, paraffin-embedded, or frozen samples can be used, but for this dissertation, frozen samples were used. After sectioning, fixing, and staining samples, the slides were imaged using a digital Keyence microscope (VHX-5000, Keyence, IL).

2.9 Conclusion

This dissertation helps to fill a knowledge gap in cardiovascular function and disease development in the female PNGR population. Cardiac dysfunction in PNGR female mice is marked by increased Ca^{2+} accumulation, reduced cardiac contractility and relaxation via echocardiogram, reduced cardiac proteins, and increased ROS production. Previous evidence reported that PNGR female mice are more susceptible to CVD than their male counterparts. It is hypothesized that sex steroids underlie the cardiac dysfunction in the female mice, specifically, reduced estrogen and increased testosterone to trigger a series of pathways that increase cardiac remodeling and reduce cardiac contractility leading to diastolic dysfunction (Figure 2.1). Since sex steroids are a key biological difference between males and females, the influence of sex steroids

must be investigated thoroughly. The literature review provides rationale to support the hypothesis and the need for research into the early postnatal life development of the cardiovascular system based on female specific mechanisms. By understanding the influence sex steroids have on early life cardiac development and function in the PNGR female population and how key cardiac proteins are altered, this dissertation will fill a gap in the literature and provide a foundation from which female-specific CVD interventions can be developed for PNGR populations.

CHAPTER 3:

THE INFLUENCE OF POSTNATAL GROWTH RESTRICTION ON ESTRADIOL AND TESTOSTERONE LEVELS IN FEMALE MICE

3.1 Abstract

Introduction: Postnatal growth restriction increases the risk of CVD by 47% and disproportionately affects females. One of the largest biological differences between males and females is sex steroids suggesting a target mechanism for cardiac development disruption in PNGR females. In the present study, I explored the effects of PNGR on the hormonal environment in female mice in adulthood. **Methods:** To isolate growth restriction to the postnatal period, a nutritive model was used. FVB mouse dams were fed a 20% normal protein (NP) diet or an 8% low-protein (LP) diet 2 weeks before mating. After pups were born, only pups born from NP fed dams were weighed, pooled, and cross fostered to either a LP fed dam (PNGR), or a different NP fed dam (CON). LP fed dams have a 20% reduction in milk production leading to permanent growth stunting in pups. On PN 21, all pups were group housed by sex and weaned onto the CON diet. Blood samples were collected from female mice at postnatal day 70 or 130, followed by serum separation. Steroids were measured using mass spectrometry and analyzed with a two-way ANOVA (diet x age) using JMP Pro (alpha level $p \leq 0.05$) and if significant Tukey's post hoc for multiple comparisons was conducted. **Results:** Testosterone was not different between CON and PNGR at either time point, $p>0.05$. Estradiol was only detected in 65% of CON and 25% of PNGR mice. 18-OH-Cortisol was elevated in PNGR mice ($p=0.0316$). No other steroids were different between CON and PNGR. **Conclusion:** The results suggest the PNGR females experience hypertension. Future research should focus on interventions to mitigate hypertension in PNGR mice to reduce CVD and myocardial stiffness.

Keywords: postnatal growth restriction, estrogen, testosterone, female, DOHaD

3.2 Introduction

As females transition into menopause, they experience more pronounced cardiac muscle stiffness, particularly LV chamber stiffness, than males that reduces diastolic function and increases their risk of CVD (287–289). Moreover, females have an increased risk of CVD from early life growth restriction compared to males without a known mechanism (11,82,120,290). As CVD is the number 1 killer of females worldwide (136,291,292), understanding the mechanism of female specific CVD is imperative (135). Human and animal models have indicated that postnatal growth restriction results in permanent changes in cardiac function that is more severe in females, increasing arterial and ventricular remodeling and cardiac stiffness leading to a diastolic dysfunction phenotype (35,39,92,293–296). The earliest manifestation of diastolic dysfunction is marked by impaired relaxation, limiting the heart's ability to relax and fill with blood in preparation for the next contraction (297). The two mechanisms underlying diastolic dysfunction are reduced active relaxation through alterations in calcium reuptake or increased passive stiffness through changes in hypertrophic gene expression (287). One of the largest biological differences between males and females that can affect relaxation and hypertrophy is sex steroids (298), suggesting a target mechanism for growth restriction to impact males and females differently.

Evidence has determined estrogen's beneficial effect on the cardiovascular system, particularly in modulating Ca^{2+} ion handling proteins and mitochondrial metabolism (51,227). E2 is the most potent form of estrogen and directly regulates Serca2 expression and activity through Pln phosphorylation and manipulation of Ca^{2+} handling (51,245). Similarly, T assists in regulating cardiac Ca^{2+} handling through Serca2-Pln modulation, suggesting any alteration to circulating levels of T or E2 will play a key role in cardiac Ca^{2+} dysregulation (61). Although E2 and T are the most abundant sex steroids in males and females, there are a series of precursor hormones that

are converted to E2 and T through enzymatic interactions that influence their [E2 and T] abundance. The steroidogenic pathways with precursor hormones are seen in Figure 3.1. All steroids are synthesized from cholesterol and the steroidogenic pathways investigated in the current study begin with the synthesis of pregnenolone from cholesterol. Pregnenolone is converted into progesterone (P4) and/or 17-hydroxyprogesterone (17-OH-P4) via different enzymes. After conversion to P4 and 17-OH-P4 there are several pathways of conversion each with unique enzymes that eventually lead to aldosterone, cortisol/cortisone, E2, and T as seen in Figure 3.1. Any disruption in the enzyme reactions and/or conversions through these pathways could lead to disruption in steroid abundance.

Data indicate that steroidogenesis is disrupted by early life growth restriction (24,299–302). In rodent models of IUGR and PNGR, female rats had abnormal maturation patterns of ovaries (303), and conflicting responses of E2 and T to the different timings of growth restriction (24,48,304,305); some studies in male rats reported either decreases in T (303) or showed no differences in T levels (306). Furthermore, in human models of growth restriction both males and females had delayed onset of puberty, suggesting early life growth restriction can program pubertal timing (47,307–309). However, a broader hormone panel of the Delta 4 pathways (Figure 3.1) is often not investigated in growth restricted cardiac investigations despite the precursor hormones influence not only on E2 and T abundance but also on cardiac function. The evidence for growth restriction's influence on organ growth and pubertal development/timing suggests that any disruption in steroidogenesis due to early life growth restriction will contribute to cardiac impairment (301,310).

Therefore, this study was designed to examine the broader steroid hormone profile in PNGR female mice and determine if postnatal growth-restriction alters steroids as compared to

CON female mice at two time points in adulthood. I hypothesized the PNGR female mice would have reduced E2, but increased T as compared to CON female mice in adulthood which would predispose females to cardiac dysfunction in adulthood.

3.3 Methods

3.3.1 Ethical Approval

The current investigation is approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University (PROTO202100230). All animals were housed in the vivarium on wood-chip bedding in a single room maintained between 18-21°C with a 12 h light/dark cycle. Mice were given access to diet specific food (8% vs. 20% protein) and water *ad libitum*.

3.3.2 Nutritive Model

The investigation used an established and reliable nutritive model to induce PNGR in mice. Two weeks prior to mating, second and third parity FVB (FVB/N; Charles River Laboratories, Wilmington, MA, USA) mouse dams were fed either a control diet (20% protein; #D06041301; Research Diets, New Brunswick, NJ, USA) or an isocaloric low protein diet (8% protein; #D06041302; Research Diets, New Brunswick, NJ, USA). After 2 weeks on the specific diets, one male was introduced into each female cage for 24 hours, then removed, to ensure all pups are born the same age. On PN 1, all pups born from dams fed the control diet were pooled and randomly reassigned to one of two experimental groups: (1) CON, pups born from a control diet dam and cross fostered to a different control diet fed dam or (2) PNGR, pups born from control diet fed dam but cross-fostered to a dam fed the low protein diet, isolating growth restriction to the early postnatal window. Litters were used as a statistical unit, as pups within a litter are not statistically different. A power analysis indicated an n=6 litters per group at each time point (PN 70 and 130) was required for a medium effect size with a β of 0.8 and a $p \leq 0.05$. Thus, the total sample size

was 24 mice. All litters were standardized to similar body masses and sex ratio (4 males and 4 females per litter) to minimize differences between litter weights. If necessary, litter size was maintained throughout lactation by introducing 'donor' pups of similar age, but donor pups were never studied. The pups suckling from a low protein fed dam experience growth restriction during the postnatal development through a ~20% reduction in milk volume and a reduction in amino acid availability in the milk (264,272,273). On PN day 21, all pups were weaned and placed on the normal protein (NP) diet. To optimize animal use, this study only investigated female mice who have previously shown cardiac impairments while male mice were allocated to other studies of skeletal muscle function. From PN 1 – 35, mice were weighed twice weekly on a small animal weighing scale (Ohaus Corporation, Parsippany, NJ; CS Series) then weekly from PN 21-130.

3.3.3 Sample Collection

On PN 70 and on PN 130 one female from each litter were euthanized for tissue collection. Before collection, mice were weighed and anesthetized with 2% isoflurane. After ensuring no response, mice were euthanized with cervical dislocation. The heart was quickly excised to encourage pooling of blood in the trunk. Pooled blood was quickly collected with disposable pipettes and stored in 2mL tubes. Whole blood sat at room temperature for 45 minutes, after which blood was disrupted by a glass cylindrical rod to detach blood from walls and disrupt blood clots. Tubes were then centrifuged at room temperature for 15 minutes at 2500xg. After centrifugation, serum and plasma were separated, serum was collected and stored in a new 1 mL tube and frozen at -80C to be analyzed with mass spectrometry as described below. Plasma was stored at -80C for later analysis.

3.3.4 Sex Steroid Analysis

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was performed at the University of Michigan Biomedical Research Core Facilities (BRCF) to quantify the Delta-4 (D4) steroidogenic pathway in mouse serum as previously described in detail (283,311,312). I specifically targeted estradiol, testosterone and their precursors as seen in Figure 3.1 and used the BRCF E2 Hisense and D4 steroid panels (University of Michigan) conducted on an Agilent 6495 triple quadrupole mass spectrometer and Agilent 1260 and 1290 dual front-end HPLC/UPLC. All values were reported as total pG/mL with limits of quantitation defined as the lowest level that can be detected with a CV of <10% as seen in table 3.1. The D4 steroid panel measures 24 steroids in the pathway, seen in Figure 3.1 and Table 3.1. The E2 Hisense panel was used to target E2, T, E1, and P4 with greater sensitivity.

3.3.5 Statistics

All data were analyzed via JMP v. 14.0 (SAS, Cary, NC) and presented as means ± standard deviations (SD). Outliers were detected by Grubb's Outlier test and removed. Body mass data were analyzed with an unpaired two-tailed Students' t-test at each time point, with an alpha level set a priori $p \leq 0.05$. Steroid levels were assessed with a two-way ANOVA (main effects of diet and age). An alpha level $p \leq 0.05$ was set a priori for the two-way interaction and main effects and if significance was found, a Tukey's HSD post hoc for multiple comparisons was used.

3.4 Results

3.4.1 Body Mass

At birth no differences existed in body mass between CON and PNGR mice (p=0.5498, Figure 3.2). Starting at PN 4 the PNGR mice displayed growth stunting and were 25% smaller than CON mice (p=0.0006). After weaning to the control diet, the PNGR mice were 40% smaller

than CON ($p<0.0001$). The PNGR mice remained small throughout the investigation as seen in Figure 3.2. At PN 70 the PNGR mice were 21% smaller than CON mice ($p=0.0015$) and at the end of the investigation (PN 130) the PNGR mice were still 21% smaller than CON ($p=0.0100$).

3.4.2 Sex Steroids

Testosterone ($p=0.5854$, T), Estrone ($p=0.4233$, E1), Androstenedione ($p=0.5385$), 18-hydroxycorticosterone ($p=0.2840$), Aldosterone ($p=0.3832$), Corticosterone ($p=0.3559$), 16-hydroxyprogesterone (0.5441), 11-hydroxyprogesterone ($p=0.7691$), Androstenedione-AD ($p=0.4658$), and Progesterone ($p=0.7952$, P4) were detected in all samples but were not different between CON and PNGR mice at either time point (Table 3.2).

There was a diet effect on 18-hydroxycortisol (18-OH-Cortisol) such that PNGR mice had higher circulating levels ($p=0.0316$, Table 3.2) and a trend for increased circulating levels of 11-deoxycorticosterone ($p=0.0618$, 11-DOC, Table 3.2) as compared to CON mice. There was a trend for reduced 17-hydroxyprogesterone (17-OH-P4) in PNGR mice ($p=0.0858$, Table 3.2).

E2 was only detected in 25% of PNGR samples and 66% of CON samples such that groups could not be statistically analyzed, but Figure 3.3 provides descriptive data.

Estriol, Dihydrotestosterone, Cortisone, Cortisol, 18-Oxocortisol, 21-Deoxycortisol were not detected in PNGR or CON mice (data not shown).

3.5 Discussion

The current investigation was successful in inducing postnatal growth restriction as evident by a permanent reduction in body mass throughout the lifespan (Figure 3.2). At PN 1 mice were not significantly different in body mass indicating no growth restriction occurred during gestation, but as of PN 4 CON mice gained weight more quickly than PNGR mice. The PNGR mice remained smaller throughout the study demonstrating permanent growth stunting as reported in the literature

(35,39,150,164). Despite the success of growth restriction during the postnatal window, counter to our hypothesis, PNGR females showed no differences in serum T and E2 was only detected in half of the female mice using LC-MS/MS. The additional precursor steroids measured that were not different included E1, Androstenedione, 18-OH-Corticosterone, Aldosterone, Corticosterone, 17-OH-Progesterone, 11-OH-Progesterone, Androstenedione-AD, and P4. Interestingly, circulating 18-hydroxycortisol was elevated in the PNGR as compared to CON, suggestive of hypertension. I was unable to detect several precursor steroids in the current investigation (data not shown).

The elevation of 18-OH-Cortisol in the PNGR mice is supported by the literature, as PNGR mice are reported to suffer from hypertension (158,313). 18-OH-Cortisol is secreted by the adrenal cortex and is often an aldosterone related disease marker, suggesting hypertension (314). Hypertension via aldosterone has also been linked with left ventricular hypertrophy and diastolic dysfunction supporting the proposed phenotype of stiffness in the PNGR mice (158,313–315). Chronic hypertension increases wall thickness to counteract increased pressure overload but leads to reduced cardiac compliance, LV diastolic filling and eventually heart failure (316,317). This result supports the hypothesis that chronic hypertension may cause the diastolic impairments present in PNGR female mice. Furthermore, the PNGR mice tended to have higher levels of 11-DOC than CON mice which acts as a mineralocorticoid to assist in sodium reabsorption but is predominantly a precursor to aldosterone (318). However, excess 11-DOC will serve as an agonist of mineralocorticoid receptors and increases hypertension, even when aldosterone levels are normal (318). Despite the indications of altered aldosterone, I did not observe an elevation of aldosterone in the current investigation. Aldosterone was not a primary interest in the current investigation but may provide important information on the PNGR hypertensive phenotype, and future research should investigate the ratio of aldosterone and renin in PNGR females.

The current literature on E2 and T in PNGR offspring is conflicting and most have not investigated any precursors (300,315,316). A precursor to both E2 and T, 17-hydroxyprogesterone tended to be lower in the PNGR mice ($p=0.0858$), suggesting a disruption in the sex steroid pathway. As seen in Figure 3.1, 17-hydroxyprogesterone gets converted to androstenedione and finally T through enzymatic interactions. Despite a trend in reduced 17-hydroxyprogesterone, Androstenedione was not different between CON and PNGR, indicating no disruption in T synthesis from PNGR in female mice. This result supports the indication that circulating T is not causing cardiac dysfunction in the PNGR female mice. Importantly, the T levels reported in the current investigation are within the published ranges when measured by LC-MS/MS (281,319). As seen in figure 3.1, androstenedione also gets converted to E1 and finally E2. As no differences were observed in androstenedione or E1, the current results propose that E2 is not different between CON and PNGR. However, the current investigation was only able to detect E2 in 66% of CON samples and 25% of PNGR samples in the current study, indicating the effect of PNGR on E2 remains to be clarified. Importantly, the E2 values detected were within the normal range of intact female mice during diestrus and proestrus (278).

LC-MS/MS is emerging as the method of choice in steroid hormone detection for its' superior specificity and sensitivity, multiplexing abilities, and small sample volume requirement (320,321) as compared to immunoassays. However, in mice, values are very low and still difficult to detect (322). The undetected hormones are listed in Table 3.1 and were below the LLOQ. With the knowledge of these difficulties, I used two protocols to generate results of E2 and T, the main steroids that influence cardiac function. The T levels were comparable between the two assays indicating our methodology was reliable and accurate. Meanwhile E2 was completely undetectable in assay 1 and detectable in 66% of CON samples and only 25% of PNGR samples in assay 2

highlighting the high sensitivity needed to detect E2. Our detection rate in CON mice is similar to the literature where Handelsman et al reported a 65% success rate of measuring E2 regardless of estrus cycle stage in healthy 3-month-old mice, using the same LC-MS/MS protocol (322). The lower detection rate (only) in PNGR mice is puzzling but is due to a lower signal-to-noise ratio that rendered samples undetectable. All samples were collected following the same protocol to minimize interference (noise) during ionization. The low detection rates of E2 highlight the need for further research into hormone quantification in mice. Due to logistical reasons, I did not control for menstrual cycle phases. However, from the literature I was able to estimate the mice were between diestrus and proestrus phases based on levels of P4 and E1, suggesting detectable levels of E2 (278). I also collected mice from several rounds of breeding to account for the variance in cycle data and to present a holistic view of sex steroids in this population.

There are numerous studies that report negative influences of early life growth restriction on organ growth and development in human and animal models (15,20,76,81,261,262,323,324), but the connection between PNGR and sex steroids remains controversial. The sex-specific association between PNGR and sex steroids has primarily investigated reproductive function (304,305,325), thus the current investigation is one of the first to explore the broader hormonal environment in mice as it relates to cardiac dysfunction from postnatal growth restriction. Here, our data show maternal protein malnutrition during lactation had no effect on T levels in female mice while the effect on E2 remains to be clarified. An early precursor for sex steroids and glucocorticoids, 17-hydroxyprogesterone, tended to be lower in PNGR mice suggesting a disruption in one of the steroidogenic pathways, but no other steroids were reduced. Through an additional pathway, the current investigation suggests a disruption in the adrenal cortex as 18-hydroxycortisol was elevated in PNGR mice. This result provides evidence that PNGR disrupts

the cortisol/cortisone pathway suggesting hypertension is likely contributing to cardiac stiffness in PNGR female mice. Future research should examine interventions to mitigate chronic hypertension in PNGR females to reduce CVD.

Figures

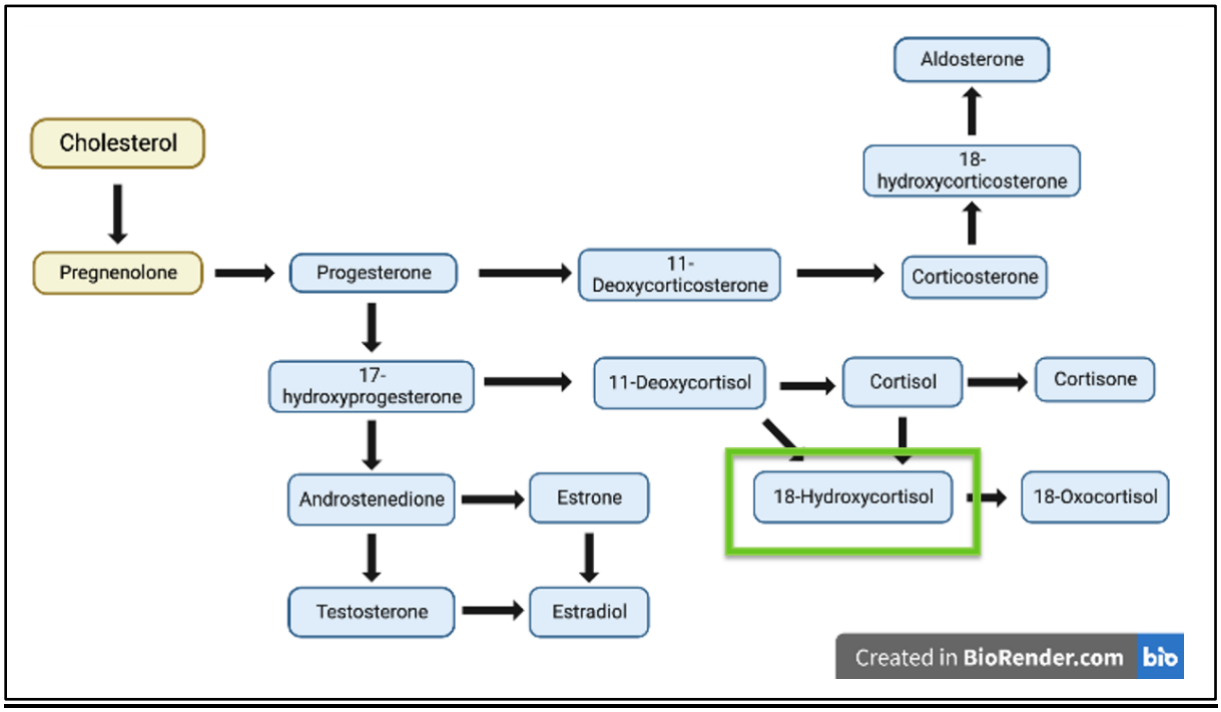

Figure 3.1 *Simplified scheme of steroidogenic pathways*
Precursor hormones regulate synthesis of final steroid products. Only steroids in the blue boxes were detected in the current study. The green box indicates significantly different steroids between CON and PNGR in the current study. Created with Biorender from Delchev et al, 2018, Holler et al, 2019 and Nounu et al, 2022 (326–328).

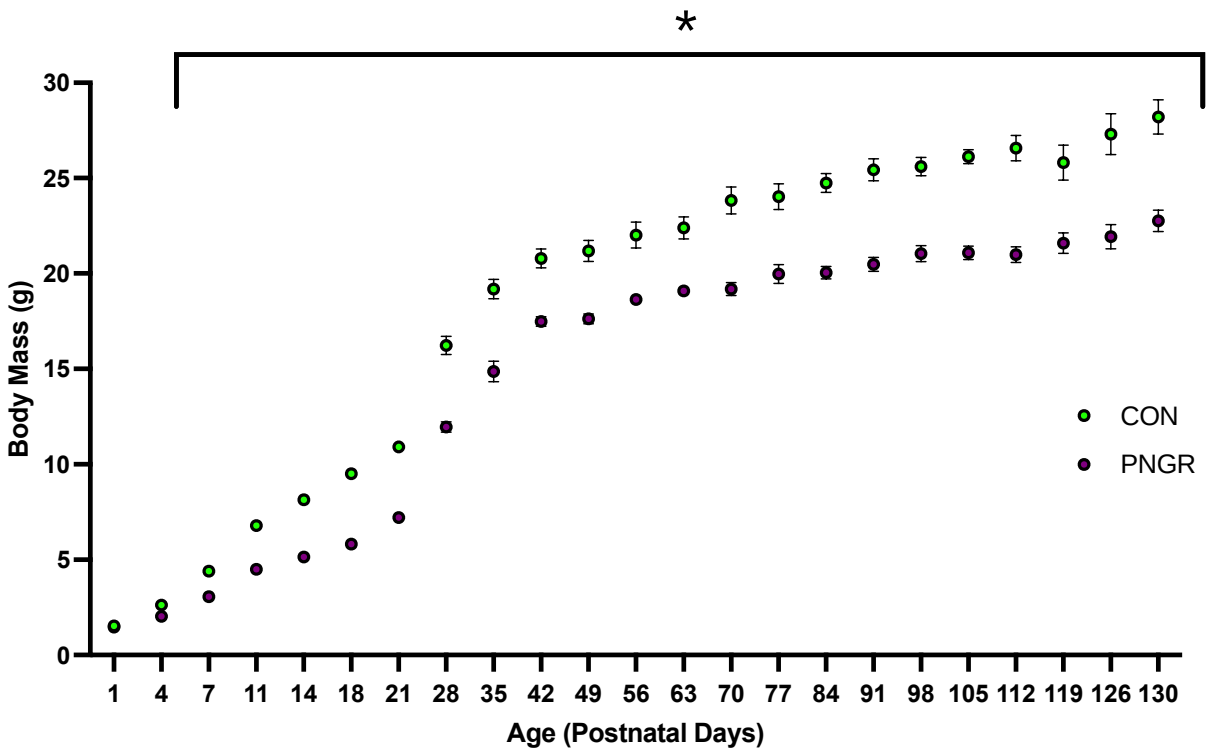

Figure 3.2 *Growth and Body Mass Data Across the Lifespan*
At each time point from PN 4 through PN 130 PNGR mice were smaller than CON mice as indicated by the bracket and asterisk (*). The following *p* values are listed respectively from PN day 1-130 and correspond to the PN days listed in figure 1, p= 0.5498, p= 0.0006, p= 0.00006, p <0.0001, p<0.0001, p<0.0001, p<0.0001, p<0.0001, p=0.0009, p=0.0064, p=0.0047, p=0.0120, p=0.0124, p=0.0015, p=0.0174, p=0.0311,p= 0.0003, p=0.0013, p<0.0001, p=0.0001, p=0.0067, p=0.0242, p= 0.0100. Values are reported as mean ± standard deviation in grams (g). Only female mice were analyzed, n=6 CON and n=6 PNGR mice.

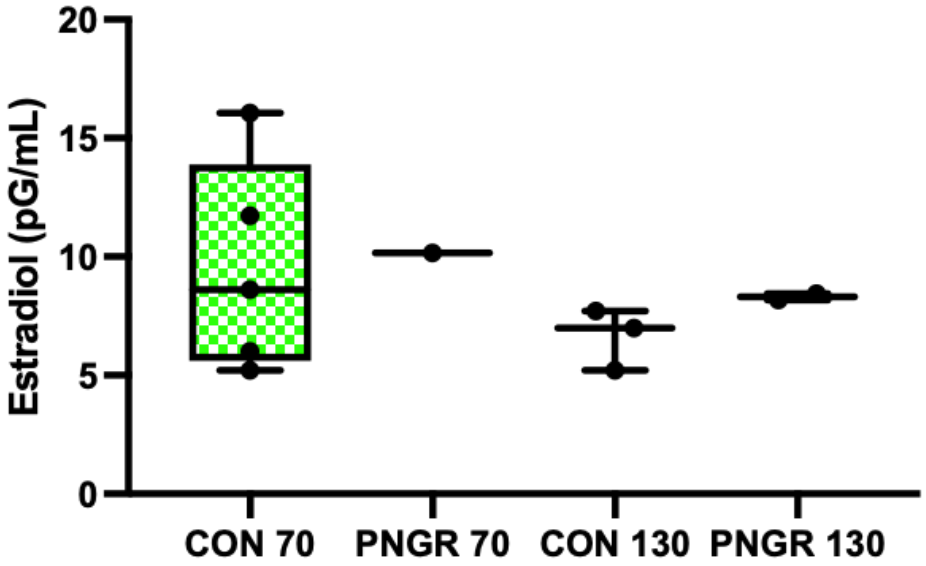

Figure 3.3 *Estradiol levels from serum*
Estradiol (E2) was only detected in 66% of CON mice and 25% of PNGR mice. Data were not statistically analyzed due to low sample size. Diet groups, pups not growth restricted (CON) and pups growth restricted during postnatal life (PNGR). Values are reported as mean ± SD in picograms/milliliter (pg/mL). Only female mice were analyzed, estradiol was only detected in some of the samples, PN 70 CON n=5, PNGR n=1 and PN 130 CON n=3, PNGR n=2.

Tables

Table 3.1 *Lower limit of quantitation for steroids*

Steroid Analyte	LLOQ (pG/mL; pG)
Aldosterone	40; 0.8
Androstenedione	5; 0.1
Cortisol	100; 2.0
11-deoxycortisol	50; 1.0
Estradiol	10; 0.2
17-Hydroxyprogesterone	13; 0.25
Testosterone	5; 0.1
11- Hydroxyandrostenedione	50; 1.0
11-Hydroxytestosterone	50; 1.0
11-Ketoprogesterone	40; 0.8
16-Hydroxyprogesterone	13; 0.25
18-Oxocortisol	25; 0.5
Androsterone	50; 1.0
Corticosterone B	50; 1.0
Cortisone	125; 2.5
11-deoxycorticosterone	18; 0.35
Estrone	10; 0.2
Progesterone	5; 0.1
Dihydrotestosterone	50; 1.0
11-Hydroxyprogesterone	40; 0.8
11-Ketoandrostenedione	50;1.0
11-Ketotestosterone	50; 1.0
18-Hydroxycortisol	25; 0.5
21-Deoxycortisol	50; 1.0

Recreated from University of Michigan Biomedical Research Core Facility for LC-MS/MS.
LLOQ: lower limit of Quantitation, pG/mL: picograms per milliliter.

Table 3.2 *Mass spectrometry measurement of steroids in female mice*

	PN 70		PN 130		p-value		
Steroids (pG/mL)	**PNGR**	**CON**	**PNGR**	**CON**	**Diet**	**Sex**	**Diet* Age**
Estrone	8.59± 2.42	7.94± 1.59	7.16± 1.48	7.65± 1.08	n.s	n.s	n.s
Testosterone	24.51± 27.24	36.38± 42.95	27.1± 12.28	30.15± 17.18	n.s	n.s	n.s
Androstenedione	23± 19.22	35.05± 40.56	22.81± 7.24	25.4± 8.18	n.s	n.s	n.s
18-OH-Cortisol	*5.61± 2.75*[a]	*3.66± 1.61*[b]	*4.33± 1.55*[a]	*3.07± 1.97*[b]	*0.0316*	n.s	n.s
18-OH-Corticosterone	1041.68± 888.24	1058.68± 713.51	1293.83± 581.43	1997.02± 794.16	n.s	n.s	n.s
Aldosterone	335.86± 289.86	320.13± 238.07	341.02± 145.37	505.49± 304.01	n.s	n.s	n.s
Corticosterone	145762.65± 67601.05	164830.65± 51434.95	157322.48± 54032.83	132710.8± 53196.27	n.s	n.s	n.s
16-OH-Progesterone	261.15± 103.33	236± 112.54	158.36± 83.76	188.51± 133.64	n.s	n.s	n.s
17-OH-Progesterone	8.29± 4.91	20.48± 31.81	6.66± 1.3	8.58± 2.19	0.0858	n.s.	n.s
11-OH-Progesterone	31.62± 29.36	29.34± 13.7	18.23± 13.8	45± 43.61	n.s	n.s	n.s
Androstenedione-AD	23.05± 16.09	33.91± 35.41	22.65± 5.76	26.27± 8.33	n.s	n.s	n.s
11-DOC	1889.94± 1772.57	1016.62± 496.68	1710.11± 1264.2	1389.67± 1661.03	0.0618	n.s	n.s
Progesterone	2720.67± 2128.26	2843.82± 3284.23	1651.09± 1923.34	1777.82± 2419.98	n.s	n.s	n.s

PN: postnatal day, CON: control, PNGR: postnatal growth restriction, pG/mL: picograms per milliliter. Values are expressed as mean ± standard deviation. Difference in superscripted letters across a row indicates statistical significance of $p<0.05$. CON; n= 6 and PNGR; n= 6.

CHAPTER 4:

THE INFLUENCE OF POSTNATAL GROWTH RESTRICTION ON CARDIAC CA^{2+} HANDLING PROTEINS IN FEMALE MICE

4.1 Abstract

Introduction: Postnatal growth restriction leads to increased risk of CVD in adulthood. More severe cardiac impairments are present in postnatally growth restricted female mice, including increased Ca^{2+} transient amplitudes, suggesting altered Ca^{2+} regulation and impaired relaxation. Thus, the current investigation hypothesized alterations in the abundance of key regulating proteins of cardiac Ca^{2+} homeostasis including reduced Serca2, P-pln and P-pka and no changes in Pln or Pka. **Methods:** To induce postnatal growth-restriction, mouse dams were fed either a 20% normal protein (NP) diet or 8% low-protein (LP) diet 2 weeks before mating. Only pups born to NP fed dams were cross-fostered on PN 1 to either a LP fed dam (PNGR) or a different NP fed dam (CON). LP fed dams produce ~20% less milk leading to permanent growth restriction in pups. On PN 21, all pups were weaned onto the NP diet. On PN 21, 70 or 130 mice were euthanized, and hearts were collected and analyzed via immunoblot. JMP Pro was used to run a two-way ANOVA (diet x age) with an alpha level of $p \leq 0.05$ set *a priori* and if significance was found, Tukey's post hoc for multiple comparisons was conducted. **Results**: Serca2 was reduced in PNGR mice as compared to CON mice (p=0.0005). No differences existed between diet groups for Pln, P-pln, Pka or P-pka at any time point (p>0.05). **Conclusion:** The reduction in Serca2 indicates a disruption in cardiac Ca^{2+} reuptake suggesting a mechanism for impaired relaxation in the PNGR mice.

Keywords: postnatal growth restriction, female, DOHaD, calcium handling proteins

4.2 Introduction

Nearly 160 million children under the age of 5 years old experience growth restriction worldwide due to a poor nutritive environment annually (2). Early life growth-restriction leads to CVD in adulthood (11,20,27). The postnatal period is a critical window of development for the heart, as cellular maturity and functionality develop and become permanent (329,330). Evidence from the Neonatal Nutrition and Exercise Research Laboratory (NNERL) and others has shown females are more severely affected by postnatal growth restriction than males (35,39,293–295,331). Specifically, PNGR female mice (and not PNGR male mice) have altered heart cell maturation, reduced cardiomyocyte size and binucleation, and impaired Ca^{2+} regulation, which results in cardiac stiffness and impaired diastolic function (35,36). Ferguson et al., reported PNGR females had increased Ca^{2+} transient amplitude and a matched increase of Ca^{2+} decay rate in binucleated cardiomyocytes. When those cardiomyocytes were stimulated the PNGR hearts could not increase reuptake further indicating a maximal elevation in Ca^{2+} cycling and sympathetic activity at baseline, which suggests the PNGR hearts work harder than CON and have compromised Ca^{2+} transient function (35).

Ca^{2+} is integral in regulating excitation contraction coupling (ECC) for cardiac contractility and relaxation (170,332,333). Alterations in cardiac Ca^{2+} regulation (as observed in female PNGR mice) are primarily regulated by Serca2. Serca2 is the key protein that increases Ca^{2+} transients by increasing Ca^{2+} reuptake and induces relaxation. Unphosphorylated phospholamban inhibits Serca2, while the phosphorylation of Pln relieves Serca2 inhibition, increases the affinity for Ca^{2+}, and allows for the initiation of muscle relaxation (173,333,334). A secondary protein that indirectly regulates Serca2, called Pka, is responsible for the phosphorylation of Pln (334). Thus,

a reduction in any of these key proteins leads to a decreased Ca^{2+} reuptake pathway and impaired cardiac contraction and relaxation dynamics (169,170).

Therefore, the purpose of the present investigation was to determine the effect of postnatal growth restriction on key cardiac Ca^{2+} handling protein expression in PNGR female mice. I hypothesized that the PNGR females would have reduced Serca2, P-pln and P-pka with no changes in Pln and Pka as compared to the control mice because PNGR mice have compromised Ca^{2+} transient function and Serca2 is the central regulator in Ca^{2+} reuptake and relaxation.

4.3 Methods

4.3.1 Ethical Approval

The present investigation is approved by IACUC at Michigan State University under Protocol 202100230. All animals were housed on wood-chip bedding in the vivarium maintained between 18-21°C with a 12 h light/dark cycle. Mice had ad libitum access to food and water.

4.3.2 Nutritive model

The mouse model in the current investigation is a validated nutritive model for inducing postnatal growth restriction in mice (35,38,39,150,335). Two weeks before breeding, female mice (dams) are fed *ad libitum* either a reduced protein diet (8% protein; LP) or normal protein isocaloric diet (20% protein; NP). LP dams have reduced amino acid availability and produce 15-20% less milk during lactation thus pups nursed by LP dams experience postnatal growth restriction (264,272,273). Mating is timed to allow all pups to be born the same age by introducing a male into the female cage for 24 hours. Following birth, on PN 1, all the pups from NP fed dams were combined and then cross fostered to either a different NP fed dam, generating a CON group or to a LP dam, generating a PNGR group. Litter size was standardized to 8 pups/dam (4 males, 4 females) and was maintained using “donor pups” (donors were not studied). On PN 21, mice were

weaned and fed the NP diet until PN130, isolating growth restriction to the postnatal window. From PN 1 to PN 35, each mouse was weighed every 4th day, after which mice were weighed 1x weekly until PN 130. To optimize the use of animals according to ethical guidelines for animal studies, only female mice, who have previously showed impairments were used (35,36,39). Sample size was calculated by power analysis using previous growth restricted studies. For this study the number of litters needed to achieve significance with a medium effect size (β of 0.8 and $P<0.05$) and the main effect of diet (CON vs PNGR) is n=6 litters per age and diet group with a total sample size of 36 mice.

4.3.3 Immunoblotting- Cardiac Ca^{2+} Handling Protein Analysis

All mice were euthanized on either PN 21, PN 70 or PN130 by cervical dislocation under 2% isoflurane. Each heart was excised and immediately frozen in liquid nitrogen and stored at -80C. Following which each sample was homogenized using a tissue homogenizer (Omni THQ Homogenizer, cat# 12-500, Omni, Kennesaw, GA) while submerged in C. Norris buffer (0.745g HEPES, 0.135g B-glycerophosphate, 0.014g ATP, 0.75mL protease inhibitor cocktail, 0.489g Benzamidine, 0.044g PMSF, 0.25mL DMSO, 0.186g EDTA, 0.254g $MgCl_2$). Protein was isolated using 1 mL of C. Norris buffer per sample (336). Tubes were then centrifuged for 15 minutes at 13,000 x g to separate supernatant and pellet. The supernatant was collected and a Pierce BCA protein assay (Thermo Fisher Scientific Cat# 23225, Rockford, IL, USA) was utilized to determine protein concentrations and ensure equal protein loading of protein for SDS-PAGE.

Protein extracts were separated by electrophoresis on SDS polyacrylamide gels and transferred onto Millipore Immobilon-FL polyvinylidene difluoride membrane (PVDF) Membranes (Millipore Sigma Cat# IPFL00010; Serca2, Pka) or nitrocellulose membranes (PALL Life Sciences, Bio Trace NT, #66489, .2um; P-pka, Pln, P-pln). Following the transfer, the PVDF membranes were air-dried, re-wet with methanol and stained with a total protein stain (LI-COR

Cat# 926-11021) for 5 minutes, washed and imaged for total protein loading control. Nitrocellulose membranes were not air dried but instead, immediately following the transfer were incubated with total protein stain (LI-COR Cat# 926-11021) and imaged. After imaging, both membranes were washed, de-stained, and blocked at room temperature for 1 hour in 5% proteomic grade non-fat dried milk (NFDM) for non-phosphorylated proteins or 5% bovine serum albumin (BSA) for phosphorylated proteins. The blocking step was followed by incubation in primary antibodies for Ca^{2+} proteins: Serca2 (8% gel, 1:1000 in 5%NFDM), Pln (12% gel, 1:1000 in 5% NFDM), P-pln (12% gel, 1:500 in 5% BSA), Pka (10% gel, 1:1000 in 5% NFDM) and P-pka (10% gel, 1:1000 in 5% BSA) (Cell Signaling Technology Cat# 8575) overnight at 4°C. The next day membranes were washed 3 times for 5 minutes each and incubated with infrared-labelled secondary antibody (800 CW Goat Anti-Rabbit IgG, LI-COR Biosciences Cat# 926-32211, 1:10000) for 1 hour at room temperature. Infrared images were collected using the Odyssey XF Imaging System at 700nm and 800nm. Individual protein bands were then standardized to their respective total protein stain and finally analyzed with densitometry software (Empiria Studio Software v.1.3, LI-COR, AU: arbitrary units) (276). Phosphorylated proteins were also normalized to their total protein counterpart.

4.3.4 Statistics

In line with ethical standards set forth by the Guide for the Care and Use of Laboratory Animals, only the minimal number of mice were used to achieve significance as determined by a power analysis. Statistical analysis was performed using JMP v14.0 (SAS, Cary, NC). Data were evaluated for normality with Shapiro-Wilk Normality test and outliers determined from Grubbs Outlier test (JMP script Version 2). Once outliers were removed all data passed Shapiro-Wilk Normality test and were analyzed with a Two-way ANOVA, with main effects of diet and age. All

significant multiple comparisons were then assessed with a Tukey's HSD post hoc test. Alpha level was set at $p \leq .05$ for all analyses.

4.4 Results

4.4.1 Body Mass

Growth restriction was achieved as seen in Figure 3.2; body mass was reduced in PNGR mice starting at PN day 4 and remained reduced throughout the entire study ($p<0.05$).

4.4.2 Interaction Effect

There was a trend for PNGR to have lower Serca2 abundance as compared to CON mice at PN130 ($p=0.0810$, Figure 4.1). There were no other interaction effects.

4.4.3 Diet Effect

Serca2 was reduced in PNGR mice as compared to CON mice ($p=0.0005$, Figure 4.1). There was no diet effect on Pln, P-pln, Pka, or P-pka ($p>0.05$, Figures 4.2-4.6). As a result, there was no diet effect on the ratio of phosphorylated to non-phosphorylated proteins P-pln/Pln or P-pka/Pka ($p>0.05$, Figures 4.4 & 4.7).

4.4.4 Age Effect

Serca2 abundance was the lowest in PN 21 mice as compared to PN 70 and 130 mice ($p=0.0146$, Figure 4.1). PN 21 had the lowest abundance of Pln that increased with age ($p<0.0001$, Figure 4.2). There was an age effect on P-Pln where mice at PN 21 had the lowest abundance of P-pln that increased at PN 70 and again at PN 130 ($p<0.0001$, Figure 4.3). There was no age effect on P-Pln/Pln ($p>0.05$, Figure 4). The Pka abundance was lower in mice at PN 21 and increased with age to PN 70 but then decreased at PN 130 (Figure 4.5, $p<0.0001$). The PN 21 mice had the highest abundance of P-pka that decreased with age (Figure 4.6, $p<0.0001$). When P-pka was

analyzed against total Pka there was an age effect in which mice aged 21 days old and 70 days old had a lower abundance than 130-day old mice (Figure 4.7, $p<0.0001$).

4.5 Discussion

The results of this study provide new information with respect to elucidating a pathway for impaired relaxation in the PNGR heart including: 1) Serca2 is reduced in PNGR female mice and 2) the key regulators of Serca2 [Pln and Pka] were not disrupted by growth restriction. This study thus builds on work by Barker and colleagues who confirmed in 1986 that early life growth restriction leads to a 47% increased risk of CVD in adulthood (10). More recently, evidence in mice show females are more severely affected by postnatal growth restriction than males which presents as impaired relaxation and suggests diastolic dysfunction marked by reduced E/A ratio, increased IVRT and DT (35,39,293–295,331). The growth-restricted female mice have altered heart cell maturation and reduced cardiomyocyte size and nucleation with impaired Ca^{2+} transient amplitude. As Ca^{2+} is integral in regulating the ECC (168) and [Ca^{2+}] is disrupted in PNGR mice, the current study investigated the effect of PNGR on Ca^{2+} handling proteins in the heart.

Growth restriction was successfully implemented in the postnatal window as seen in figure 3.2. PNGR female mice had reduced body mass from PN 4 to PN 130 indicating a permanent disruption in growth and increased risk of CVD. In congruence with the success of our growth restriction, the current study determined PNGR female mice have reduced Serca2 proteins across the lifespan (Figure 4.1). Serca2 is the primary regulator of Ca^{2+} reuptake into the sarcoplasmic reticulum (SR), where Ca^{2+} is stored until needed for the next contraction. Serca2 removes ~90% of Ca^{2+} from the cytosol and deactivates the contractile apparatus to induce relaxation (337,338). Thus, any reduction in Serca2 would lead to excess Ca^{2+} in the cytosol, resulting in impaired/delayed relaxation (158). According to He et al, reductions in Serca2 (regulated by Pln

and Pka) have been linked with impaired contractile performance and reduced filling, suggesting a phenotype for the PNGR population (339–344). Lacombe et al. reported a reduction in Serca2 proteins in hearts from a rat model of impaired relaxation and diastolic dysfunction (341). The current results also add confirmation of a study published by Ferguson et al. in 2019, showing an increased Ca^{2+} transient amplitude and faster rate of Ca^{2+} decay in PNGR female mouse hearts that was not able to increase uptake upon stimulation, indicating the PNGR hearts are working harder than CON hearts at rest (35). Although an increased Ca^{2+} decay indicates faster reuptake by the sarcoplasmic reticulum (SR), the PNGR mice are hypothesized to have greater free Ca^{2+} in the cytosol which is supported by increased spontaneous Ca^{2+} waves and sparks (35). The excess free Ca^{2+} supports a disruption in reuptake associated with Serca2 proteins.

Serca2 activity is regulated through several direct and indirect pathways including modulatory proteins Pln, P-pln, Pka, and P-pka and post translational modifications. Pln has a direct effect on Serca2 activity, increasing its affinity for Ca^{2+} when phosphorylated (P-pln) and decreasing the affinity when unphosphorylated (Pln) (345). Thus, I hypothesized a reduction in P-pln protein abundance to support impaired relaxation in the PNGR female mice. However, there were no differences in Pln or P-pln at any of the three time points measured in the PNGR female mice (Figures 4.2 & 4.3). The lack of change in Pln suggests no disruption in each Serca2 protein's affinity for Ca^{2+}, but rather the concern for PNGR female mice is with the total reduction of Serca2 abundance in the cardiomyocytes. This hypothesis is supported by Nagai et al that determined in a rabbit model the expression of Serca2 and Pln mRNA may not be coordinately regulated during myocardial adaptation to different physiological conditions, despite their connection in function (346).

As there were no changes in P-pln, I did not expect to see a change in Pka. Pka is an indirect regulator of Serca2 through phosphorylation of Pln. I did hypothesize a reduction in P-pka, as this would conformationally change Pln and subsequently alter Serca2's affinity for Ca^{2+}, but the results did not agree. There were no changes in any of the measured key regulators of Serca2 in the PNGR female mice, suggesting the reduced reuptake is not due to changes in Serca2 activity. Therefore, it is likely there is a reduced gene expression or an interruption in translation of Serca2 proteins. Ferguson et al. reported more mononucleated cardiomyocytes in PNGR females which have prolonged Ca^{2+} transients and desynchronized Ca^{2+} release/reuptake (35,347). In mononucleated cardiomyocytes the t-tubules and SR are underdeveloped and functionally immature as compared to binucleated cardiomyocytes (347–349). Therefore, the greater proportion of mononucleated cardiomyocytes in PNGR female mice is also likely underlying the reduced abundance of Serca2 and impaired reuptake properties prompting delayed relaxation in this population.

Interestingly, the PN 21 mice had the lowest abundance of Serca2 as compared to PN 70 and PN 130. Basani and Basani reported a lower reliance on Serca2 to remove Ca^{2+} in the hearts of newborn rodents as compared to adult rodents, but which increased with developmental age (350). Ziman et al. also reported lower SR Ca^{2+} loads in the hearts of rats at PN day 10-15 as compared to adults, further indicating the importance of early postnatal maturation of the SR, t-tubules, and Serca2 in cardiomyocytes of rodents (351). Due to the postnatal development of cardiomyocytes in which mice become more reliant on Serca2 and cardiomyocytes continue to mature (170), the current results indicate the permanent and detrimental effect growth restriction during the postnatal window has on cardiac development and Serca2 abundance in female mice. Furthermore, the literature suggests different mechanisms between fetal, perinatal, and mature

adult hearts for SR Ca^{2+} transport regulation due to the cardiomyocyte and SR maturation process during development (349,352). A future investigation should determine Serca2 abundance in mono- versus binucleated cardiomyocytes at different ages (including older ages) to help develop a therapeutic countermeasure and appropriate timing of an intervention.

Similar to Serca2, Pln and P-pln abundance were lowest at PN 21, increased with age to PN 70, and remained unchanged through PN 130. The current results of Pln and P-pln supports normal postnatal cardiac development (353) but suggests reduced Ca^{2+} reuptake by Serca2 proteins, as CON and PNGR had similar regulatory protein levels at PN 21 but PNGR have less Serca2 (Figure 4.1). Pka also followed a similar timeline in which PN 21 had the lowest abundance and gradually increased with age (354) but then decreased at PN 130 (Figure 4.4). P-pka however developed differently than the other proteins as P-pka reported the highest abundance at PN 21. P-pka demonstrated typical decreases in phosphorylation with aging (355) (Figure 4.5). Since Pka is phosphorylated most notably by catecholamines and P-pka is elevated in PNGR mice, this result could indicate elevated levels of catecholamines in response to early life growth restriction as has been reported in fetal growth restriction and premature offspring (152,356–360). Lastly, it is important to note that Pka plays a significant role in cardiac function beyond Pln phosphorylation, including the activation of RyR2, $Ca_v1.2$ and cTnI to modulate Ca^{2+} dynamics (361) highlighting its complexity and importance in development and across the lifespan.

4.5.1 Limitations

I did not measure NCX in the current study as NCX is only responsible for ~7-10% of Ca^{2+} reuptake (while Serca2 sequesters ~90-92% of Ca^{2+}) in mice (338). Since the PNGR female mice experience impaired relaxation and Serca2 is reduced, the current study suggests NCX is not able to compensate for the loss of Serca2 in the PNGR female mice (338). The immunoblot data provide

evidence for protein abundance in a tissue but does not elucidate a gene issue (55). Therefore, the current results provide direction for future research into the developmental disruption of Serca2.

4.5.2 Conclusion

In conclusion, this study shows postnatal growth restriction reduces Serca2 protein abundance in female mice without any recuperation in adulthood. Given the primary role of Serca2 in cardiac relaxation, a reduction in Serca2 is likely one mechanism for impaired relaxation and possibly diastolic dysfunction in the PNGR female hearts. Despite no differences in key modulators of Serca2, the reduction in Serca2 is likely due to a lower gene expression or a disruption in protein translation during SR development, which is also a result of more immature cardiomyocytes in the PNGR heart. Thus, the results from the current study suggest future research to target the restoration of Serca2 protein abundance and/or ameliorate its efficiency to normalize Ca^{2+} reuptake and mitigate cardiac dysfunction in the PNGR female mice.

Figures

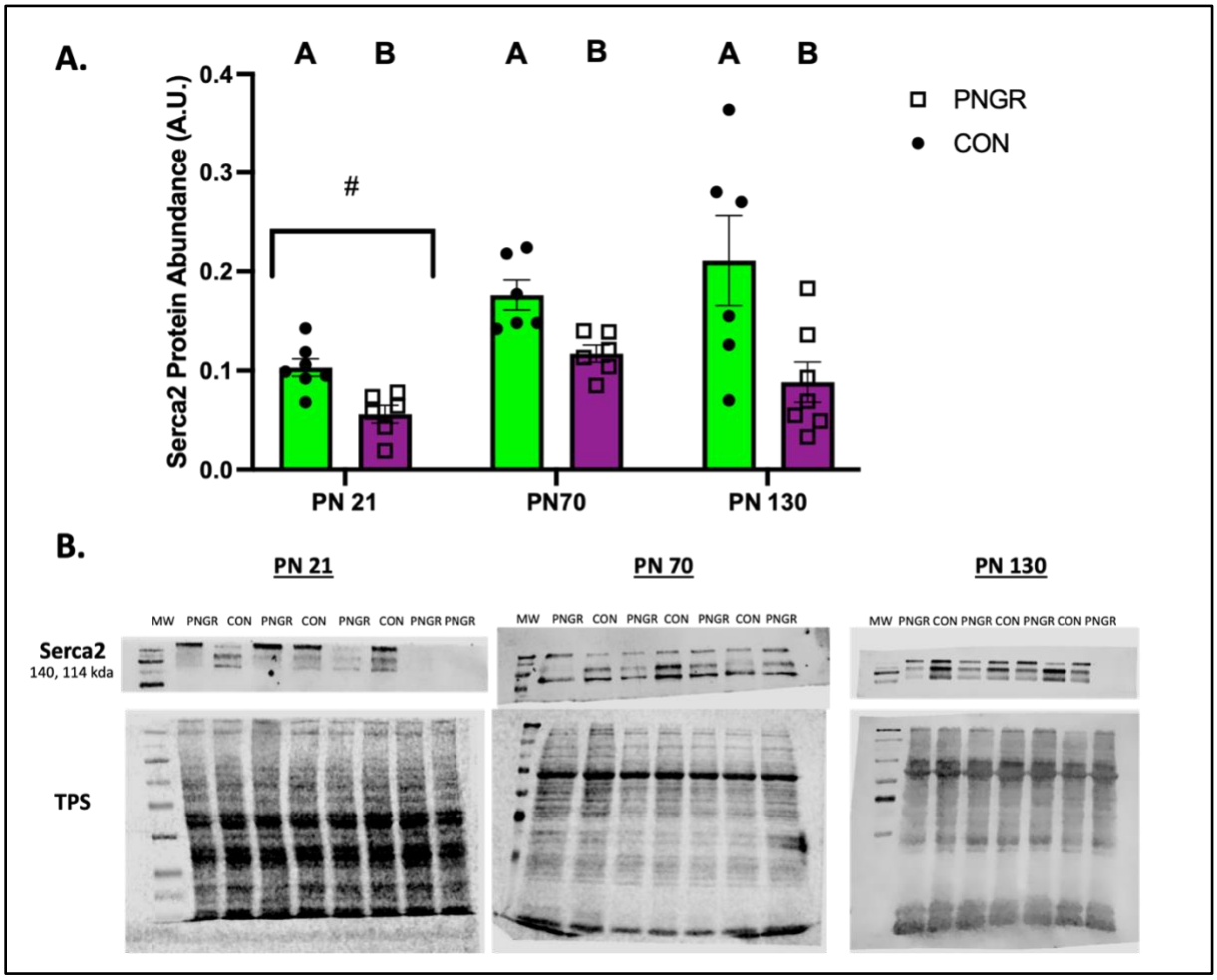

Figure 4.1 *Sarcoendoplasmic Reticulum ATPase 2 protein abundance*
A) Serca2 was reduced in all PNGR mice as compared to CON mice (p=0.0005). PN 21 mice had the lowest abundance of Serca2 proteins as compared to PN 70 and PN130 (p=0.0146). Serca2 also trended down in 130-day old PNGR mice as compared to 130-day old CON mice (p=0.0810). **B)** Representative immunoblot data for each time point. Data were analyzed with two-way factorial ANOVA (diet x age) followed by a Tukey's HSD Post Hoc test if variables were significant. Data are presented as mean ± SEM. The interaction effect was set at $p<0.05$, the diet effect between groups ($p<0.05$) is denoted by differing letters above each bar and age effect is denoted by a bracket with a '#' over the PN 21 group. Only female mice were studied, PN 21 PNGR n=7; CON n=7; PN 70 PNGR n=6; CON n=6 and PN 130 PNGR n=7; CON n=6. Postnatal growth restriction, PNGR, control, CON, postnatal day, PN, sarco-endoplasmic reticulum ATPase 2, Serca2.

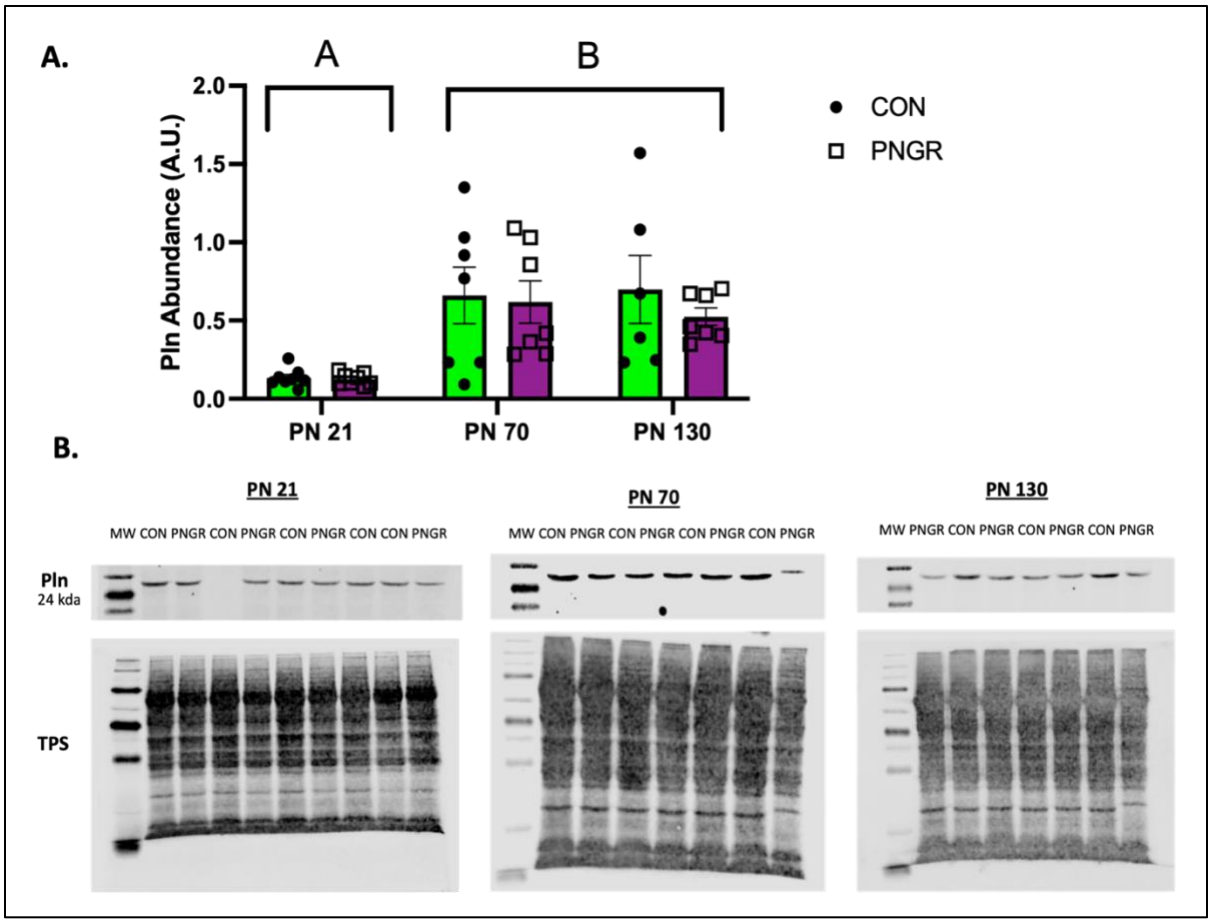

Figure 4.2 *Phospholamban protein abundance*
A) There was no diet effect on Pln levels at any time point. Pln was the lowest in PN 21 and significantly increased with age but PN70 and PN130 were not different ($p<0.0001$). **B)** Representative immunoblot data for Pln at each time point. Data were analyzed with two-way factorial ANOVA (diet x age) followed by a Tukey's HSD Post Hoc test if variables were significant. Data is presented as mean ± SEM. Age effect was denoted by bars lacking a common letter. Only female mice were studied, PN 21 PNGR n=7; CON n=7; PN 70 PNGR n=6; CON n=6 and PN 130 PNGR n=6; CON n=6. Postnatal growth restriction, PNGR, control, CON, postnatal day, PN, phospholaban, Pln.

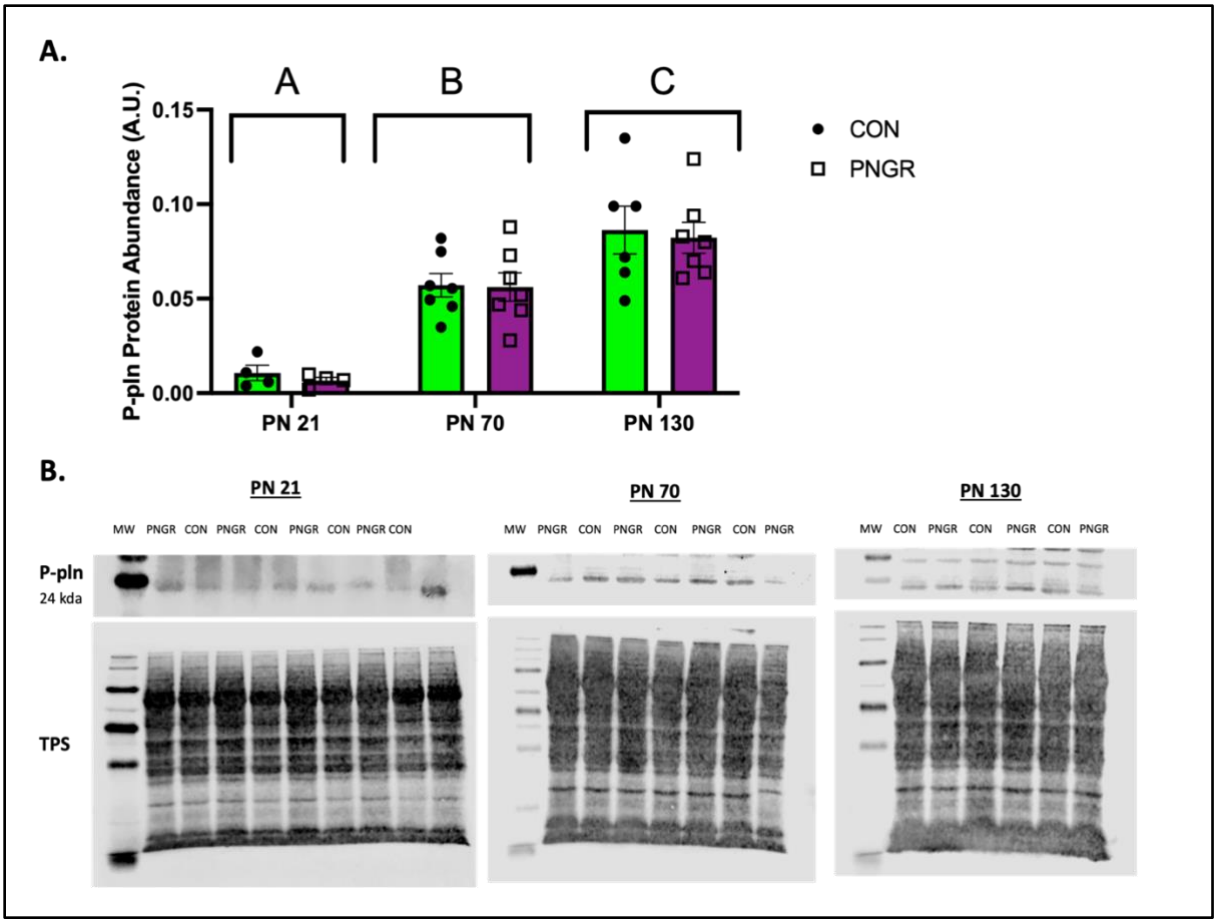

Figure 4.3 *Phosphorylated phospholamban protein abundance*
A) P-pln was not affected by PNGR in the female mice ($p>0.05$). Again PN 21 had the lowest abundance of P-pln that significantly increased at PN 70 and again at PN 130 ($p<0.0001$). **B)** Representative immunoblot data for P-pln at each time point. Data were analyzed with two-way factorial ANOVA (diet x age) followed by a Tukey's HSD Post Hoc test if variables were significant. Data are presented as mean ± SEM. Age effect was denoted by bars lacking a common letter. Only female mice were studied, PN 21 PNGR n=7; CON n=7; PN 70 PNGR n=6; CON n=6 and PN 130 PNGR n=6; CON n=6. Postnatal growth restriction, PNGR, control, CON, postnatal day, PN, phosphorylated phospholamban, P-pln.

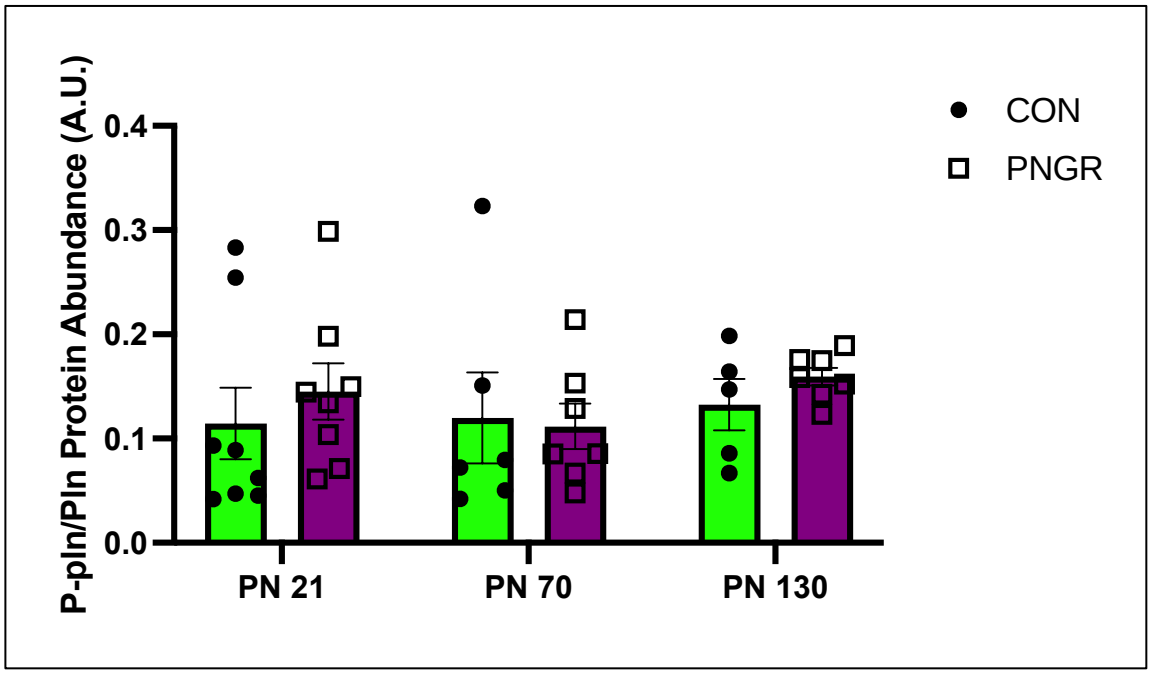

Figure 4.4 *Ratio of P-pln to Pln protein abundance*
After normalization with respective TPS, P-pln was calculated as a ratio of Pln but no diet or age effects were evident ($p>0.05$). Data were analyzed with two-way factorial ANOVA (diet x age) followed by a Tukey's HSD Post Hoc test if variables were significant. Data are presented as mean ± SEM. Age effect was denoted by bars lacking a common letter. Only female mice were studied, PN 21 PNGR n=7; CON n=7; PN 70 PNGR n=6; CON n=6 and PN 130 PNGR n=6; CON n=6. Postnatal growth restriction, PNGR, control, CON, postnatal day, PN, phospholaban, Pln, phosphorylated phospholamban, P-pln.

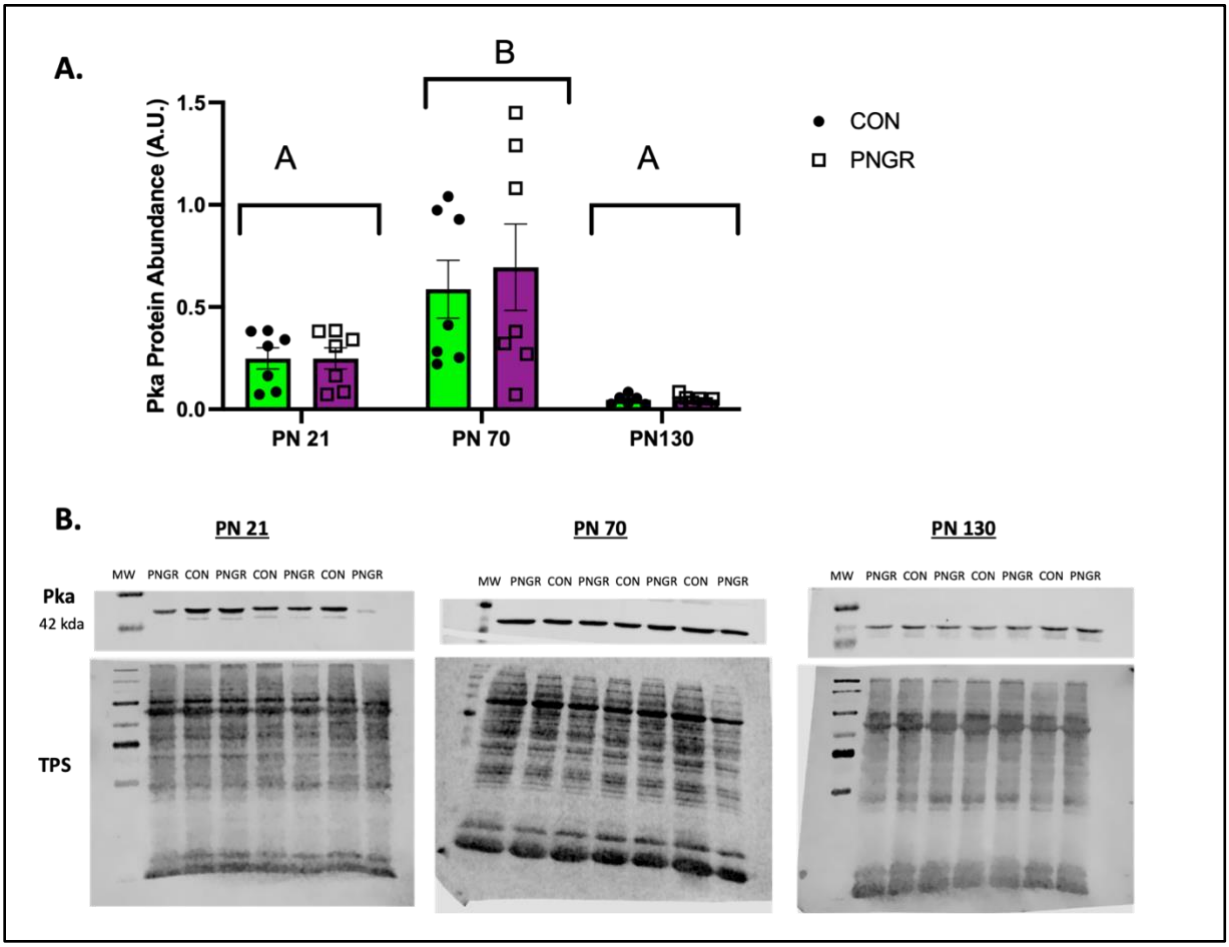

Figure 4.5 *Protein Kinase A protein abundance*
A) There was no diet effect on Pka levels at any time point ($p>0.05$). Pka was the lowest in PN 21 and significantly increased with age to PN 70 but decreased again at PN 130 ($p<0.0001$). **C)** Representative immunoblot data for Pka at each time point. Data were analyzed with two-way factorial ANOVA (diet x age) followed by a Tukey's HSD Post Hoc test if variables were significant. Data are presented as mean ± SEM. Age effect was denoted by bars lacking a common letter. Only female mice were studied, PN 21 PNGR n=7; CON n=7; PN 70 PNGR n=6; CON n=6 and PN 130 PNGR n=6; CON n=6. Postnatal growth restriction, PNGR, control, CON, postnatal day, PN, protein kinase A, Pka.

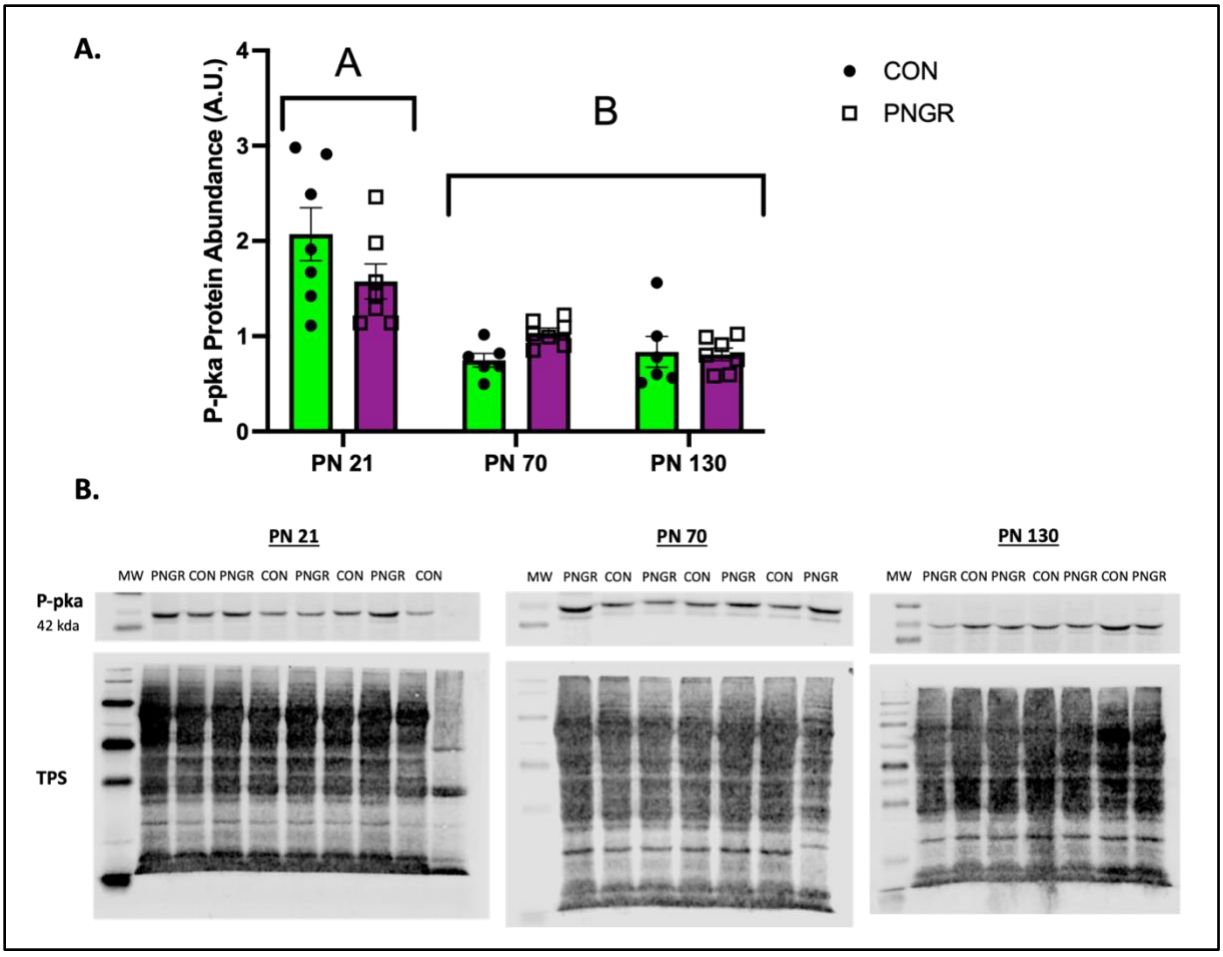

Figure 4.6 *Phosphorylated Protein Kinase A protein abundance*
A) P-pka was not affected by growth restriction in the female mice ($p>0.05$) but PN 21 mice had the highest abundance of P-pka that significantly decreased with aging ($p<0.0001$). **B)** Representative immunoblot data for P-pka at each time point. Data were analyzed with two-way factorial ANOVA (diet x age) followed by a Tukey's HSD Post Hoc test if variables were significant. Data are presented as mean ± SEM. Age effect was denoted by bars lacking a common letter. Only female mice were studied, PN 21 PNGR n=7; CON n=7; PN 70 PNGR n=6; CON n=6 and PN 130 PNGR n=6; CON n=6. Postnatal growth restriction, PNGR, control, CON, postnatal day, PN, phosphorylated protein kinase A, P-pka.

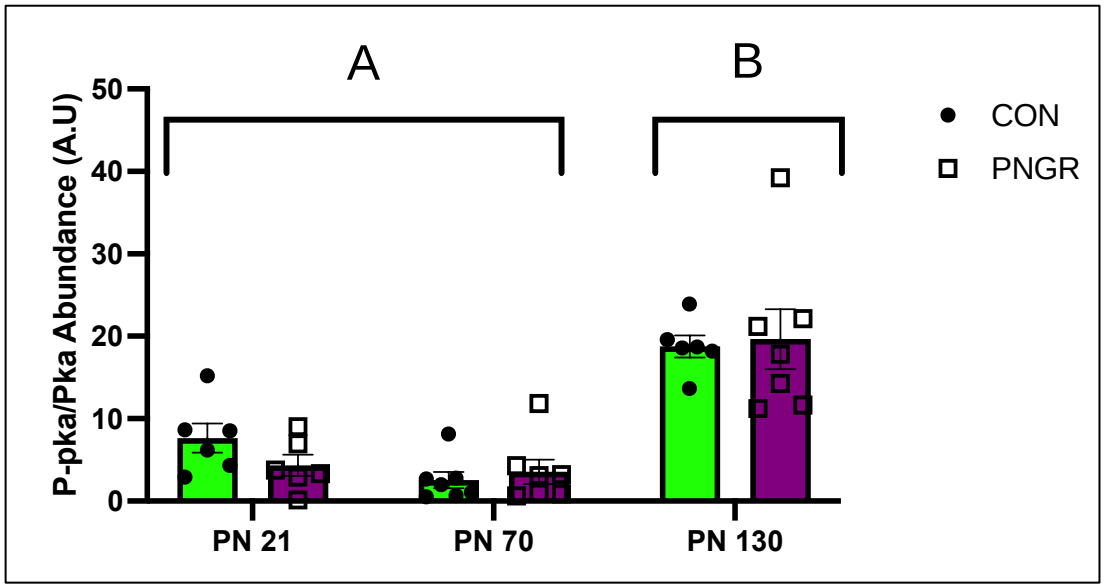

Figure 4.7 *Ratio of P-pka to Pka protein abundance*
After normalization with respective TPS, P-pka was calculated as a ratio of Pka that revealed an age effect where PN 130 mice had the greatest abundance as compared to PN 70 and 21 mice ($p<0.0001$). Data were analyzed with two-way factorial ANOVA (diet x age) followed by a Tukey's HSD Post Hoc test if variables were significant. Data are presented as mean ± SEM. Age effect was denoted by bars lacking a common letter. Only female mice were studied, PN 21 PNGR n=7; CON n=7; PN 70 PNGR n=6; CON n=6 and PN 130 PNGR n=6; CON n=6. Postnatal growth restriction, PNGR, control, CON, postnatal day, PN, protein kinase A, Pka, phosphorylated protein kinase A, P-pka.

CHAPTER 5:

THE INFLUENCE OF POSTNATAL GROWTH RESTRICTION ON CARDIAC FIBROSIS IN FEMALE MICE

5.1 Abstract

Introduction: Postnatal growth restriction leads to an increased risk of CVD in adulthood. Postnatally growth restricted female mice present with increased cardiac stiffness that suggests cardiac fibrosis, which often manifests as remodeling with increased collagen deposition through elevated AngII and ET-1. The current investigation aimed to 1) determine the presence of collagen in cardiac tissue and 2) determine if there is upregulation of ET-1. **Methods:** To induce postnatal growth-restriction, dams were fed either a 20% normal protein diet or 8% low-protein isocaloric diet 2 weeks before mating. Pups born to normal protein fed dams were cross-fostered to either a low-protein fed dam (PNGR) or a different normal protein fed dam (CON) and weaned on PN day 21. Mice were euthanized and heart tissue was collected at PN 21, 70 or 130 to assess ET-1 protein abundance via immunoblot, and a second heart was collected at PN 130 for histology analysis with Masson's Trichrome. Immunoblot data were analyzed with a two-way ANOVA (diet x age) and the histology data were analyzed with a Student's T-test (CON vs. PNGR), both with an alpha level of $p \leq 0.05$. **Results:** ET-1 protein abundance was not different between PNGR and CON mice at PN 21, 70, or 130 ($p>0.05$). Masson's Trichrome staining revealed no differences in collagen, myocardium, or interstitial space percentages between CON and PNGR groups ($p>0.05$). **Conclusion:** Together the results indicate cardiac fibrosis is not the cause of impaired relaxation and is supported by no changes in ET-1, leaving the pathology for cardiac stiffness still undetermined. Future research is needed to determine the cause of impaired relaxation in PNGR mice.

Keywords: postnatal growth restriction, female, DOHaD, fibrosis, endothelin 1

5.2 Introduction

Globally, 160 million children under the age of five experience early life growth restriction due to an inability to acquire adequate nutrition (2). Growth restriction is associated with an increased incidence of chronic diseases including CVD, type II diabetes, hypertension, and sarcopenia (11,14,27). Most of the evidence on growth restriction and CVD is collected from human epidemiological studies or animal models of IUGR with or without a combined model of postnatal growth restriction. Animal models of IUGR indicate delayed cardiomyocyte maturation, arterial and vascular stiffness, atherosclerosis, ECM remodeling, metabolic deficiencies, and hypertension (21,22,25,96,107,362,363). While cardiac growth and development also occur in the postnatal period, postnatal growth has not been studied in isolation as frequently as IUGR (271,364–366). Initial evidence from Ferguson et al. reported cardiac stiffness in PNGR female mice measured by echocardiogram as increases in IVRT and isovolumic contraction time (IVCT) and reductions in E/A ratio, whereas males did not experience stiffness. Ferguson also determined increased Ca^{2+} transient amplitude, Ca^{2+} decay rate and spontaneous Ca^{2+} waves and sparks, indicating a disruption in Ca^{2+} reuptake and impaired relaxation (35). Supporting the work of Ferguson et al., our lab determined PNGR female mice have increased IVRT and DT with a reduced EWDR, indicating abnormal filling function of the heart (manuscript currently in review), which in turn increases the risk of heart failure in adulthood (133,134,367). Despite the evidence for stiffness and relaxation abnormalities, it has yet to be confirmed if PNGR female mice develop cardiac fibrosis.

Cardiac fibrosis is an adaptive pathological process of ECM remodeling that eventually leads to abnormalities in matrix components/quality as well as impairments in cardiac function (284). The process of ECM remodeling is initially protective and vital to maintain cardiac function

across the lifespan. Unfortunately, abnormal continuous secretion of fibrotic proteins (i.e., collagen type I) results in stiffening of the myocardium, ultimately leading to worsening cardiac dysfunction (368). There are several complex, multifaceted pathways through which fibrosis is activated, including pro-fibrogenic, inflammatory, and matrix metalloproteinase mediators. Since previous evidence has determined increased renin angiotensin system (RAS) activity in growth restricted mice (369,370), this pathway is a likely candidate for cardiac fibrosis investigation. AngII is the primary effector molecule for the RAS and is mediated in part by ET-1 to elicit cardiac fibrosis. The IUGR literature confirms elevated ET-1 in offspring (192), but has not yet been examined in the PNGR model.

Despite the evidence for cardiac stiffness in the PNGR female mice, limited evidence exists to demonstrate a rate limiting step through which PNGR female mice develop fibrosis (73,195). Thus, the current investigation was performed to determine the presence of collagen type I deposition in cardiac tissue as a marker of cardiac stiffness/fibrosis and a rate limiting step in fibrosis development by examining ET-1 in cardiac tissue from the PNGR female mice. Determination of these mechanisms could help determine interventions to reduce mortality from CVD in PNGR populations. I hypothesized that PNGR female mice would have greater abundance of collagen tissue in the heart in adulthood and an upregulation of ET-1 proteins across the lifespan as compared to healthy controls.

5.3 Methods

5.3.1 Ethical Approval

Protocol number 202100230 has been approved by IACUC at Michigan State University. Animals were housed with wood-chip bedding in the vivarium maintained between 18-21°C with

a 12 h light/dark cycle. Mice were paired with a same sex partner and given access to diet specific food and water *ad libitum.*

5.3.2 Nutritive Model

The same nutritive model from study one and two was used to induce postnatal growth restriction in mice (35). Briefly, one half of the FVB (FVB/N; Charles River Laboratories, Wilmington, MA, USA) mouse dams were fed a normal protein diet (20% protein; #D06041301; Research Diets, New Brunswick, NJ, USA) and the other half of dams were fed a low protein isocaloric diet (8% protein; #D06041302; Research Diets, New Brunswick, NJ, USA) 2 weeks prior to mating. At mating, one male was introduced into a cage with two female mice for only 24 hours ensuring experimental pups were born on the same day. On PN day 1, all pups born from normal protein fed dams were pooled and randomly redistributed to one of two experimental groups. Pups born from LP fed moms were not used in the current investigation. The two groups were CON, where pups born from a normal protein fed dam were cross fostered to a different normal protein fed dam and PNGR where pups born from a normal protein fed dam were cross-fostered to a dam fed the low protein diet. Dams fed the LP diet produce ~20% less milk during lactation and have reduced amino acid availability eliciting postnatal growth restriction in the pups suckling from the LP fed dams (264,272,273). Each litter was standardized to similar body weights and maintained an equal sex ratio of 4 males and 4 females per litter. In the unexpected case of a pup death, litter size was maintained by introducing 'donor' pups of similar age, but donor pups were never studied. On PN day 21, all pups were weaned and placed on the control 20% protein diet. To maintain accordance with ethical guidelines and the 3R's of research, only female mice, who have previously showed impairments (male mice are not compromised (35,39)), were assessed in the present investigation. Male mice were allocated to other studies of skeletal muscle

structure. Mice were weighed on a small animal weighing scale (Ohaus Corporation, Parsippany, NJ; CS Series) every 3 days until PN 35 and then once per week until end of experiment. G*Power was used to calculate the sample size required to achieve a medium effect size with a β of 0.8 and $p<0.05$. For this study the number of litters needed was n=6 litters per age and diet group (CON vs PNGR), requiring a total sample size of 36 mice.

5.3.3 Sample Collection

On PN 21 and 70, one female from each litter, and on PN 130, two females from each litter were euthanized for tissue collection. Two females are needed at PN 130 to prepare histology and immunoblot samples. Before collection, mice were weighed and anesthetized with 2% isoflurane. After ensuring no response, mice were euthanized with cervical dislocation. At PN 21, 70 and 130, the heart was quickly excised and immediately frozen in liquid nitrogen for immunoblot analysis.

The second mouse at PN 130 was anesthetized with 2% isoflurane and injected intraperitoneally (IP) with a 5% hyperkalemic solution to stop the heart in diastole [148g potassium chloride (Sigma Aldrich cat#746436) dissolved in 500mL of deionized H_2O and added to 25mL of Pure 20x PBS (VWR Life Sciences cat#E703-1L)]. After 5 additional minutes under isoflurane, the mouse was euthanized by cervical dislocation. The heart was quickly excised and prepped for Langendorf reperfusion. The heart was canulated through the aorta, attached to the Langendorf apparatus and flushed with the same hyperkalemic solution used for IP injections, for 5 minutes. The flow rate was set to 8 RPM for the peristaltic pump to elicit continuous flow through the canula that does not cause any back flow out of the aorta. Once hearts have been flushed with hyperkalemic solution, the heart was detached from the aortic cannula, placed in a cryomold, and covered with optimal cutting temperature solution (OCT, Sakura, cat# 4583). Once covered, the

cryomold was submerged in a container with liquid nitrogen cooled with 2-methylbutane, to quickly freeze the sample. Once frozen, samples were stored at -80C until staining.

5.3.4 Immunoblotting

Each heart sample was manually homogenized using a tissue homogenizer (Omni THQ Homogenizer, cat# 12-500, Omni, Kennesaw, GA) while submerged in 1mL of C. Norris buffer (336) (0.745g HEPES, 0.135g B-glycerophosphate, 0.014g ATP, 0.75mL protease inhibitor cocktail, 0.489g Benzamidine, 0.044g PMSF, 0.25mL DMSO, 0.186g EDTA, 0.254g $MgCl_2$). Tubes were then centrifuged for 15 minutes at 13,000 x g to separate supernatant and pellet. To determine protein concentration and allow for equal protein loading for SDS-PAGE, a Pierce BCA protein assay (Thermo Fisher Scientific Cat# 23225, Rockford, IL, USA) was used.

Homogenized protein extracts were separated by electrophoresis on SDS polyacrylamide gels (12% gel) and transferred onto nitrocellulose membranes (PALL Life Sciences, Bio Trace NT, #66489, .2um). After transfer, membranes were briefly rinsed with water and stained with a total protein stain (LI-COR Cat# 926-11021) for 5 minutes, followed by imaging for total protein loading control. Following total protein stain imaging, membranes were washed, de-stained, and blocked at room temperature for 1 hour in 5% proteomic grade non-fat dried milk, followed by incubation in primary antibody for endothelin 1 (1:5000, Abcam Cat# ab117757, ET-1) in Intercept Protein-Free Blocking Buffer (LI-COR Cat#927-80001) on a rocker, overnight at 4°C. The next day membranes were washed 3x and incubated with infrared-labelled secondary antibody (800 CW Goat Anti-Rabbit IgG, LI-COR Biosciences Cat# 926-32211, 1:10000) for 1 hour at room temperature. An Odyssey XF Imaging System was used to collect infrared images and Empiria Studio Software v. 1.3 (LI-COR) used to analyze individual bands. Target protein intensities were standardized to their respective total protein stains (276) (AU: arbitrary units).

5.3.5 Masson's Trichrome Staining

Frozen cryomold samples were transported with dry ice to the cryostat room for sectioning. Samples equilibrated to -20C for ~30 minutes inside cryostat chamber. The sample was removed from the mold and attached to the cryostat arm to begin sectioning. Heart samples were sectioned at 8 um thickness with three sections per slide. Two slides were collected per mouse (a total of 6 sections per mouse) to ensure appropriate and consistent depth was achieved to visualize left and right ventricles. All slides were kept at -20C until ready for staining.

Masson's Trichrome (HT15, Sigma Aldrich) is a multi-step staining process that stains collagen fibers blue, myocardium red, and nuclei black. First the sections were brought to room temperature. A pap pen (Abcam, cat# ab2601) was used to draw a circle around each sample on the slides to contain liquid solutions. Reagents were prepped in coupling jars and aligned under a fume hood. The prepped slides were first fixed in 10% formalin for 1 hour and then incubated in Bouin's solution (Sigma Aldrich, cat# HT 101128) at room temperature, overnight. The next day, slides were washed with RO water for 10 minutes to remove excess Bouin's stain and rinsed in distilled water. Slides were then stained in a 1:1 ratio of hematoxylin A and B solution (Sigma Aldrich, cat# HT1079) for 5 minutes. Hematoxylin was removed with a 10-minute wash of running RO water. Slides were then stained with Biebrich Scarlet-Acid Fuchsin solution (Sigma Aldrich, cat# HT15) for 5 minutes followed by a distilled water wash x3. Next, slides were treated with Phosphotungstic/ Phosphomolybdic acid (Sigma Aldrich, cat# HT15) for 10 minutes, and immediately after, stained with Aniline Blue (Sigma Aldrich, cat# HT15). The slides were washed with distilled water x2 and differentiated in 1% acetic acid for 2 minutes. Slides were dehydrated with 95% ethanol for 2 minutes, followed by 100% ethanol for 1 minute and finally cleared in xylenes for 2 minutes. Finally, a mounting based medium (cat #24176-120, Polysciences,

Warrington, PA) was used to finalize slides for imaging. 3 Images were collected per mouse with a Keyence Digital Microscope (VHX-7000S, Itasca, IL). Images were collected at 150x magnification, using auto focus, and setting the brightness to visualize all structures. Digitally stitched images were generated for quantification with MATLAB.

After collecting and uploading the images to MATLAB Version 9.14 (R2023a, MathWorks Inc., Nattick, MA, 2023), a peer-reviewed script (371–373) was used to quantify the myocardial volume in each image and calculated percentage of myocardial tissue (red/pink), collagen (blue) and interstitial space (white) from each image. The script allows the threshold to be set for each image by selecting the colors white, red/pink, and blue, in each image (threshold is individualized for each image). Lastly, the region of interest (ROI) was manually selected. The output was reported as a percentage of each color. Each sample is the average of 3 stained and quantified images.

5.3.6 Statistics

In order to maintain accordance with the Guide for the Care and Use of Laboratory Animals only the minimal number of mice were used to achieve significance. As these mice are genetically homologous and not statistically different within litters, litters were used as the statistical unit. All statistical analysis was performed using JMP v14.0 (SAS, Cary, NC) and GraphPad Prism 9.3 (GraphPad Software, San Diego, CA). A Shapiro Wilk's Normality test was used to check the data and any outliers were determined with a Grubbs Outlier test (JMP script Version 2). No outliers were detected but ET-1 failed normality, therefore a log transformation was used before running a two-way ANOVA (main effects diet and age) for the immunoblot data. The alpha level was set at $p \leq 0.05$. If significance was found, a Tukey's HSD post hoc for multiple comparisons was used. The histology data only included 1 time point (PN 130) so an unpaired two-tailed Students' t-test

was used to determine significant differences between diet groups, with an alpha level set a priori $p \leq 0.05$.

5.4 Results

5.4.1 Body Mass

The PNGR mice were smaller than CON mice beginning at PN 4 and remained smaller throughout the study (see Figure 3.2). There was no difference in body mass at birth or PN 1.

5.4.2 Immunoblot

The interaction of Diet x Age was not significant for ET-1 (Figure 5.1A, p=0.5835). Protein expression of ET-1was not different between diet groups at any time point (Figure 5.1A, p=0.9182). There was an age effect, where PN 21 had greater abundance of ET-1 than PN 70 and PN 130 (Figure 5.1A, p< 0.0001).

5.4.3 Histology

Masson's Trichrome staining revealed no differences in collagen (p=0.1729), myocardium (p=0.5544), or interstitial space (p=0.7360) between CON and PNGR mice (Figure 5.2).

5.5 Discussion

The results of this study shown that ET-1 is not upregulated in the adult PNGR female mice and, surprisingly, the PNGR female mice do not have excess collagen as compared to the CON mice, leaving the pathology for cardiac stiffness still undetermined. The role of early life growth restriction and increased risk of chronic diseases in adulthood is well established (15,20,76,120,140,323). Postnatal growth restriction in particular leads to impaired relaxation and reduced diastolic function in females (35,39). Our lab has shown changes in Ca^{2+} flux and echocardiogram measurements that indicate cardiac stiffness in the PNGR females, (35,39) however the underlying mechanism of this pathology remains unclear. As excess collagen

accumulation leading to fibrosis is a common mechanism of cardiac stiffness, this study investigated collagen accumulation in older adulthood PNGR female hearts and ET-1 as a rate-limiting step for collagen deposition through AngII.

The nutritive model used in the present investigation was successful in inducing permanent growth restriction in the PNGR mice as seen in Figure 3.2. In agreement with literature, the CON and PNGR mice were not different at PN 1 (35–37,374) and all mice had similar body mass at birth, indicating no intrauterine growth restriction. However as early as day 4, the growth curves begin to separate, demonstrating the CON mice begin to gain mass and develop more quickly than the PNGR mice. Despite ad libitum access to the control food diet after weaning, the PNGR group remained smaller throughout the lifespan. The current body mass results indicate postnatal growth restriction induces permanent growth stunting as the PNGR mice do not recuperate the lost growth despite re-feeding. This permanent growth stunting suggests irreversible programming occurs during the postnatal development leaving this population susceptible to chronic diseases in adulthood. The heart specifically continues to develop and mature during the postnatal window in mice (equating to 3rd trimester in a fetus (34)) and the literature supports the detrimental effect of postnatal growth restriction on the heart (35–39,296).

The aim of the current study originated from preliminary evidence in our lab and work by Ferguson et al. that suggested cardiac fibrosis as the underlying cause of stiffness in PGNR female mice identified through echocardiogram changes and elevations in AngII (39). Despite success in inducing postnatal growth restriction, the results were surprising as ET-1 was not different between CON and PNGR at any time point (Figure 5.1) and PNGR mice did not have greater abundance of collagen in histology staining (Figure 5.2). These results are informative for the growth restricted field as the two results are in agreement with one another and provide foundational

information for future studies. The current results rule out a fibrotic explanation for cardiac stiffness such that other mechanisms for altered blood flow velocities and impaired relaxation can and should be investigated.

Despite a rejection of the original hypotheses, the ET-1 results are noteworthy as PN 21 mice had the highest levels of ET-1 protein. As ET-1 is an important cardiac growth factor, the higher ET-1 abundance in PN 21 mice as compared to PN 70 and PN 130 is supported by the literature (375–378), and indicates appropriate markers of development are present (for both groups). However, PNGR females experience cardiac dysfunction in adulthood, indicating the PNGR hearts still do not develop appropriately (35–37,375,378). As PNGR female mice are known to have a majority of immature mononucleated cardiomyocytes (35), the current results suggest an alternative marker of development, such as the Salvador-Warts-Hippo (HIPPO) pathway, may be altered during development that leads to impaired relaxation.

The current investigation was designed and conducted with rigor and results are in line with publication standards (379–383). Previous studies in this field have been conducted at PN 70 but I included an older time point (PN 130) to ensure appropriate analysis of aging and cardiac adaptations. A power analysis was conducted a priori and in line with the growth restricted literature (24,35,37,306,384,385), revealing n=6 litters per group was required to reach significance with a medium effect size. Our methods were up to date as we used the infra-red western blot technique and each sample was normalized to their total protein stain, reducing the variability in individual loading controls like GAPDH and beta-Actin (354,386,387). Lastly, I used Masson's Trichrome to quantify collagen tissue. Unlike Picrosirius red staining, Masson's can quantify collagen, myocardium, and the interstitial space to ensure an adequate measure of the

whole heart, rather than just collagen (388,389). Therefore, the study was rigorously designed, and the results provide novel information for the postnatally growth restricted field.

5.5.1 Conclusion

Although one of the most obvious reasons for impaired relaxation is collagen deposition leading to fibrosis, the current results confirm PNGR female mice do not suffer from excess collagen accumulation. The current study ruled out a fibrotic explanation for cardiac stiffness and suggests rather, the previously documented relaxation impairment in PNGR mice is the result of a different mechanism related to the myocardium such as impaired excitation-contraction coupling.

Figures

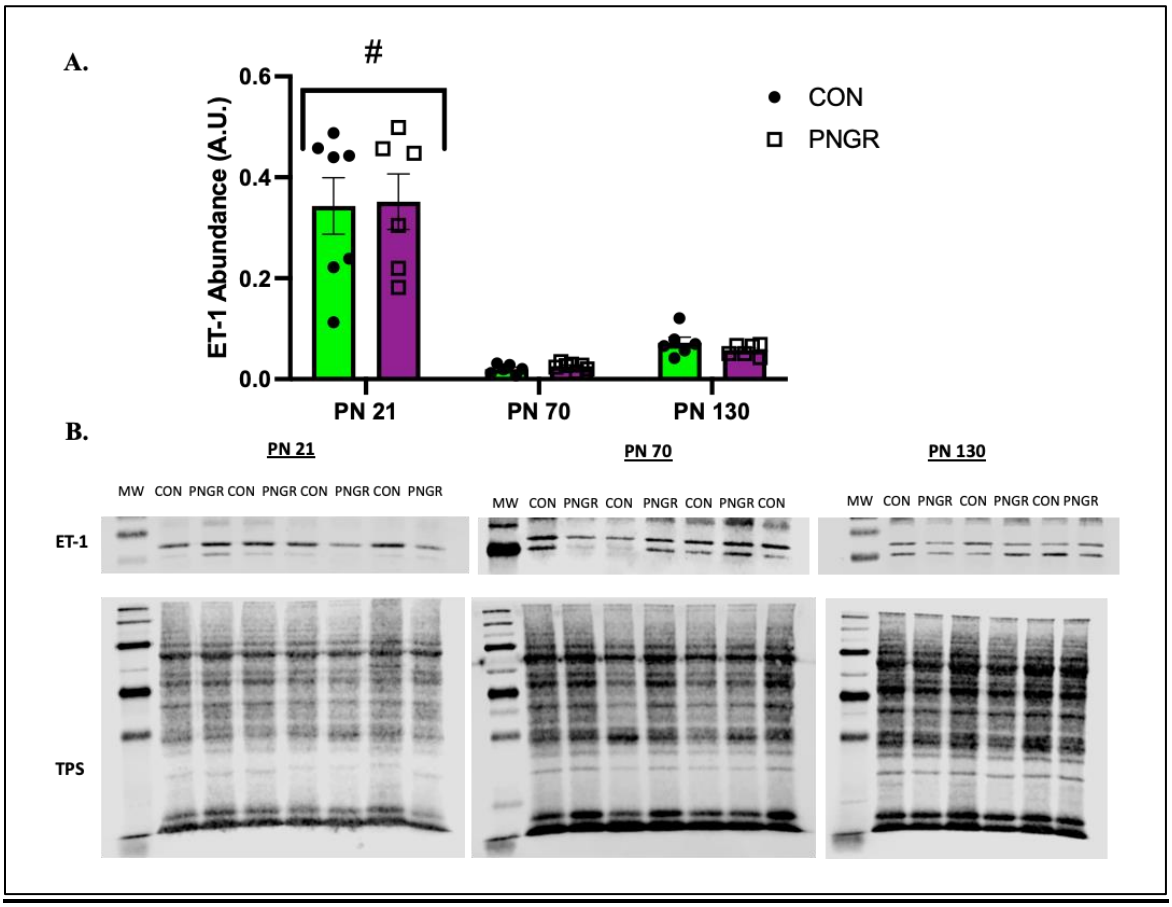

Figure 5.1 *Immunoblot analysis of Endothelin-1*
(A) Representative images of immunoblot analysis at PN 21, PN 70 and PN 130. All bands were normalized to their respective total protein stain (TPS). **(B)** ET-1 levels (~24 kda) were not different between CON and PNGR mouse samples at any time point ($p > 0.05$). The PN 21 mice presented with more ET-1 abundance than PN 70 and PN 130 ($p < 0.0001$). Only female mice were analyzed. Values are reported as mean +/- SEM as arbitrary units, n= 6 CON and n=6 PNGR biologically independent animals.

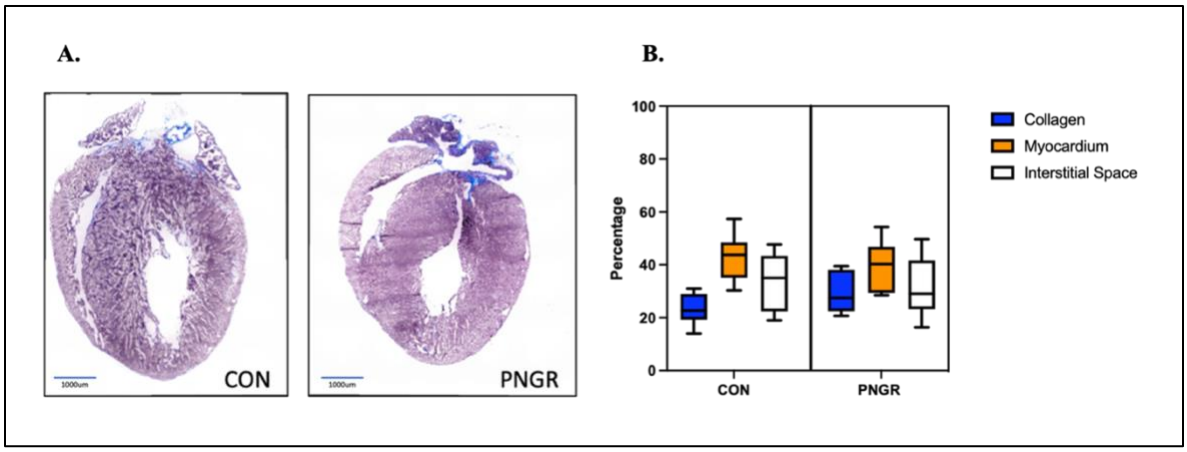

Figure 5.2 *Collagen staining with Masson's Trichrome*
(A) Representative images of Masson's Trichrome stains of the coronal area of the left and right ventricles. Pink indicates myocardium, blue indicates collagen, and white indicates interstitial space. **(B)** Quantitative analysis of Masson's trichrome stained cardiac sections. Percent volume of myocardium (pink), collagen (blue), and interstitial space (white) in CON and PNGR female mouse hearts. There were no differences between CON and PNGR for collagen (p=0.1729), myocardium (p=0.5544), or interstitial space (p=0.7360) percentages. Only female mice were analyzed. Values are presented as percentages and reported as average of 3 samples per mouse, n = 6 CON and n=6 PNGR biologically independent animals.

CHAPTER 6:

SUMMARY AND CONCLUSIONS

6.1 Summary

In summary, the three studies of this dissertation aimed to fill a gap in the literature of the female-specific effects of PNGR in a mouse model. Evidence has shown that those who experience undernutrition in early developmental windows have permanently altered cardiovascular development and increased risk of CVD mortality (15,120,140,390). It has been well documented that PNGR female mice suffer from increased CVD risk as marked by cardiac stiffness, impaired relaxation, and reduced exercise capacity, while male mice do not (35,37,38). Diastolic dysfunction is associated with cardiac stiffness and impaired relaxation and characterized predominantly as a result of one or more of the following three mechanisms: (1) impaired ventricular relaxation, (2) increased ventricular wall thickness, or (3) the accumulation of myocardial interstitial collagen (316,337). However, the mechanisms underlying this dysfunction in PNGR mice are not fully understood. Based upon the available evidence, stiffness and impaired relaxation was hypothesized to be a result of changes in Ca^{2+} handling proteins and cardiac fibrosis by way of altered sex steroids (35). The three studies in this dissertation I was able to successfully achieve the aims and confirm an impairment in Ca^{2+} handling proteins, rule out a fibrotic mechanism for stiffness, and provide initial evidence for the effect of postnatal growth restriction on steroid hormones in PNGR female mice.

In all three studies the mice were growth restricted during the postnatal period through a validated nutritive model. All mice were born from normal protein fed dams and on PN 1 all mice were weighed and cross-fostered to either a different normal protein fed dam or a low protein fed dam. After 21 days all mice were weaned onto the control diet, limiting the growth restricted period to the postnatal window. As evidence demonstrated the female mice are more severely affected, this dissertation only investigated the female mice, focusing on a female-specific mechanism. The

success of our nutritive cross-fostering model is evident by body mass data where PNGR female mice were smaller than CON beginning at PN 4 and remained smaller even after refeeding with the normal protein diet. This result is in line with the literature, confirming postnatal growth restriction. The three studies summarized below used the same nutritive model and various advanced laboratory techniques to answer the research questions.

The NNERL has accumulated evidence that female mice are more severely affected by postnatal growth restriction than males as evident by decreases in cardiac function and reduced exercise capacity (35,36,38). Therefore, the purpose of study one was to examine the effect of PNGR on sex steroids in the female mice to determine a female-specific mechanism. Estradiol is difficult to measure in mouse samples due to their low values, but this dissertation used one of the most sensitive techniques, LC-MS/MS to assess 24 different steroids to examine a holistic view of the hormonal environment. With this technique I was able to detect and determine no differences in T between PNGR and CON female mice and no differences in several of the precursor steroids in the pathway. Only 18-hydroxycortisol was different between PNGR and CON, where PNGR had higher levels. 18-hydrocycortisol is a marker of hypertension and supports the literature that PNGR female mice suffer from high blood pressure. Chronic hypertension can lead to diastolic dysfunction through increased LVH and ventricular stiffness. The additional steroids seen in Figure 3.1 also provided information on the phase of the cycle and suggested female mice were in diestrus and proestrus at the time of sample collection. Both diestrus and proestrus report higher, detectable levels of estradiol when using LC-MS/MS (278). Despite the menstrual phase data, I was not able to detect estradiol in all of the female samples, 65% in CON and 25% in PNGR. The results from study one provide a holistic view of the hormonal environment in PNGR female mice

and new evidence for hypertension in PNGR female mice which suggests an opportunity for intervention.

In study two I investigated the effect of PNGR on Ca^{2+} handling proteins, Serca2, Pln, Pka, P-pln and P-pka, as previous work by Ferguson et al determined increased Ca^{2+} transient amplitude indicating a disruption in Ca^{2+} reuptake (35). Study two determined a reduction in Serca2, the key Ca^{2+} regulating protein for contraction and relaxation in the PNGR female mice using infrared immunoblotting. Serca2 was reduced in PNGR at all three time points indicating developmental programming in early life has permanent consequences. Surprisingly there were no changes in the regulating proteins Pln and Pka, suggesting a lower abundance of the Serca2 gene or a disruption in translation from Serca2 RNA to protein. The reduction in Serca2 is also likely the result of the greater number of immature, mononucleated cardiomyocytes in PNGR mice. Study two supports previous evidence of impaired myocardial relaxation and compromised Ca^{2+} transient function and suggests new treatment interventions to increase Serca2 abundance in the PNGR female mice.

Lastly, PNGR female mice experience impaired relaxation and cardiac stiffness which suggests diastolic dysfunction. One of the components of diastolic dysfunction is ventricular wall thickness, which can be related to collagen accumulation and/or cardiomyocyte hypertrophy. Ferguson et al reported smaller cross-sectional area for cardiomyocytes in PNGR mice at PN 70 (35), indicating hypertrophy is not likely responsible for increased wall thickness. This result led to the hypothesis of increased collagen accumulation and cardiac fibrosis in the PNGR female mice. However, in study three I ruled out cardiac fibrosis as a mechanism for stiffness as myocardial collagen accumulation was not higher in the PNGR mice; nor was ET-1 upregulated, an upstream marker of collagen synthesis. The current literature has contrasting reports when comparing wall thickness in growth restricted mice at different ages; At PN 70 PNGR female mice

have reduced wall thickness (35,36,38) while at PN 130 PNGR female mice have increased wall thickness (manuscript currently in review). These results indicate the adaptations of the cardiovascular system through aging can be influenced by different mechanisms. Specifically, the results from study three determined impaired relaxation in PNGR female mice is not from cardiac fibrosis but rather the dysfunction is likely caused by the alterations in Ca^{2+} homeostasis reported in study two and increased hypertension from study one.

6.1.1 Future Research

Despite filling a gap in the literature and confirming altered Ca^{2+} homeostasis in the PNGR females, this dissertation also recommends future research to focus on interventions to mitigate CVD in this population. The rate of Ca^{2+} decline in the cytosol (169,391) specifically stands out as an area of focus as it is primarily controlled by Serca2 and Serca2 is reduced in PNGR female mice hearts. A reduction in Serca2 causes impaired rate of Ca^{2+} decline in the cytosol and altered Ca^{2+} homeostasis which leads to prolonged relaxation (337,345,392–394). Future investigations to increase Serca2 abundance via gene transfer therapy (currently under clinical investigation) or to increase the affinity of Serca2 for Ca^{2+} in PNGR female mice will help to reduce cardiac dysfunction and mortality in this population.

Secondly, this dissertation revealed maternal protein malnutrition during lactation had no effect on T levels in female mice, increased 18-hydroxycortisol while the effect on E2 remains to be clarified. However, one of the precursory steroids, 17-hydroxyprogesterone, tended to be lower in PNGR mice which suggests a disruption in one of the steroidogenic pathways from figure 3.1 that requires further investigation. Lastly, elevated 18-hydroxycortisol suggests a disruption in the adrenal cortex as this steroid is a marker of hypertension. Hypertension has been reported in the PNGR literature and may be contributing to cardiac dysfunction in PNGR female mice as

hypertension is associated with increased wall thickness and cardiac stiffness. Future research should investigate interventions to mitigate chronic hypertension in PNGR females to reduce LVH and CVD.

6.2 Conclusions

In conclusion, this dissertation successfully achieved the aims and filled existing gaps in the literature and suggests future research to implement therapeutic interventions for the PNGR population. In agreement with the DOHaD literature, this dissertation confirmed postnatal growth restriction causes alterations in cardiac development that permanently alters cardiovascular function in adult aged female mice. Specifically, my novel findings were PNGR female mice have a lower abundance of Serca2 proteins and PNGR mice do not have cardiac fibrosis, which together suggests a mechanism for altered Ca^{2+} transients and impaired relaxation that is reported in the PNGR females. Additionally, this dissertation determined the PNGR mice have an increase in 18-hydroxycortisol, a disease marker of hypertension. This novel finding is foundational for future research into the broader mouse hormonal environment and adds evidence to support hypertension in the PNGR female mice. Therefore, we confirm that growth restriction during the postnatal window reduces Serca2 protein abundance and causes hypertension which will likely lead to diastolic dysfunction in the female mouse and recommend future research is focused on interventions to improve Ca^{2+} reuptake and reduce hypertension to alleviate cardiac dysfunction and decrease mortality in this population.

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APPENDIX A: IMMUNOBLOT PROTOCOL

Create Running Gel

1. Different percentages can be made depending on the target protein.
2. Once mixed, use 1000uL pipette tip to our gel solution into gel caster, fill a little more than ¾ of the plate surface (level with bottom of the green bar)
3. If gel is wavy, use ethanol to even out gel (1-2mL)
4. Gel hardens in approximately 15-20 minutes
5. After hardened, dump excess ethanol, rinse with water, and let dry
6. Mix up stacking gel and use 1000uL pipette tip to our gel solution into gel caster

Create Gel Wells

1. 10 well green combs are used (should match gel thickness)
2. Gently and neatly push combs into gel caster
3. Wells will harden and comb can be carefully removed
4. Gels can be stored overnight I Di water for use the next day
4. Wash gels with Di water & wells before loading samples

Sample Prep

1. **Perform dilution curve to determine correct protein concentration**
    a. Let sample thaw on ice.
    b. Each well will receive 20 ul of fluid which will contain sample, water, and 7.5 ul of 4x sample buffer.
    c. Adjust sample volume and water based on Bradford (for Dangott lysis buffer) or BCA (for C. Norris buffer).
    d. Typically add 3-5 ug of protein.
    e. Vortex samples
2. Incubate at 90-95 C for ~7 minutes
    a. Mini-spin samples for 30 seconds at 5.0 rcf
    b. can freeze at –20 after this if prep the night before
    c. Thaw next morning & centrifuge before loading

Electrophoresis Set-up

3. Secure the gel plates (tall glass facing you) into the electrophoresis chamber with
4. Fill buffer chamber (in front and behind gel) with 1x electrobuffer
    a. (diluted from 10x buffer kept in fridge, MAKE FRESH) *should be cold*
5. fill sample wells with 20 ul each
    c. Be careful as you load, do not blow sample into next lane
    d. Use special loading tips if available (20 uL in each well)
    e. 4ul of ladder loaded in well 1
    f. Leave first well and last well blank
6. Samples go in lanes 2-8
7. Connect buffer chamber to voltage system, set at 70V, constant voltage (V) for 10-20 minutes to move through stacking gel, then increase to 80 V ad 90V, until orange protein marker line reaches bottom of gel plates

a. ~90-120 minutes
b. Does not need to be cold

Protein Transfer

1. Cut low-fluorescent PVDF membrane to match roughly same size as gels
    a. Place in Tupperware and soak in methanol for 2 minutes
    b. If using nitrocellulose membranes, they do not need to be activated with methanol, ca go directly into buffer
2. Soak the membranes, filter paper and transfer cassettes foam in transfer buffer for 20 minutes ***(in fridge/freezer to keep tris-glycine transfer buffer cold***).
    a. The actual transfer buffer should be made new.
3. Once proteins electrophorese take the glass plates apart gently using the red wedge tool
4. Place glass with gel face down onto filter paper & gently lift glass so gel is on filter paper
    a. Can use silver container to soak membrane, filter and sponges and to move gels to transfer cassette
5. Place the extra filter paper on the sponge under the gel
6. Place membrane on top of gel and gently push any air bubbles/foam out with side of hand.
6. Place 1 filter paper on top and again make sure no air bubbles, the place another sponge.
7. -Sandwich Cassette = black side --> Sponge, Filter Paper, Filter Paper, Gel, Membrane, Filter Paper, , Sponge --> clear side
9. Fill the chamber with (made fresh) **TRANSFER** buffer
10. Put ice block inside transfer chamber to help stay cold
10. Lock the case and place it vertically in the blotting chamber.
    a. place the hinges of the cassette facing downwards
    b. Black to Black & Red to Red
    c. Gel to the black and Membrane to the red electrodes
    d. Place the black part of the cassette to the black electrodes
    e. Place the clear part of the cassette to the red electrodes
12. Leave the chamber connected at 100 V for 60 minutes.
    a. Time and voltage can be optimized for different protein sizes
    b. Place chamber inside container and fill with ice to keep cold

Total Protein Stain

1. After transfer, let membrane dry for 30 minutes on filter paper
    a. You can reuse one big piece of filter paper.
2. Re-wet membrane in methanol ~30 seconds
3. Nitrocellulose does not need to dry after transfer
    a. Rinse with DI water (3 x 20 seconds)
    b. Add 6 mL of 700 channel Total protein stain and rock for 5 minutes
    c. 2x 30 second washes with Licor wash solution
        i. Rinse briefly again with Di water (3x 20 seconds)
4. Log into computer: **NNERL, PW: Ferguson1**

5. Open Image Studio and select NNERL workspace
    e. Image in **700nm channel** & **800nm channel** on Image Studio for 2 minutes each
    f. Click Acquire image.
    g. Then de-stain in 6mL Revert De-stain solution, rocking, for 7 minutes. **(no longer than 10 minutes**)**
    h. Rinse briefly in DI water (3 x 20 seconds) and block immediately

Blocking & Primary Antibody Prep

7. Block for 1 hour in **5% Non-fat dried milk in TBST (25 grams in 500 ml of TBST)**
    a. **Phosphorylated proteins use 5%BSA (made fresh) for blocking**
8. Prepare primary antibody diluent – **5% BSA or 5% NFDM (gentle shake/rock)**
9. Wash membranes 3 x for 5 mins in TBST
10. Add primary antibody according to dilution from manufacturer **1:1000 is typical** (5uL in 5mL)
11. Seal membranes in baggies (remember to label)
12. Place on rocker in mini fridge and leave overnight at 4°C for **18-20 hours**.
    a. Can also take out of fridge & rock for 1 hour at room temperature if low binding affinity

Secondary Primary Prep

10. Remove membranes from fridge. Wash membrane 3x 5 minutes in TBST.
    a. Can increase wash time if lots of binding
11. Prepare secondary diluent (IBB, tween & 10% SDS)
    a. 100ml IBB, 200ul Tween, 10ul of 10% SDS.
    b. If using BSA for block & primary, use BSA for 2ndary
12. Add secondary antibody (Licor anti-rabbit secondary ([1:15,000]0.33ul in 5ml in secondary diluent) --> **1:10,000 = .5ul in 5mL**
13. Seal membranes in baggies (light sensitive*)
14. Rock vigorously in dark for 60 minutes (use tinfoil, black out box, etc to keep light out)
    a. Some can be rocked in secondary for 1.5 hours
15. Wash 3x5 minutes in TBST (still need to keep covered)
16. Wash 2x5 minute additional in 1X TBS
17. Image in **800 channel** (10 mins) & 700nm (2 mins) channel in ImageStudio
    a. Membrane can sit in TBS while imaging other membranes
18. Analyze images in Empiria Studio

1. **BCA Protein Assay [Thermo Scientific Protocol] (Plate reader login: 00000)**
2. Dilute the BSA Standard
3. Prepare BCA Working Reagent
    a. Combing reagents, A+B at a 50:1 ratio
        i. It will be a light green solution
4. Pipette 25 microliters of each standard or sample into a microplate well
5. Pipette 200 microliters of BCA working reagent into same wells

6. Gently mix the components for 30 seconds
7. Incubate the plate at 37 degrees for 30 minutes
8. Cool the plate at room temperature for 5 minutes
9. Put into the plate reader and measure the absorbance at or near 562nm

SOLUTIONS/BUFFER RECIPES

C Norris Buffer

0.745g HEPES
0.135g B-glycerophosphate
0.014g ATP
0.75mL protease inhibitor cocktail
0.489g Benzamidine
0.044g PMSF
0.25mL DMSO
0.186g EDTA
0.254g $MgCl_2$

SDS Running 10X Buffer [5 L]

Tris [150 g]
SDS [25 g]
Glycine [720 g]

10X Transfer Buffer [1 L]

Tris [24.7 g]
Glycine [112.6 g]
Do not pH the transfer buffer

1X Transfer Buffer [1 L]

10X Transfer Buffer [100 mL]
Methanol [200 mL]
Dionized water [700mL]

Tris-Saline [1 L]

1M Tris, pH 7.3 [5 mL]
NaCl [8.77 g]

Tris-Saline 0.1% Triton [2 L]

1M Tris, pH 7.2 [5 mL]
1M Tris, pH 7.4 [5 mL]
5M NaCl [60 mL]
Triton X-100 [2 mL]

1X TBS-T [1 L]

TBS [667 mL]
Tween-20 [333 mL]

30% Acrylamide/Bis-Acrylamide [500 mL]
Acrylamide [150 g]
Bis-acrylamide [4 g]

10% [w/v] Ammonium Persulfate [500 mL]
Ammonium persulfate [50 g]
Make 50 mL aliquots. Store at -20°C.

APPENDIX B: HEART HISTOLOGY COLLECTION, FREEZING, & SECTIONING

Preparation

1. Run ultrapure water through Langendorff for 10 minutes, remove all bubbles from circulation using valves on upper mechanism
2. Fill syringe with 3mL 5% hyperkalemia solution and attach mouse cannula
3. Loosely tie silk suture around the cannula, leaving room to slide whole knot over aorta after cannulation
4. Fill two petri dishes with ultrapure water

Collection

1. Place mouse in isoflurane for 2 minutes until mouse is sedated. Inject 1 mL of 5% hyperkalemic solution intraperitoneally per mouse, and return to isoflurane for 2-3 minutes.
2. Use cervical dislocation by holding the bottom of the skull and pulling the tail while the mouse is in prone position.
3. Cut open the thoracic cavity of the mouse, starting at the lower abdomen and working your way up to the neck.
4. Remove the heart by lifting at the lungs, and cutting the aorta and vena cava posterior the heart. Be sure not to touch the heart during this process.
5. Place the heart in one of the dishes of water and locate the aorta. You can do this by trimming away fat and extra lung tissue until proper visualization. If the buffer in the plate gets too foggy, transfer heart to the other plate for clarity.
6. Use the tweezers to insert the cannula into the aorta, and secure with sutures. Secure by tying a tight overhand knot.
7. Gently press the buffer through the heart to clear of blood. Check to make sure that the buffer is going through the heart properly. Watch for unwanted bubbles and buffer going through the aorta. If this is the case, you probably have a hole in the aorta and need to start again (Keep in mind this is all time sensitive).
8. Transfer cannula to Langendorf, and perfuse heart at a rate of 8rpm using 5% hyperkalemic solution for 5 minutes
9. After flushing the heart, prepare the histology area

Freezing Heart Samples

10. Prepare your histology area, by taking a tin can and filling it 2-methylbutane (in the flammables cabinet) to about a quarter high. Grasp this can with the lab clamp, and submerge it slowly into your jar of liquid nitrogen, which can be obtained from the BMB research store.
11. Prep cyo-mold by filling up half-way with OTC
12. Remove the cannula from the heart gently and align in cryo-mold base at top and apex at bottom
13. Label the cyro-mold with which muscle it is, the mouse number, and any other identification that is needed to properly know what it is just by looking.
14. Cover the heart in OTC, making sure to cover it entirely (no bubbles), as well as fill the mold.
15. Grip the cyro-mold with long tweezers, and dip directly into the 2-methyl butane until the OTC has properly cooled and is opaque.

16. Wrap the cyro-mold in tin foil (labeled with mouse number to allow for easy identification), and place directly into the liquid nitrogen.
17. Store at –80C until histology staining

Supplies needed:
2-methylbutane, liquid nitrogen, cryo-stat molds, OTC, long tweezers, tin-can set-up, tin foil

Frozen Heart Sectioning (must obtain access to cryostat in Plant Bio or Pharm Tox)

1. Equilibrate the heart molds, paint brushes, and blades to -20C by sitting in cryostat chamber for 20-30 minutes
2. Set microtome to 8um
3. Slice sections of the heart to get to middle
4. Once in the middle of the heart and both ventricles are visible begin collecting 8um thick section on the slide
5. Each slide will have 3 sections, each mouse should have 2 slides for a total of 6 sections per mouse
6. Keep slides stored at -20 until ready to stain

Masson's Trichrome Staining Protocol

Prep:

1. Make working solution of phosphotungstic/ phosphomolybdic acid solution
    a. Mix 1 volume of phosphotungstic acid & 1 volume of phosphomolybdic acid with 2 volumes of Di water (discard after 1 use)
2. Make working Weigert's Iron hemotoxylin solution
    a. Mix equal parts Solution A & Solution B
3. Prepare 1% Acetic Acid
    a. Dilute 8.8 mL of 1N acetic acid with 41.2 mL of Di water

Day 1

1. Use pap pen to circle the samples
2. Fix sections in 10% formalin for 1 hour
    a. 100mL H2O + 10mL Formaldahyde
3. Treat sections in Bouin's solution overnight under fume hood at room temperature (~12 hours)

Day 2

4. Wash sections in running RO water for 5-10 minutes to remove yellow color.
    a. Could use shaker/rocker to wash sections
5. Stain with Weigert's iron hematoxylin solution for 5-10 minutes.
6. Rinse the stain with running tap water for 5-10 minutes.
7. Rinse in distilled water.
8. Stain with the Beibrich-Scarlet Acid Fuschin solution for 5-10 minutes.
9. Rinse in distilled water.

10. Place sections in the phosphomolybdic-phosphotungstic acid solution for 5-10 minutes
    a. or until the collagen loses its red color.
11. NO WASH... transfer the stained section to aniline blue solution and stain for 5-10 minutes.
12. Rinse the stained section briefly in distilled water and differentiate with 1% acetic acid solution for 2-5 minutes. (discard solution)
13. Wash in distilled water.
14. Quickly dehydrate through 95% ethyl alcohol.
15. Clear in xylene.
16. Mount with a mounting medium.
    a. Place medium on cover slip & then place slide upside down on cover slip
    b. Helps to prevent bubbles

Day 3

1. Log into Keyence digital microscope (no password)
2. Set lighting & focus before saving image
3. Upload images to ImageJ to calculate blue area as percentage of red
4. Can use MATLAB as well to calculate percentages
    1. Find script on OneDrive in folder labeled: MATLAB Histology

APPENDIX C: SERUM COLLECTION PROTOCOL

Prep:

Label & autoclave tubes (x2)

- 1 set of 2mL tubes
- 1 set of 1mL tubes

70% ethanol to clean stir rod between samples

Procedure:

1. Sacrifice mice according to protocol
    a. Should collect heart first (this will help blood pool in chest cavity)
2. Use small disposable pipette to suck up blood *quickly* can do many small pipettes to get as much blood as possible
    a. Ideally ~200ul of whole blood per mouse
    b. PN 21 will need 2 mice from same litter
3. Once blood is in tube, sit in tube rack for 30-45 minutes to allow to clot (usually can get 2-3 done in 45-minute window so all have been sitting for about >30 minutes)
4. Before centrifuge, you need to disrupt clots from wall.
    a. Roughly swirl glass rod around tubes wall (both directions) and make sure all blood is free from the wall
5. Then centrifuge tubes at room temperature for 15 minutes at 2000xg
6. Finally, aliquot serum from tube into new clean 1mL autoclaved labeled tubes
    a. Use old 100uL pipette, **be very careful** not to get any blood into pipette, just serum***
    b. Will be pinkish in color on top of plasma
    c. Can discard plasma left in tube
7. Store all samples at –80 for future analysis