Improving Fetal Health Outcomes: Investigating the Impact of Gestational Obesity and NAD+ Supplementation on Cardiometabolic Health of Offspring

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A thesis submitted to the University of Ottawa in partial fulfillment of the requirement of the master's degree of Science in Cellular and Molecular Medicine

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List of Abbreviations

ACC – Animal Care Committee

ADP – Adenosine Diphosphate

ATP – Adenosine Triphosphate

BAT – Brown Adipose Tissue

BDNF – Brain-Derived Neurotrophic Factor

BMI – Body Mass Index

BSA – Bovine Serum Albumin

cAMP – Cyclic Adenosine Monophosphate

CLAMS – Comprehensive Lab Animal Monitoring System

CNTRL – Control

COPD – Chronic Obstructive Pulmonary Disease

CRP – C-Reactive Protein

CTB – Cytotrophoblast

DNA – Deoxyribonucleic Acid

DOHaD – Developmental Origins of Health and Disease

DMSO – Dimethyl Sulfoxide

ETC – Electron Transport Chain

EVTs – Extravillous Trophoblasts

FFA – Free Fatty Acids

gWAT – Gonadal White Adipose Tissue

GP – Gastrocnemius-Plantaris

GPS – Gastrocnemius–Plantaris–Soleus complex

H&E – Hematoxylin and Eosin

HFHS – High-Fat High-Sugar (diet)

HSL – Hormone-Sensitive Lipase

iWAT – Inguinal White Adipose Tissue

IKK – IκB Kinase

IL – Interleukin

JAK/STAT – Janus Kinase / Signal Transducer and Activator of Transcription

JNK – c-Jun N-terminal Kinase

LGA – Large for Gestational Age

LPL – Lipoprotein Lipase

mRNA – Messenger Ribonucleic Acid

MVM – Microvillous Membrane

NAD^+ – Nicotinamide Adenine Dinucleotide

$NADP^+$ – Nicotinamide Adenine Dinucleotide Phosphate

NA – Nicotinic Acid

NAM – Nicotinamide

NE – Norepinephrine

NF-κB – Nuclear Factor Kappa-light-chain-enhancer of Activated B cells

NMN – Nicotinamide Mononucleotide

NR – Nicotinamide Riboside

OXPHOS – Oxidative Phosphorylation

OCT – Optimal Cutting Temperature (compound)

PAR – Poly-ADP Ribose

PARP – Poly-ADP Ribose Polymerase

PARylation – Poly(ADP-ribosyl)ation

PFA – Paraformaldehyde

PlGF – Placental Growth Factor

PKR – Protein Kinase R

RER – Respiratory Exchange Ratio

ROS – Reactive Oxygen Species

SCT – Syncytiotrophoblast

SD – Standard Deviation

SGA – Small for Gestational Age

SIRT – Sirtuin

SOCS-3 - Cytokine signaling 3

sFlt-1 – Soluble fms-like Tyrosine Kinase-1

TA – Transversus Abdominis

TAC – Thesis Advisory Committee

TG – Triglyceride

TNF-α – Tumor Necrosis Factor Alpha

VO_2 – Oxygen Consumption

11β-HSD1 - 11β-hydroxysteroid dehydrogenase 1

Abstract

Introduction: Gestational obesity is an increasing concern in North America, associated with adverse pregnancy outcomes and long-term health risks in offspring. Chronic low-grade inflammation and placental dysfunction are thought to mediate these effects, partly through depletion of nicotinamide adenine dinucleotide (NAD^+), a vital coenzyme in cellular metabolism and mitochondrial function.

Objective: This study aimed to investigate how maternal obesity affects offspring cardiometabolic health and to assess whether supplementation with the NAD^+ precursor nicotinamide riboside (NR) during pregnancy and lactation can mitigate these effects.

Methods: Female C57BL/6N mice were fed either a control (CNTRL) or high-fat high-sugar diet for 15 weeks prior to and during pregnancy and lactation. During gestation, mice were either treated with water (CNTRL H_2O = 20, HFHS H_2O = 7) or NR (400 mg/kg/day: CNTRL NR = 16, HFHS NR = 7). Offspring were weaned onto a standard chow diet and assessed for cardiometabolic outcomes between 12 and 47 weeks of age, including glucose tolerance, fat distribution, organ histology, and lipolysis response.

Results: Offspring from HFHS-exposed dams showed early-onset weight gain, elevated fasted blood glucose (specifically in females), and impaired lipolysis in adipose tissues. No changes were observed in blood pressure through to adulthood. NR supplementation mitigated early weight gain and improved glucose handling in a sex-specific manner but did not rescue lipolytic defects or prevent long-term fat accumulation.

Conclusion: Gestational obesity leads to sex-specific, long-term metabolic impairments in offspring, potentially mediated by NAD^+ depletion. While NR supplementation during pregnancy provided early metabolic protection, it did not fully prevent the long-term consequences. These findings support the potential of NAD^+ modulation as a therapeutic

strategy but highlight the need for further investigation into timing, dosage, and sex-specific responses.

Keywords: Gestational Obesity, Chronic Inflammation, Fetal Programming, Cardiometabolic Health, Nicotinamide Adenine Dinucleotide (NAD+), Nicotinamide Riboside (NR).

Acknowledgements

I would first like to thank my supervisor, Dr. Shannon Bainbridge, whose guidance was instrumental in the completion of this thesis. As an expert in placental and reproductive health, Dr. Bainbridge has been an incredibly supportive mentor throughout my master's project.

I would also like to acknowledge my Thesis Advisory Committee (TAC), Dr. Barbara Vanderhyden and Dr. Keir Menzies, for their thoughtful feedback and the time they dedicated to my TAC meetings and progress reports. I'm deeply grateful for their insight and encouragement.

This two-year combined BSc/MSc journey would not have been possible without the support of the Placenta Lab and the Menzies Lab at the University of Ottawa. I am especially thankful for the continued encouragement and the many good laughs we shared in the lab. A special thanks to Yusmaris Cariaco and Alexander Green for their help with animal experiments, dissections, and experimental design.

I would also like to thank the following core facilities for their technical support: the Louise Pelletier Imaging Core in the Department of Pathology and Laboratory Medicine for assistance with placental paraffinization, sectioning, and staining; and the Animal Behaviour and Physiology Core for their help with EchoMRI and CLAMS testing.

Finally, to my family, friends, and partner—thank you for putting up with me over the last few years. Your motivational words and unwavering support meant the world to me. I truly couldn't have done this without you.

Authorship Contribution

The principal investigator for the described studies was Dr. Shannon Bainbridge, who was responsible for the conceptualization, funding, and oversight of the study. The Louise Pelletier Imaging Core in the Department of Pathology and Laboratory Medicine at the University of Ottawa was responsible for all paraffinization/ deparaffinization, sectioning, and staining of organ samples. The Animal Behaviour and Physiology Core (BEH core) at the University of Ottawa was responsible for the completion of all EchoMRI testing and CLAMS testing. Yusmaris Cariaco was responsible for assistance with animal dissections. Jade Gamelin Kao was responsible for the development and maintenance of the animal model, all dissections pertaining to the animal model, and all other experiments/analysis pertaining to the animal model. The written thesis was prepared by Jade Gamelin Kao and revised by Dr. Shannon Bainbridge.

Ethics, Animal Care Committee, and Protocol Review:

There was no human recruitment, samples, or data used. Therefore, ethics approval was not required for this study. An animal care committee (ACC) protocol (#3937) was approved for this study with a copy of the protocol included in the Appendix.

Chapter 1 - Introduction

1. Introduction

1.1 Obesity

Obesity is a global health crisis marked by excessive fat accumulation, defined by The World Health Organization as having a body mass index (BMI) of > 30 kg/m [1]. Obesity is a leading risk factor for non-communicable diseases and premature mortality. Its prevalence has surged over the past 5 decades, with one in eight individuals affected globally as of 2022[2]. North America faced particularly high rates, between 25% and 35%[3], contributing to increased incidence of cardiometabolic, cognitive, and psychological disorders.

1.2 Gestational Obesity

Gestational obesity, defined as entering pregnancy with a BMI > 30 kg/m^2, affects over 13% of pregnant individuals in Canada, with nearly half exceeding recommended gestational weight [4]. This condition heightens the risk of complications such as preeclampsia, gestational diabetes, and pre-term birth[5–7]. It also influences fetal programming, referring to permanent changes in structure and/or function of fetal organ systems during critical windows of development. This is evidenced by the increased likelihood of offspring being born large-for-gestational-age (LGA; > 4000g[8] or > 90th birth weight centile[9]) or small for gestational age (SGA; < 2500g[10] or < 10th birthweight centile), both linked to long-term cardiovascular and metabolic complications[11,12 13,14]. The prevailing theory suggests that SGA results from significant abnormalities in placental development[15], while LGA is more commonly linked to a disrupted maternal metabolic phenotype, characterized by elevated levels of glucose and free fatty acids[16]. LGA infants, with more adipose tissue [17], may exhibit elevated fasting glucose and insulin resistance [18], especially if they fail to normalize their growth trajectory [8]. Conversely, SGA infants often undergo rapid catch-up growth in the first two years of life [19], predisposing them to central fat accumulation [20], insulin resistance, and short stature. These distinct patterns underscore the lasting impact of gestational obesity on offspring health.

1.3 Pathophysiology of Obesity in Pregnancy

Gestational obesity disrupts endocrine and metabolic homeostasis during pregnancy [21], contributing to complications such as gestational diabetes, hypertensive disorders of pregnancy, and thromboembolism. Excess adipose tissue leads to dysregulated secretion of cytokines, chemokines, and adipokines. Adipokines are essential for glucose and lipid metabolism, therefore their dysregulation is key to poor metabolic function and cardiovascular disease risk[22]. For example, elevated leptin and decreased adiponectin levels impair insulin signaling and glucose homeostasis [23], promoting early and excessive gestational insulin resistance [24]. These hormonal imbalances, mediated through pathways like JAK/STAT and AMP-kinase[25], increase the risk of hyperglycemia and metabolic dysfunction in both the mother and fetus[25,26].

1.4 Inflammatory Profile in Gestational Obesity

Maternal obesity is associated with a chronic, low-grade systemic inflammatory state that negatively impacts fetal development. Inflammation is largely driven by dysfunctional adipose tissue, which not only secrete adipokines but also become a major source of proinflammatory cytokines such as IL-1β, IL-6, IL-18, and TNF-α [27]. As adipocytes enlarge and become hypertrophic, they experience hypoxia and cellular stress, triggering apoptosis and necrosis and attracting immune cells. This leads to an increased infiltration of macrophages, particularly of the proinflammatory M1 phenotype, further amplifying cytokine production and perpetuating the inflammatory cycle[28]. Concurrent metabolic disturbances, including leptin and decreased adiponectin, exacerbate the systemic state of insulin resistance and contribute to β-cell dysfunction [28,29], compounding the inflammatory environment. Leptin resistance, which often occurs with maternal obesity, further amplifies the inflammatory response by enhancing the secretion of proinflammatory cytokines. This promotes a feedback loop that exacerbates insulin resistance by impairing the action of insulin on target tissues. Adiponectin, on the other hand, normally plays a role in increasing insulin sensitivity, and its decreased levels in maternal obesity contribute directly to insulin resistance [28]. The hormonal imbalance

induced by obesity disrupts normal metabolic signaling, reinforcing the insulin resistance cycle [28,29].

In gestational obesity, elevated free fatty acids (FFAs) serve as potent activators of intracellular inflammatory signaling pathways. These FFAs stimulate stress-responsive kinases such as c-Jun N-terminal kinase (JNK), protein kinase R (PKR), and IκB kinase (IKK), which are central to the propagation of metabolic inflammation. Dietary intake, particularly of high-fat, high-sugar diets, exacerbates the accumulation of FFAs, making them a crucial mediator in the progression of insulin resistance. JNK and PKR activation converge on the phosphorylation of insulin receptor substrate-1 (IRS-1) at serine residues, impairing insulin signaling and promoting insulin resistance. These pathways enhance nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, a key transcriptional regulator of inflammation [29]. IKK specifically phosphorylates the inhibitor of NF-κB (IκB), targeting it for degradation and enabling NF-κB to translocate to the nucleus, where it upregulates proinflammatory genes such as TNF-α, IL-1β, and IL-6, thereby exacerbating systemic inflammation. This heightened inflammatory response is further amplified by adipose-derived IL-6, which acts systemically to induce hepatic C-reactive protein (CRP), a clinical marker and mediator of inflammation [30,31]. The persistent state of low-grade inflammation, combined with the hormonal and dietary factors, creates a chronic insulin-resistant state. Together, these signaling cascades create a self-sustaining loop of immune activation and metabolic disruption. The resulting proinflammatory environment impairs insulin signaling, disrupts placental function, and compromises fetal nutrient and oxygen exchange, ultimately contributing to adverse metabolic programming of the offspring.

1.5 The Placenta

The placenta is a transient yet vital organ that supports fetal development by facilitating nutrient and gas exchange, hormone production, immune tolerance, and waste elimination between mother and fetus. Placentation begins with blastocyst implantation into the uterine wall, establishing dual maternal-fetal circulation by the end of the first trimester, followed by expansion of the exchange surface area in the second and third trimesters.

Structurally, the placenta consists of both fetal and maternal components: the fetal side includes the chorionic plate and branching villi that extend into the intervillous space, while the maternal side is formed by the decidua basalis [32]. The villi—lined by inner cytotrophoblasts (CTBs) and outer syncytiotrophoblast (SCTs) – serve as the primary units for nutrient and gas exchange, with SCTs directly interfacing with maternal blood[33]. The SCT also function as a dynamic, selective barrier [34,35], regulating the passage of molecules such as glucose, amino acids, and maternal antibodies, while limiting fetal exposure to harmful agents [32,34]. Extravillous trophoblasts (EVTs) migrate from anchoring villi into the decidua to remodel maternal spiral arteries into high-capacity, low-resistance vessels essential for adequate perfusion. Inadequate EVT invasion is linked to complications such as pre-eclampsia and fetal growth restrictions[33]. The amnion and chorion enclose the fetus as protective membranes, while the umbilical cord connects the fetus to the chorionic plate, containing vessels that sustain fetal circulation. Together, these integrated structures form a dynamic interface that ensures fetal support throughout pregnancy.

1.6 Gestational Obesity and The Placenta

The placenta serves as a critical interface between the mother and fetus, mediating nutrient and gas exchange, immune regulation, and endocrine signaling. In pregnancies complicated by gestational obesity this interface can become profoundly disrupted. Exposure to excess nutrients, inflammatory mediators, and metabolic stress alters both placental structure and function, creating an intrauterine environment that may drive adverse fetal programming, and predisposing the offspring to long-term health risks.

Structurally, gestational obesity is associated with increased placental weight and altered histomorphology, including the presence of syncytial knots, calcifications, infarcts, and excessive fibrinoid deposition [36–38]. Interestingly, these effects appear to be more pronounced in placentas of female fetuses[39]. These features often reflect maladaptive responses to hypoxia and nutrient oversupply. Additionally, angiogenic signalling within the placenta appears dysregulated in many cases, characterized by decreased placental growth factor (PIGF) and increased soluble fms-like tyrosine kinase-1 (sFlt-1), resulting in an elevated sFlt-1/PIGF ratio – a clinically used biomarker of placental dysfunction.

Although the placenta may enlarge in response to overnutrition, its efficiency often declines, contributing to fetal growth abnormalities.[40,41].

These structural changes are accompanied by functional adaptations that further disrupt maternal-fetal exchange. In particular, glucose and lipid transport are significantly enhanced. Gestational obesity is associated with an upregulation of the glucose transporter protein-1 (GLUT-1) on the apical microvillous membrane (MVM) and basement membrane of the SCT cells[42], which facilitates greater glucose flux to the fetus[43–45]. In parallel, elevated maternal triglycerides (TGs) and FFAs, coupled with increased placental lipoprotein lipase (LPL) activity[46], promote greater lipid transfer to the fetus [47 48]. These nutrient fluxes are strongly associated with increased fetal adiposity and may initiate early-life metabolic programming that predisposition offspring to obesity and metabolic dysfunction in life[46].

Maternal obesity also impairs the immunological environment of the placenta. Under normal conditions, pregnancy promotes a TH2-dominant, anti-inflammatory profile [49,50] that facilitates immune tolerance and tissue remodeling [51]. However, in the context of obesity, this balance shifts toward a Th1-dominant state, characterized by increased M1 macrophage activation and elevated levels of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β[52,53]. This inflammatory phenotype is compounded by elevated oxidative stress and reactive oxygen species (ROS), further compromising placental health [54]. Notably, these effects appear largely restricted to the placenta itself, with minimal evidence of direct fetal inflammatory exposure, suggesting that the placenta acts as both sensor and mediator of metabolic stress[54,55].

Central to the functional decline of the placenta in gestational obesity is mitochondrial dysfunction[56]. Placentas from mothers with obesity exhibit reduced mitochondria density, abnormal mitochondrial morphology, impaired oxidative phosphorylation (OXPHOS)[57]. lower β-oxidation capacity and decreased ATP production [58]. Dysfunction is particularly evident in respiratory complex I activity and overall electron transport chain performance [59–61]. These impairments reduce placental energy efficiency and limit its adaptive capacity

under metabolic stress, increasing the risk of adverse pregnancy outcomes and suboptimal fetal development[56].

Given the central role of mitochondria in placental function, recent evidence suggests that maternal obesity may impair placental metabolism through the depletion of intracellular nicotinamide adenine dinucleotide (NAD^+). NAD^+ is a critical cofactor for mitochondrial respiration, redox balance, and sirtuin activity. Chronic inflammation and oxidative stress may accelerate NAD^+ consumption, leading to energetic deficits and disrupted cellular signaling. This thesis explores the hypothesis that NAD^+ depletion is a mechanistic link between gestational obesity and adverse fetal cardiometabolic programming, and whether supplementation with NAD^+ precursors can restore placental function and improve offspring outcomes.

1.7 NAD^+ Biology, Inflammation and Mitochondrial Function

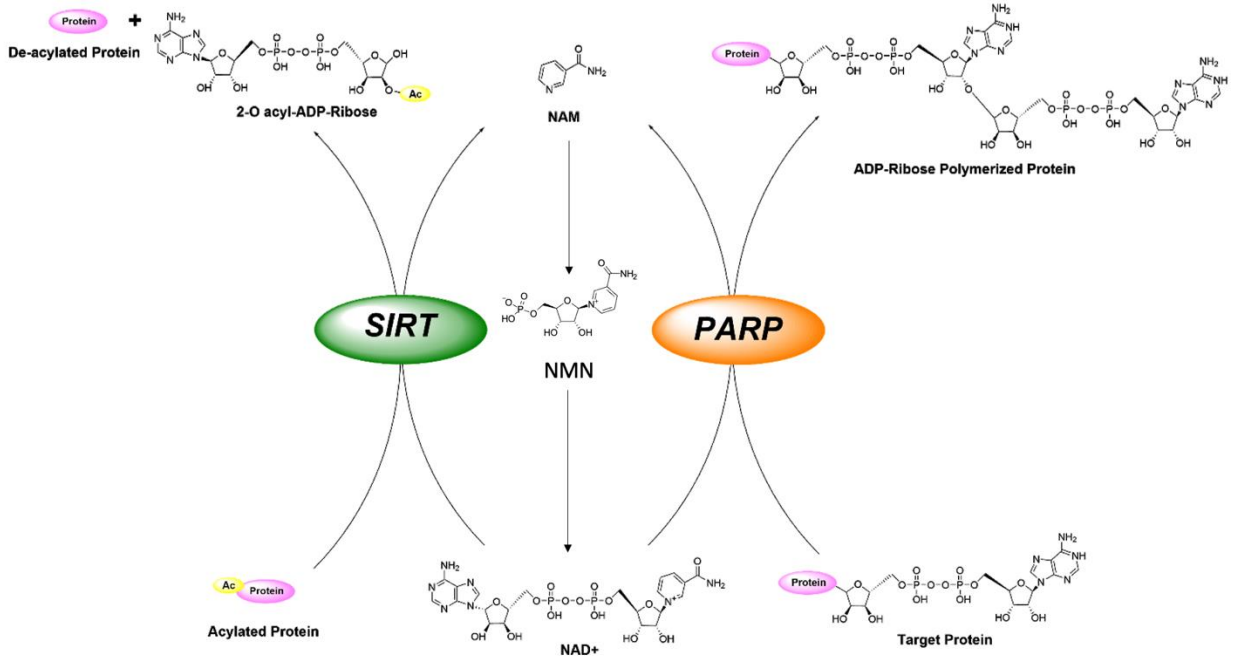

Figure 1.1 NAD^+ functions as a co-factor in enzymatic reactions catalyzed by SIRTs and PARPs.
When PARPs locate DNA strand breaks, it attaches to them, and uses NAD^+ to produce ADP-ribose, which then gets polymerized to nuclear acceptor proteins, such as histones and transcription factors. On the other hand, SIRTs use NAD^+ as a cofactor to remove acyl groups from their substrates, yielding a deacylated

substrate, 2-O-acyl-ADP-ribose, and nicotinamide (NAM) as end products. SIRT= sirtuin, PARP = poly-ADP ribose polymerase, NAM = nicotinamide, NAD^+ = nicotinamide adenine dinucleotide, NMN = nicotinamide mononucleotide. This figure was created using ChemDraw 23.1.2.

NAD^+ is an essential coenzyme involved in a wide array of metabolic and cellular functions, including redox reactions, DNA repair, gene expression, and mitochondrial function [62]. In its oxidized form, NAD^+ acts as a hydride acceptor during glycolysis, fatty acid oxidation, and the citric acid cycle, facilitating the transfer of electrons to the electron transport chain (ETC) for ATP synthesis [63]. Additionally, its reduced form, NADPH, is crucial for antioxidant defense and lipid biosynthesis [63].

Beyond its role in redox balance, NAD^+ is also a substrate for several classes of signaling enzymes, most notably such as sirtuins (SIRTs) and poly-ADP ribose polymerases (PARPs)[62] - among the largest consumers of NAD^+ (**Fig 1.1**), competing for its availability [64].

The SIRT family comprises seven NAD^+-dependent deacetylases (SIRT1–7) with diverse cellular localizations and functions. Of these, SIRT1 (nuclear) and SIRT3 (mitochondrial) are the most well-characterized and play pivotal roles in regulating mitochondrial biogenesis, oxidative phosphorylation, antioxidant defense, and metabolic adaptation[65,66]. SIRT3, in particular, deacetylate and activate key mitochondrial enzymes involved in the TCA cycle, fatty acid oxidation, and ETC function, making it essential to mitochondrial health and energy production [67]. SIRT1 also interacts with transcription factors such as PGC-1α to promote mitochondrial gene expression and suppress inflammation through inhibition of NF-κB.

The PARP family consists of 17 members, but PARP1 is the most predominant isoform responsible for NAD+ consumption in response to DNA damage, accounting for the majority of cellular PARP activity. PARP1, and to a lesser extent PARP2 and PARP3, catalyze poly(ADP-ribosyl)ation (PARylation) of target proteins by cleaving NAD^+ into nicotinamide and ADP-ribose (**Fig 1.1**) [64,68]. This process forms long PAR chains on histones and other nuclear proteins, serving as recruitment platforms for DNA repair enzymes [64]. While PARylation is critical for maintaining genome stability, sustained PARP activation under

chronic inflammatory conditions can rapidly deplete cellular NAD^+ stores, impairing metabolic function and silencing other NAD^+-dependent enzymes, such as SIRTs.

Under conditions of chronic inflammation, over-activation of PARP1 leads to excessive NAD^+ consumption[69], which impairs SIRT activity and disrupts cellular energy homeostasis[70]. NAD^+ depletion compromises mitochondrial respiration, reduces ATP production[71], increases ROS generation, and enhances NF-κB signalling[72]. These changes drive a proinflammatory state and exacerbate tissue dysfunction. Such mechanisms are well-documented in non-pregnant inflammatory diseases, including inflammatory bowel disease[73], cardiovascular disease[74], and chronic obstructive pulmonary disease[75] (COPD).

1.8 NAD^+ depletion in Gestational Inflammation and Maternal Obesity

Emerging evidence suggests that NAD+ depletion may play a central role in mediating the adverse effects of inflammation on placental dysfunction, particularly in the context of pregnancy complications such as preeclampsia and gestational obesity. In placentas from women with inflammation-driven preeclampsia, elevated PARP activity has been linked to increased NAD^+ consumption, reduced intracellular NAD+ availability, and impaired mitochondrial respiration capacity[76]. These molecular changes correlated with lower birth weights, suggesting a link between placental metabolic dysfunction and impaired fetal growth. Supporting these findings, a rodent model of lipopolysaccharide (LPS)-induced inflammation recapitulated this phenotype, showing marked NAD^+ depletion in the placenta, mitochondrial dysfunction, reduced placental efficiency, and fetal growth restriction[76]. Most importantly, restoring placental NAD^+ stores through nicotinamide riboside (NR) supplementation, a NAD^+ precursor, during pregnancy in this model effectively rescued mitochondrial function, enhanced placental performance, and normalized fetal growth parameters—highlighting the essential role of NAD^+ availability in maintaining placental health during inflammatory pregnancy states. [76].

Our group has recently established and characterized a mouse model of gestational obesity using a high-fat, high-sugar (HFHS) diet, which closely mimics the metabolic and inflammatory features observed in human gestational obesity. In this model, we observe

both systemic and placental inflammation, along with a marked increase in placental protein PARylation and significant depletion of NAD+ levels[77]. These molecular alterations are accompanied by poor placental efficiency and reduced fetal growth, suggesting that NAD^+ depletion may likewise be a key mediator of placental dysfunction in the setting of maternal obesity.

1.9 Therapeutic Targeting of NAD^+ Depletion with Nicotinamide Riboside

Given the detrimental effects of NAD^+ depletion on mitochondrial function, inflammation, and cellular health, intracellular NAD+ stores have gained attention as novel therapeutic targets across a range of inflammatory and metabolic conditions [78]. Several NAD+ precursors – namely nicotinamide (NAM), nicotinamide mononucleotide (NMN), nicotinic acid (NA), and NR, have been investigated for their ability to restore intracellular NAD^+ levels and improve metabolic function. Among these, NR has emerged as a particularly promising candidate due to it efficacy and favorable safety profile[79].

NR, a naturally occurring NAD+ precursor found in foods such as milk, has demonstrated the ability to increase NAD^+ concentrations in multiple tissues, enhance SIRT activity, and improve mitochondrial function[80]. Importantly, unlike some other precursors, NR is not associated with adverse effects such as flushing. In human studies involving non-pregnant individuals with obesity, NR supplementation has been well-tolerated. For instance, a study investigating the safety of NR supplementation and its effects on metabolism administered 2000 mg of NR daily for 12 weeks to otherwise healthy obese men, yielding no adverse effects[81]. Importantly, additional studies have reported improvements in markers of NAD+ biosynthesis, metabolic rate, and fat-free mass and skeletal muscle metabolism following shorter courses of NR supplementation [82]. These findings, combined with NR's established safety and bioavailability, position it as a compelling candidate for investigation in the context of maternal obesity, where NAD+ depletion may underly placental dysfunction and subsequent adverse fetal programming events.

1.10 The DOHaD Paradigm and Implication of Placental NAD+ Depletion in Gestational Obesity

The Developmental Origins of Health and Disease (DOHaD) paradigm proposes that environmental exposures during critical windows of fetal development can result in long-lasting changes to organ structure and function, thereby influencing disease risk in adulthood[83]. While initially established through studies of maternal undernutrition, DOHaD is now widely applied to various environmental stressors including inflammation, metabolic dysfunction, and obesity. Gestational obesity, characterized by chronic low-grade inflammation, has emerged as a potent *in-utero* stressor capable of reprogramming fetal development through both direct and indirect mechanisms—chiefly via placental dysfunction.

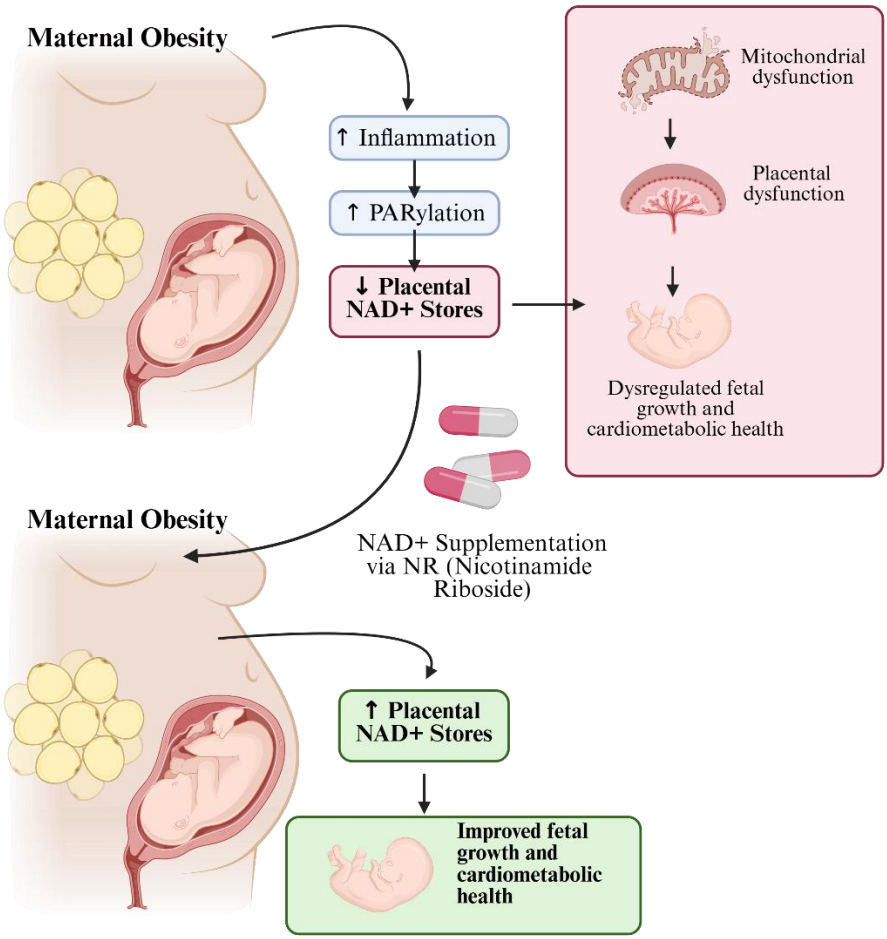

Figure 1.2 Mechanism of chronic inflammation imparted by gestational obesity and NAD+ supplementation with NR.

Chronic inflammation induced by gestational obesity elevates PARylation activity, depleting NAD^+ reserves and impairing mitochondrial function, which contributes to placental dysfunction and disrupted fetal growth (A). Supplementation with nicotinamide riboside (NR) replenishes NAD^+ levels, thereby restoring normal placental and mitochondrial function (B). This figure was created using BioRender.com.

As discussed, maternal obesity leads to systemic and placental inflammation. In our established rodent model of gestational obesity, this is coupled to increased PARP activation, NAD^+ depletion, disrupted mitochondrial function and compromising the placenta's capacity for efficient nutrient and gas exchange. This impairment occurs during critical periods of organogenesis and may restrict or dysregulate the delivery of essential metabolic signals to the fetus. As a result, fetal tissues—particularly those involved in

energy regulation, such as the liver, pancreas, and cardiovascular system—may undergo permanent adaptations that predispose offspring to cardiometabolic diseases, including obesity, insulin resistance, and hypertension later in life.

Evidence from both human and animal studies supports this link. Infants born to obese mothers are more likely to present with abnormal growth trajectories, such as being SGA or LGA, both of which are associated with increased risk of adverse cardiometabolic outcomes [84–86]. Longitudinal data show that maternal obesity is correlated with elevated offspring BMI, blood pressure, and diabetes risk in adolescence and adulthood, with sex-specific effects observed in some cohorts [87]. These epidemiological trends underscore the public health importance of understanding and potentially interrupting this programming pathway[88].

This thesis aims to build upon existing research by examining the impact of maternal obesity on offspring cardiometabolic health during adolescence and adulthood. Furthermore, it explores the potential to counteract these effects by targeting placental NAD+ depletion through NR supplementation during pregnancy and lactation (**Fig 1.2**).

Chapter 2 – Hypothesis and Research Aims

2.1 Hypothesis

The overarching hypothesis of the current MSc thesis is that ***gestational obesity-mediated inflammation leads to placental NAD^+ depletion and results in altered fetal organ development which pre-disposes the offspring to cardiometabolic disorders in later life.*** More specifically, we hypothesize that offspring from dams fed a high-fat high-sugar diet throughout pregnancy and lactation would have poor fetal cardiometabolic health and organ development. We propose that supplementing maternal NAD^+ levels throughout pregnancy will improve placental function and, in turn, promote healthier fetal organ development and cardiometabolic outcomes in offspring exposed to gestational obesity.

2.2 Specific Research Aims

In the current MSc thesis, the previously stated hypothesis will be tested through the completion of the following research aims:

Aim 1. Measure metrics of cardiometabolic health in adolescent and adult offspring exposed to an obesogenic environment in utero, with and without NAD^+ supplementation.

Aim 2. Determine the programming effects of an in utero obesogenic exposure, with and without NAD^+ supplementation, on the structural and metabolic development of key organ systems in adult offspring.

Chapter 3 - Methods

3 Methods

3.1 Mouse Model for Studying Offspring Exposed to Gestational Obesity

All animal experiments were performed in accordance with the University of Ottawa Animal Care Committee and Protocol Review (protocol# HS3937). C57BL/6N mice (Charles River Laboratories International, Inc.) were housed at 23 ℃ and kept on a 12:12-hr light-dark cycle. To create a maternal model of gestational obesity, beginning at 6 weeks of age, female mice were maintained on either a control (CNTRL) diet (standard chow) or high-fat high-sugar (HFHS) diet (42% kcal fat, 34% sucrose by weight, TD.88137 Harlan Tekklad) for 15 weeks and weighed weekly. Our previous studies, using the same mouse model, showed that the HFHS diet resulted in significant weight gain, increased fat mass, and elevated fasting blood glucose levels in dams prior to pregnancy (**Fig 3.1**), thus establishing the maternal obesity model[77].

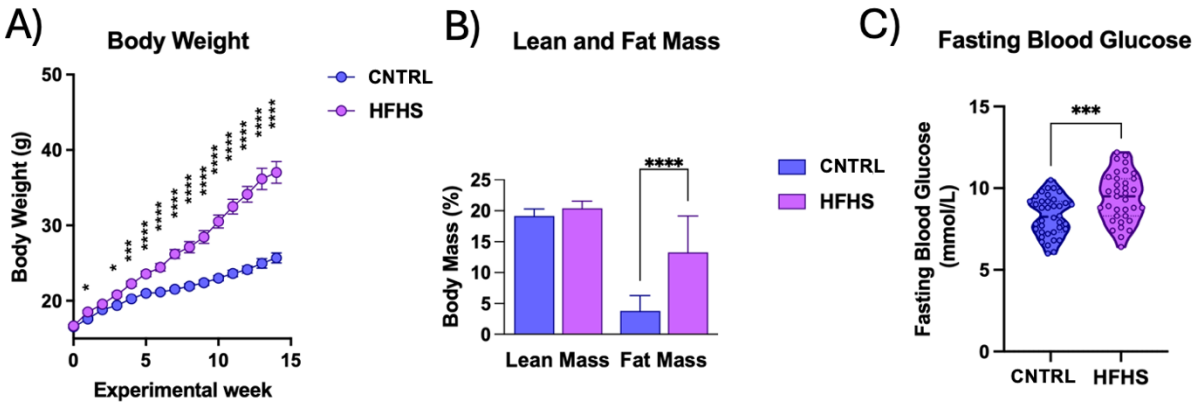

Figure 3.1 Dam measurements in a HFHS diet-induced model of obesity.

Pre-gestational body weight over 15 weeks of diet exposure (A), fat mass percentage measured via Echo-MRI after 12 weeks of diet (B), and fasting blood glucose after 12 weeks of diet (C). Differences in diet exposure assessed via unpaired t-test with Bonferroni post-hoc test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. CNTRL = regular chow; HFHS= high fat high sugar diet.

At 18 weeks of age (12 weeks after diet administration), female mice were mated with 10-week-old C57BL/6N male mice maintained on a control diet. Vaginal plugs were checked daily to confirm pregnancy, with the presence of the plug denoted as gestational day (E) 0.5. Maternal body weights were recorded daily to track gestational weight gain. Pregnant dams were maintained on their assigned pre-pregnancy diet (CNTRL or HFHS) and

beginning at E0.5, received either 400 mg/kg/day NR or sterile water (H_2O) via oral gavage daily for the duration of gestation. This created four distinct maternal treatment groups. The rationale for NR treatment during pregnancy is supported by our previous maternal obesity studies, in which we observed significant NAD+ depletion in the placenta using the same model of maternal obesity[77] (**Fig 3.2**).

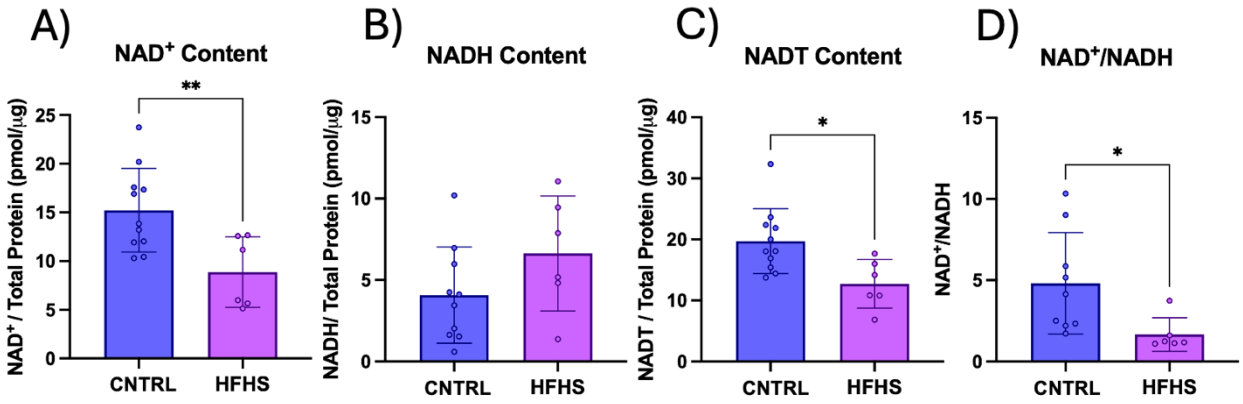

Figure 3.2 Effect of HFHS diet-induced obesity on NAD+ content.

NAD^+ (A), NADH (B), and total NAD content (C), as well as NAD^+/NADH ratio (D) calculated from a colorimetric NAD^+ assay. Differences in diet exposure assessed via unpaired t-test with Bonferroni post-hoc test. *p<0.05, **p<0.01. CTRL = control chow diet, HFHS = high-fat high-sugar diet, NADH = nicotinamide adenine dinucleotide + hydrogen, NAD^+ = nicotinamide adenine dinucleotide, NADT = total nicotinamide adenine dinucleotide.

Following the birth of the pups (approximately 18-21 days after a confirmed plug), dams remained on their assigned diets and treatments throughout the 4-week lactation period. At four weeks of age, the pups were weaned and transitioned to a standard control chow diet (**Fig 3.3**). This resulted in four distinct experimental offspring groups. The sample sizes were derived from two separate cohorts (cohort 1 and cohort 2) initiated six months apart. Sample sizes are listed in **Table 3.1,** with cohort 1 in black and cohort 2 in red.

1) **CNTRL-H_2O (n=21):** Offspring from dams that were maintained on CNTRL chow diet and received sterile water via oral gavage from gestational day 0.5-18.5.
2) **HFHS-H_2O (n=7):** Offspring from dams that were maintained on HFHS diet and received sterile water via oral gavage from gestational day 0.5-end of lactation.

3) **CNTRL-NR (n=16):** Offspring from dams that were maintained on CNTRL chow diet and received NR via oral gavage from gestational day 0.5- end of lactation.
4) **HFHS-NR (n=7):** Offspring from dams that were maintained on HFHS diet and received NR via oral gavage from gestational day 0.5- end of lactation.

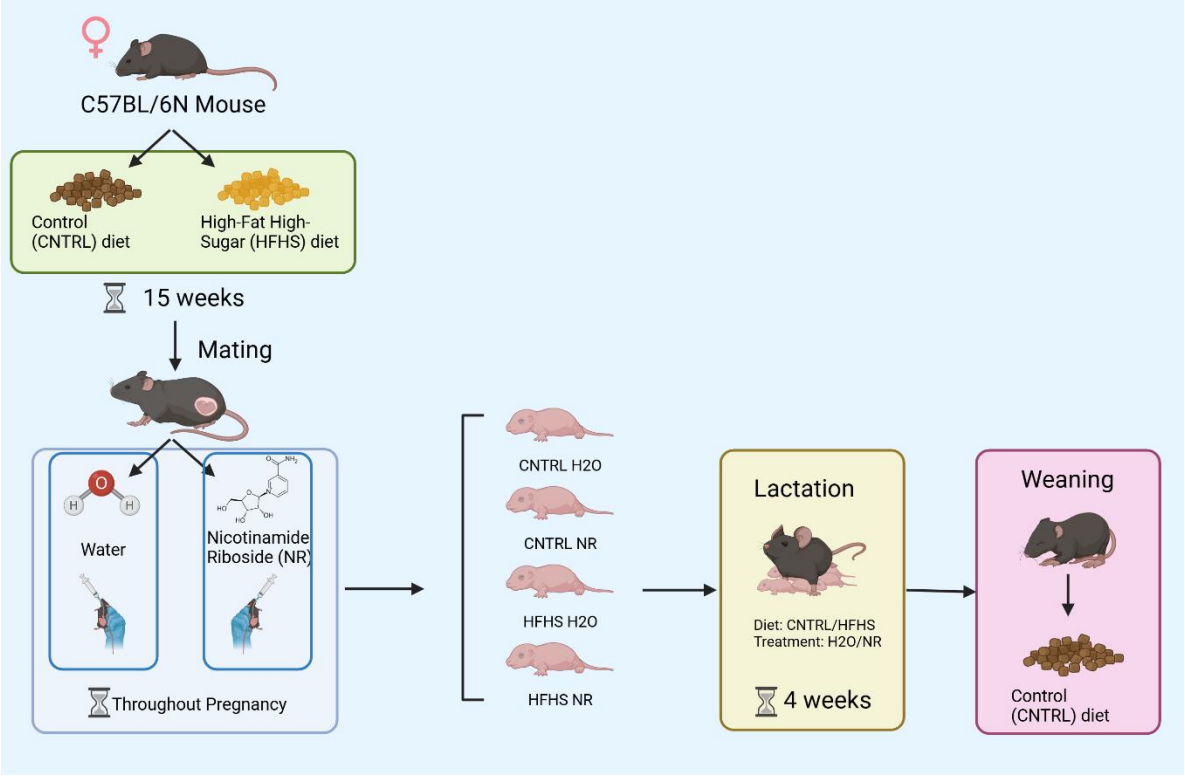

Figure 3.3 Experimental design for generating offspring exposed to gestational obesity.
Female mice were fed either a control (CNTRL) or high-fat high-sugar (HFHS) diet for 15 weeks prior to mating and throughout pregnancy and lactation. Upon pregnancy confirmation, dams were treated daily with either sterile water (H_2O) or nicotinamide riboside (NR) until birth. Offspring were exposed to maternal diet and treatment during gestation and lactation, then weaned onto standard chow.

Table 3.1 Sample sizes separated by cohort.

	All Offspring			Male Offspring			Female Offspring		
	Cohort 1	**Cohort 2**	**Total**	**Cohort 1**	**Cohort 2**	**Total**	**Cohort 1**	**Cohort 2**	**Total**
CNTRL H_2O	4	16	20	2	8	10	2	8	10
CNTRL NR	16	0	16	8	0	8	8	0	8
HFHS H_2O	7	0	7	4	0	4	3	0	3
HFHS NR	0	7	7	0	2	2	0	5	5

3.2 Measuring Offspring Cardiometabolic Health

Offspring from all four treatment groups were weighed weekly. Beginning at 12 weeks of age, blood pressure was measured every three months at noon using the BP-2000 Blood Pressure Analysis System™, with a one-week acclimatization period followed by one week of measurements. Both systolic and diastolic blood pressure were measured, with the data representing the weekly average of daily measurements, each consisting of 10 individual readings. Body composition was assessed at 12- and 24-weeks using EchoMRI to determine lean mass and fat mass ratios. Glucose handling was evaluated through fasted blood samples collected at 12 and 19 weeks of age, after a 6-hour fasting period. At 21 weeks, whole-body metabolism was assessed using a Comprehensive Lab Animal Monitoring System (CLAMS) to measure oxygen consumption, respiratory exchange ratio, overall activity, and ambulatory movement. The CLAMS analysis included a 48-hour acclimatization period, followed by a 24-hour measurement phase. All data were analyzed based on time of day, distinguishing between light hours (7 AM–7 PM) and dark hours (7 PM–7 AM).

3.3 Analyzing Offspring Organ Function/Health – Kidney Histomorphology

Kidneys fixed in paraformaldehyde (PFA) were embedded in paraffin, sectioned, stained, and imaged. Histological sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome following standard protocols. Collagen content in the renal tissue was

quantified using QuPath[89], applying automated color detection to Masson's trichrome-stained sections. Three anatomically equivalent sections were analyzed per kidney, sampled from the superior, mid, and inferior regions. The percentage of collagen-positive area was calculated to assess fibrosis levels.

The average glomerular size was determined using H&E-stained kidney sections. Twenty glomeruli per kidney were randomly selected and annotated in QuPath, and the area of each glomerulus was measured and expressed as a percentage. Glomeruli were equally sampled from the superior, mid, and inferior regions of the kidney. The final glomerular size was expressed as the average percent area of all selected glomeruli per kidney.

H&E-stained kidney sections were used to quantify the average number of nuclei per glomerulus. Twenty glomeruli were randomly selected and annotated in QuPath, and the cell count function was used to determine the number of nuclei within each glomerulus. Results were expressed as the average number of nuclei per glomerulus per kidney.

3.4 Analyzing Offspring Organ Function/Health – Liver Histomorphology

Liver samples fixed in optimal cutting temperature (OCT) compound were cryosectioned, stained, and imaged. Sections were stained with Oil Red O following standard protocols. Stained slides were analyzed using ImageJ Fiji[90], where the color threshold function was applied to quantify fat content. Three anatomically equivalent regions—superior, mid, and inferior—were selected for analysis per liver sample. The final fat content was expressed as the average percent area of Oil Red O staining across the selected regions.

Additional liver samples were fixed in PFA, embedded in paraffin, sectioned, stained, and imaged. These histological sections were stained with Masson’s trichrome following standard protocols. Collagen content was quantified using QuPath[89], with automated color detection. Although the entire liver section was analysed, collagen levels were normalized to total tissue area and expressed as a percentage of collagen-positive area to assess fibrosis levels.

3.5 Norepinephrine Sensitivity/Lipolysis Activity in Fats

During dissections, norepinephrine (NE) stimulation assays were performed. HKRB buffer was prepared in advance with 4% fatty acid-free BSA (Sigma A7030), 10 mM HEPES, and KRB powder (Sigma K4002). L-(−)-Norepinephrine (+)-bitartrate salt monohydrate (NE; Sigma N5785) was dissolved in DMSO and supplemented into HKRB solutions at 0 µM (Basal), 0.1 µM (Submaximal), or 10 µM (Maximal NE Signaling). Each solution (1000 µL) was aliquoted into wells of a preheated 24-well plate.

Gonadal (gWAT), inguinal (iWAT), and brown adipose tissue (BAT) explants (>10 mg) were dissected, weighed, and immediately incubated in the prewarmed buffer. Explants were maintained at 37°C in a shaking incubator, and media aliquots (50 µL) were collected at 1, 2, 3, and 4 hours and stored at −20°C for glycerol analysis. At 24 hours, remaining explants were similarly processed and stored. Glycerol release was quantified using the Sigma MAK117 kit, following the manufacturer's protocol. Glycerol levels were determined through colorimetric analysis, normalized to tissue mass, and expressed relative to baseline.

3.6 Data Analysis

All statistical analyses were performed using GraphPad Prism (version 10.3.2). Data are presented as mean ± standard deviation (SD). A two-way ANOVA with Bonferroni post-hoc multiple comparisons were used to assess the effects of diet and treatment. Statistical significance was set at $p < 0.05$. All data was normally distributed.

Chapter 4 - Results

4 Results

4.1 Gestational obesity induces early-onset weight gain in offspring, mitigated by NR supplementation

Offspring from a previously established maternal HFHS diet-induced obesity model and corresponding control groups, with or without NR treatment, were weighed weekly starting at weaning (4 weeks of age,) till 47 weeks of age (Fig 4.1A-B). At weaning, both male and female pups exposed to the HFHS diet *in-utero* and during lactation displayed significantly higher body weights compared to control offspring (Fig 4.1C). Notably, maternal NR supplementation significantly attenuated this early excess weight gain in HFHS-exposed offspring (Fig 4.1C).

Interestingly, offspring born to dams on a CNTRL chow diet who received NR supplementation also showed increased body weight at weaning relative to untreated controls (Fig 4.1A–C). However, these weight differences resolved by adolescence (12 weeks; Fig 4.1D) and were no longer apparent in adulthood (24 and 36 weeks; Fig 4.1E). An exception was observed in male HFHS + NR offspring, who displayed a steeper trajectory of weight gain over time (Fig 4.1B) and remained significantly heavier at 36 weeks of age.

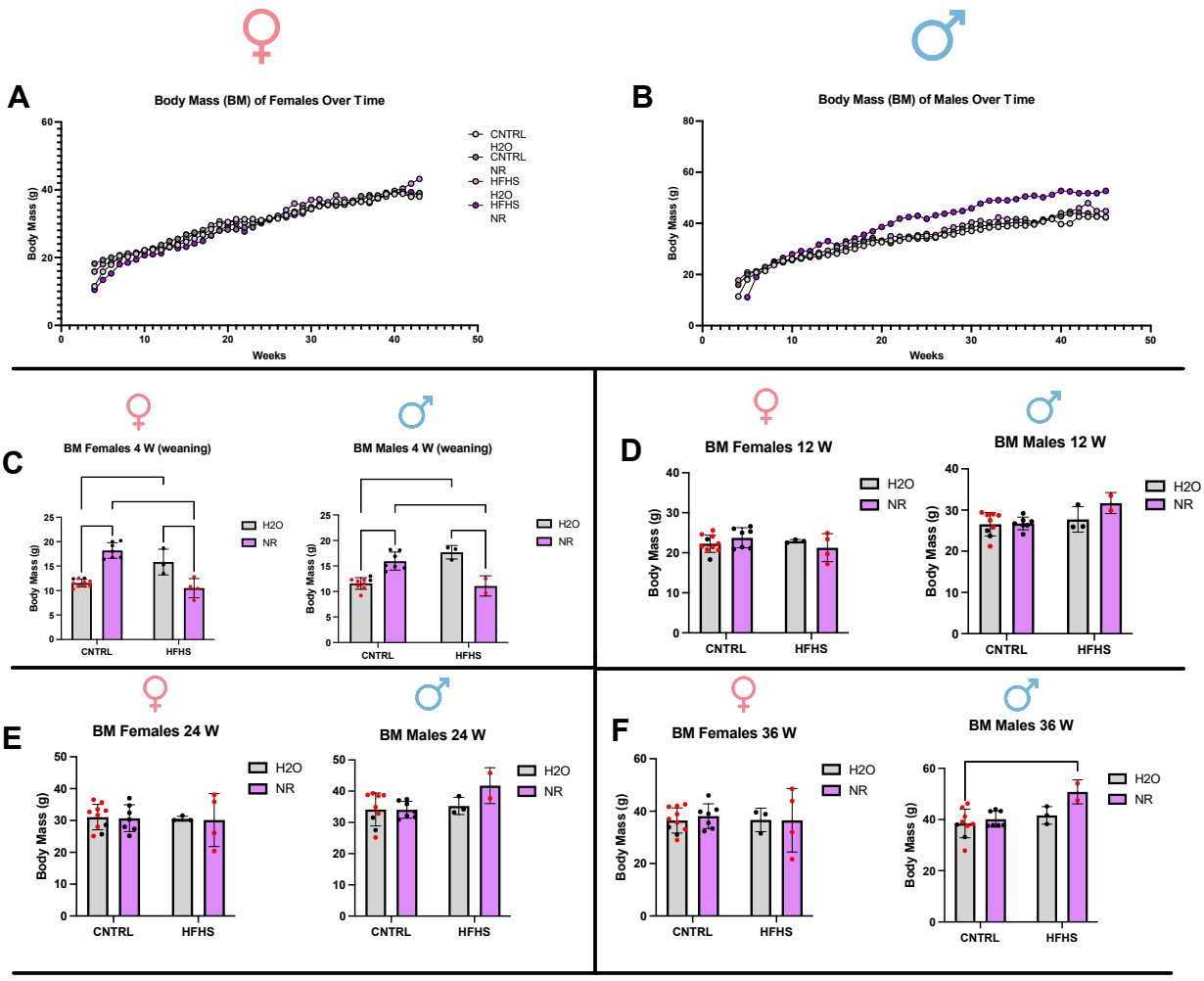

Figure 4.1 Offspring weights from weaning to late adulthood. Offspring body weights trajectories for females (A) and males (B), with group means at 4 weeks (C), 12 weeks (D), 24 weeks (E), and 36 weeks of age (F). Black and red points indicate cohort 1 and 2, respectively. Two-way ANOVA with Bonferroni post-hoc test assessed effects of diet and NR treatment. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. CNTRL = regular chow; HFHS= high fat high sugar diet; NR = nicotinamide riboside; H_2O = water vehicle.

4.2 Early life gestational obesity exposure elevates fasting glucose in female offspring, with NR showing sex-specific protective effects

At 12 weeks of age, female offspring exposed to a HFHS diet *in-utero* and during lactation demonstrated a trend towards increased fasting blood glucose levels compared to chow-exposed CNTRLs (p = 0.0503), while NR supplementation appeared to exert a protective effect in this group (Fig 4.2A). In contrast, male offspring showed no significant differences in fasting glucose across exposure groups at the same age (Fig 4.2B). A similar trend was observed in females at 19 weeks of age, with HFHS + H_2O offspring showing higher fasting

glucose levels than controls (p = 0.0672, Fig 4.2D), and NR supplementation again appeared protective (p = 0.0712, Fig 4.2D). When male and female data were pooled at 19 weeks, early-life HFHS exposure was associated with increased impaired glucose handling, which was partially ameliorated by NR treatment (p = 0.0611, Fig 4.2F).

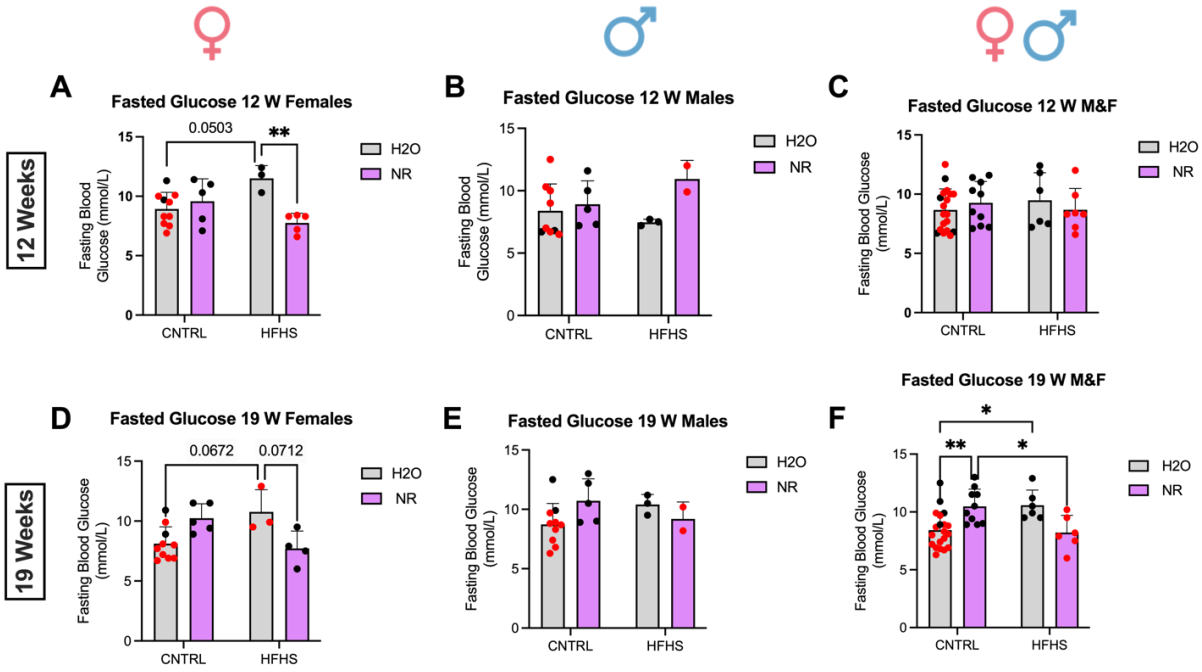

Figure 4.2 Effect of maternal obesity on fetal fasted glucose levels**.** Fasted blood glucose levels at 12 and 19 weeks in females (A, D), males (B, E), and combined (C, F). Black and red points denote cohorts 1 and 2, respectively. Two-way ANOVA with Bonferroni post-hoc test assessed effects of diet and treatment. *p<0.05. CNTRL = chow-diet; HFHS= high fat high sugar; NR = nicotinamide riboside; H_2O = water vehicle.

4.3 Exposure to maternal obesity leads to no changes in body composition and blood pressure

Although increased body weight was observed at 4 weeks of age in both sexes and at 36 weeks HFHS + NR exposed males, no significant differences in body fat percentage were detected during adolescence (12 weeks of age) or adulthood (24 weeks of age) in either sex across all exposure groups (Fig 4.3.1A-F).

Likewise, systolic, and diastolic blood pressure measurements taken at 12, 24, and 36 weeks revealed no statistically significant differences between groups at any timepoint. However, a trend toward elevated systolic ($p = 0.0700$; Fig 4.3.2N) and diastolic ($p = 0.0563$; Fig 4.3.2Q) blood pressure was noted in HFHS + H_2O-exposed male offspring compared to controls.

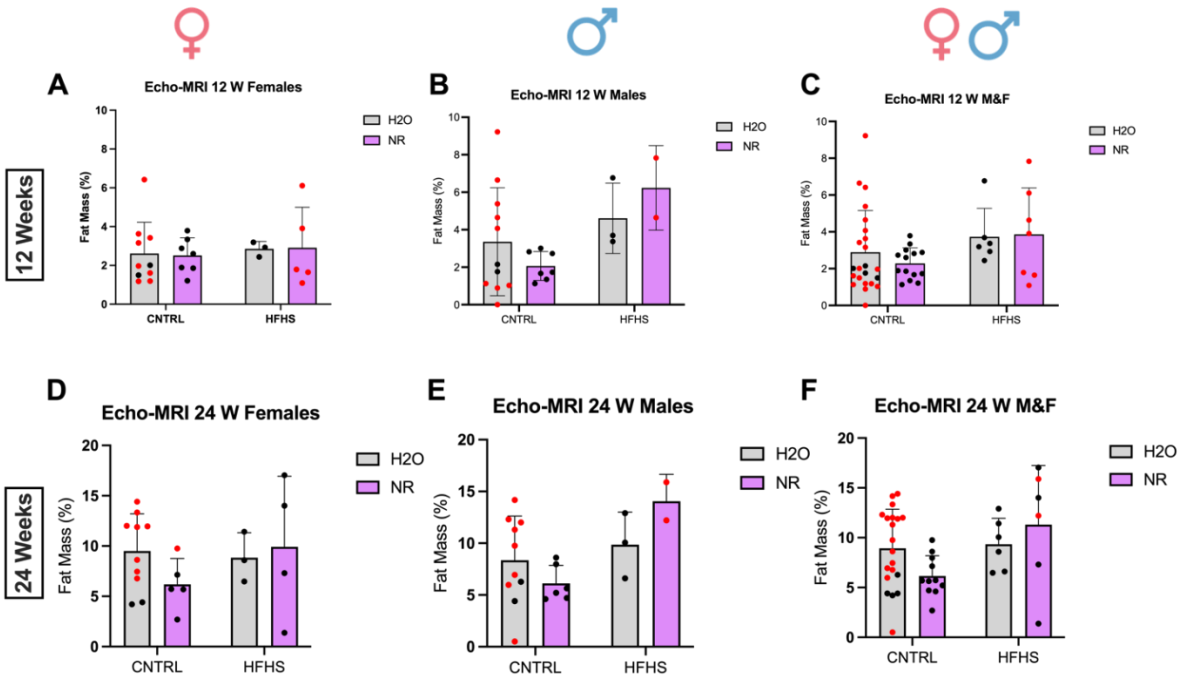

Figure 4.3.2 Effect of maternal obesity on offspring body composition. Offspring body fat percentage at 12 and 24 weeks of age for females (A, D), males (B, E), and combined (C, F). Body composition was assessed by Echo-MRI. Black and red points denote cohorts 1 and 2, respectively. Two-way ANOVA with Bonferroni post-hoc test assessed effects of diet and treatment. CNTRL = chow-diet; HFHS= high fat high sugar diet; NR = nicotinamide riboside; H_2O = water vehicle.

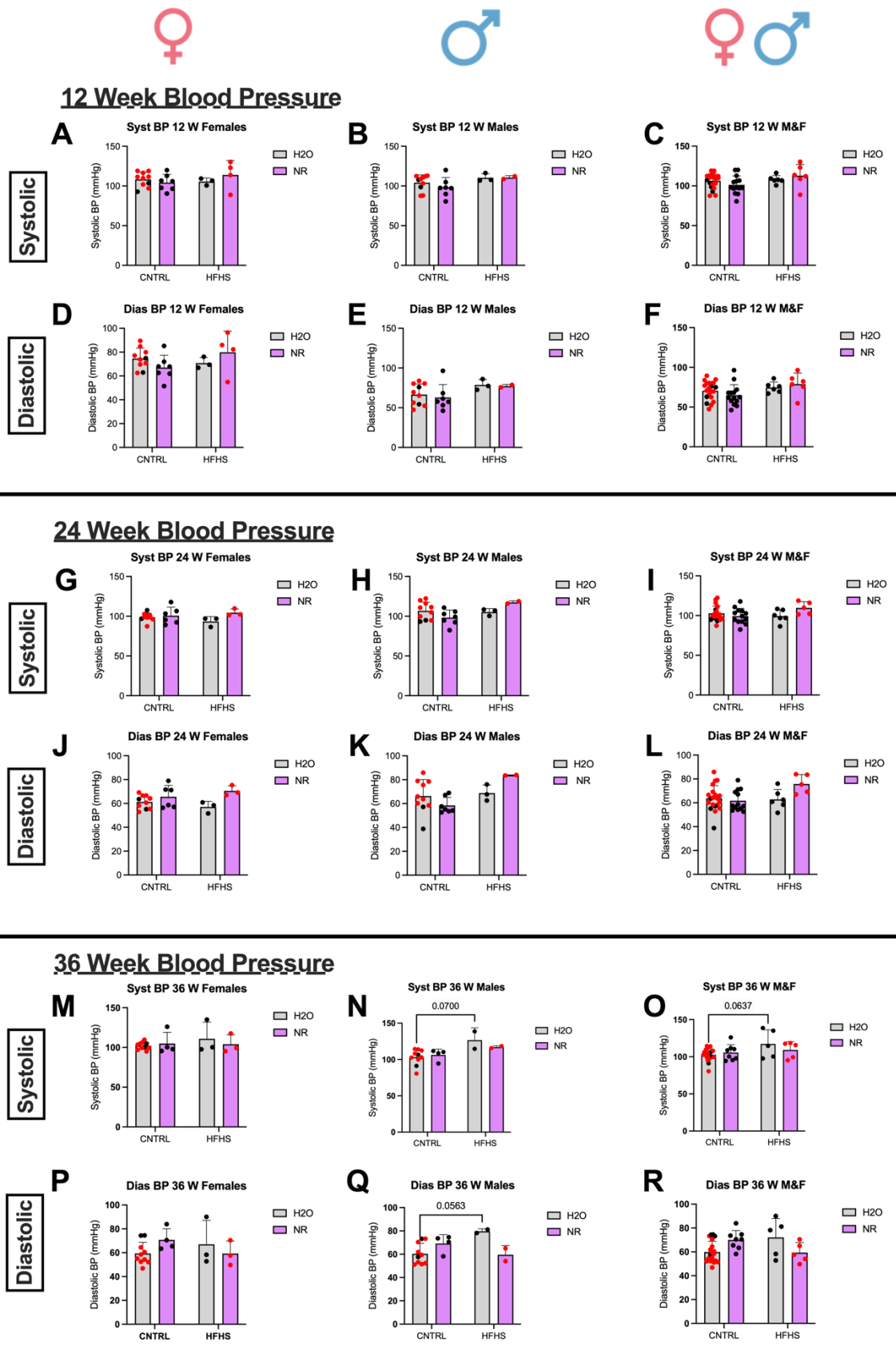
12 Week Blood Pressure
Systolic
Diastolic
A
Syst BP 12 W Females
B
Syst BP 12 W Males
C
Syst BP 12 W M&F
D
Dias BP 12 W Females
E
Dias BP 12 W Males
F
Dias BP 12 W M&F
24 Week Blood Pressure
G
Syst BP 24 W Females
H
Syst BP 24 W Males
I
Syst BP 24 W M&F
J
Dias BP 24 W Females
K
Dias BP 24 W Males
L
Dias BP 24 W M&F
36 Week Blood Pressure
M
Syst BP 36 W Females
N
Syst BP 36 W Males
0.0700
O
Syst BP 36 W M&F
0.0637
P
Dias BP 36 W Females
Q
Dias BP 36 W Males
0.0563
R
Dias BP 36 W M&F
Systolic BP (mmHg)
Diastolic BP (mmHg)
CNTRL
HFHS
H2O
NR

Figure 4.3.2 Effects of maternal obesity on offspring blood pressure. Blood pressure measurements at 12, 24, and 36 weeks of age in female (Systolic - A, G, M; Diastolic – D, J, P), male (Systolic - B, H, N; Diastolic – E, K, Q), and combined (Systolic - C, I, O; Diastolic – F, L, R) offspring. Measurements were collected using the BP-2000 tail-cuff system at noon daily for one week. Black and red points denote cohorts 1 and 2, respectively. Two-way ANOVA with Bonferroni post-hoc test assessed effects of diet and treatment. CNTRL = chow-diet; HFHS= high fat high sugar diet; NR = nicotinamide riboside; H_2O = water vehicle.

4.4 Exposure to maternal obesity leads to no significant changes in whole-body metabolism

Assessment of whole-body metabolism at 21 weeks of age revealed no significant differences in oxygen consumption rates (VO_2) or respiratory exchange ratios (RER = VCO_2/VO_2) across male and female offspring in any of the exposure groups (Fig 4.4.1A-L). Horizontal movement patterns – such as small positional shifts and repetitive behaviors like grooming - were evaluated using X activity measurement, demonstrating no significant exposure-related effects (Figure 4.4.2A-L). However, reduced X activity was noted in male offspring exposed to the HFHS diet in utero and during lactation ($p>0.999$ Fig 4.4.2E), which appeared to improve with maternal NR treatment during pregnancy ($p=0.594$ Fig 4.4.2E). X activity is specifically measured by counting the amount of beam breaks that occur in the CLAMS system. X ambulatory activity, defined as locomotor movements such as traveling from one point to another, exhibited an opposite trend. Female offspring exposed to HFHS showed increased movement ($p=0.374$, Figure 4.4.2G, H, I), which significantly decreased with NR treatment, particularly during light phase of the day (Fig 4.4.2H). X ambulatory is specifically measured by counting the amount of footsteps the mouse takes within the CLAMS system.

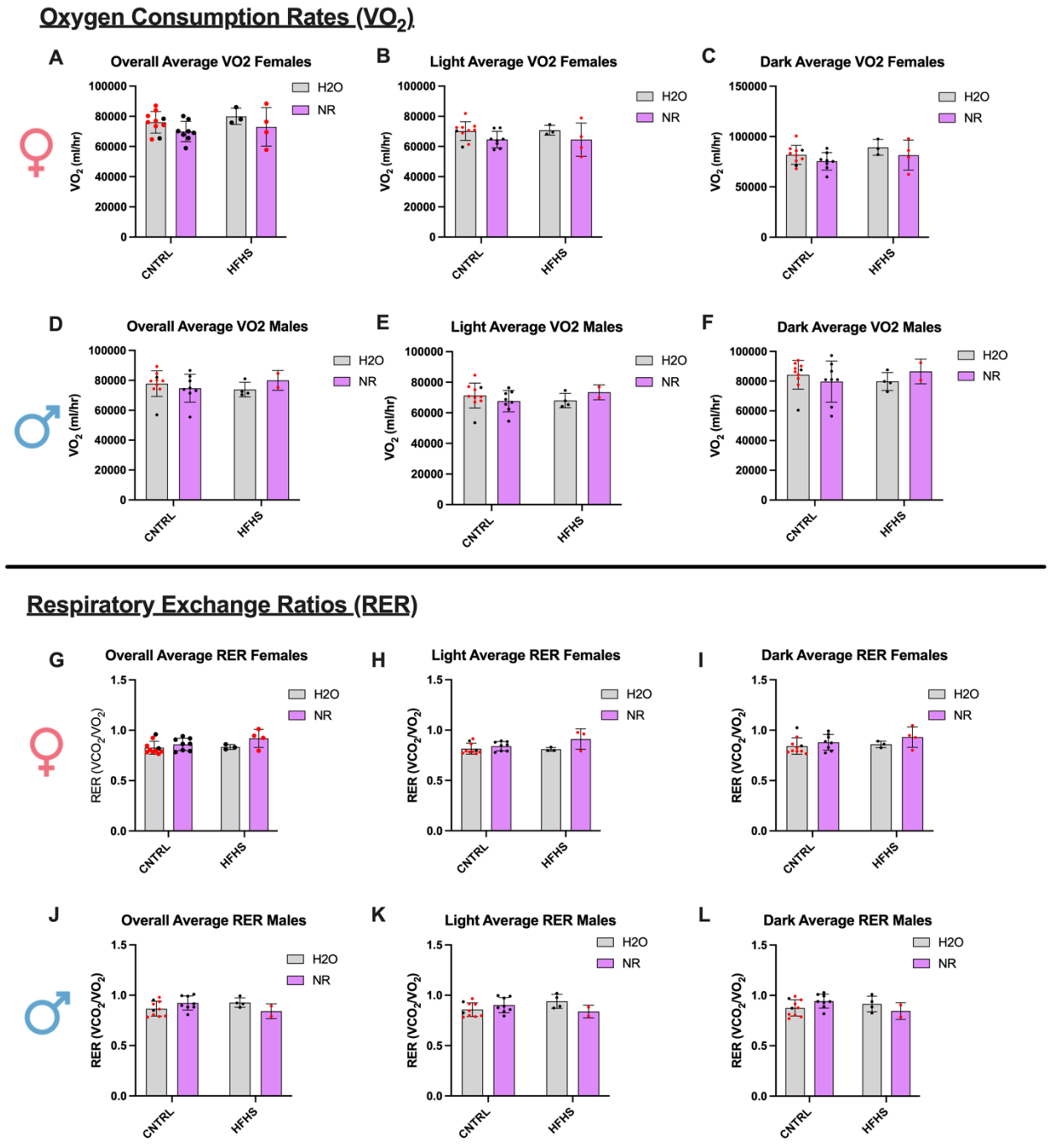

Figure 4.4.1 Effect of maternal obesity on offspring average oxygen consumption and respiratory exchange ratios. Average oxygen (VO2) consumption for female (A, B, C) and male (D, E, F) offspring, further broken down according to daytime ('light', 7am to 7pm; B, E) and nighttime ('dark', 7pm to 7am; C, F) measurements. Average respiratory exchange ratio (RER) for female (G, H, I) and male (J, K, L) offspring, further broken down according to daytime ('light', 7am to 7pm; H, K) and nighttime ('dark', 7pm to 7am; I, L) measurements. Measurements were taken using the CLAMS, with presented data collected during the final 24 hours of data collection. Black points denote offspring from cohort 1 while red points denote offspring from cohort 2. Two-way ANOVA with Bonferroni post-hoc test assessed differences by diet and treatment.

CNTRL = chow-diet exposed offspring; HFHS= high fat high sugar diet exposed offspring; NR = nicotinamide riboside exposed offspring; H_2O = water vehicle exposed offspring.

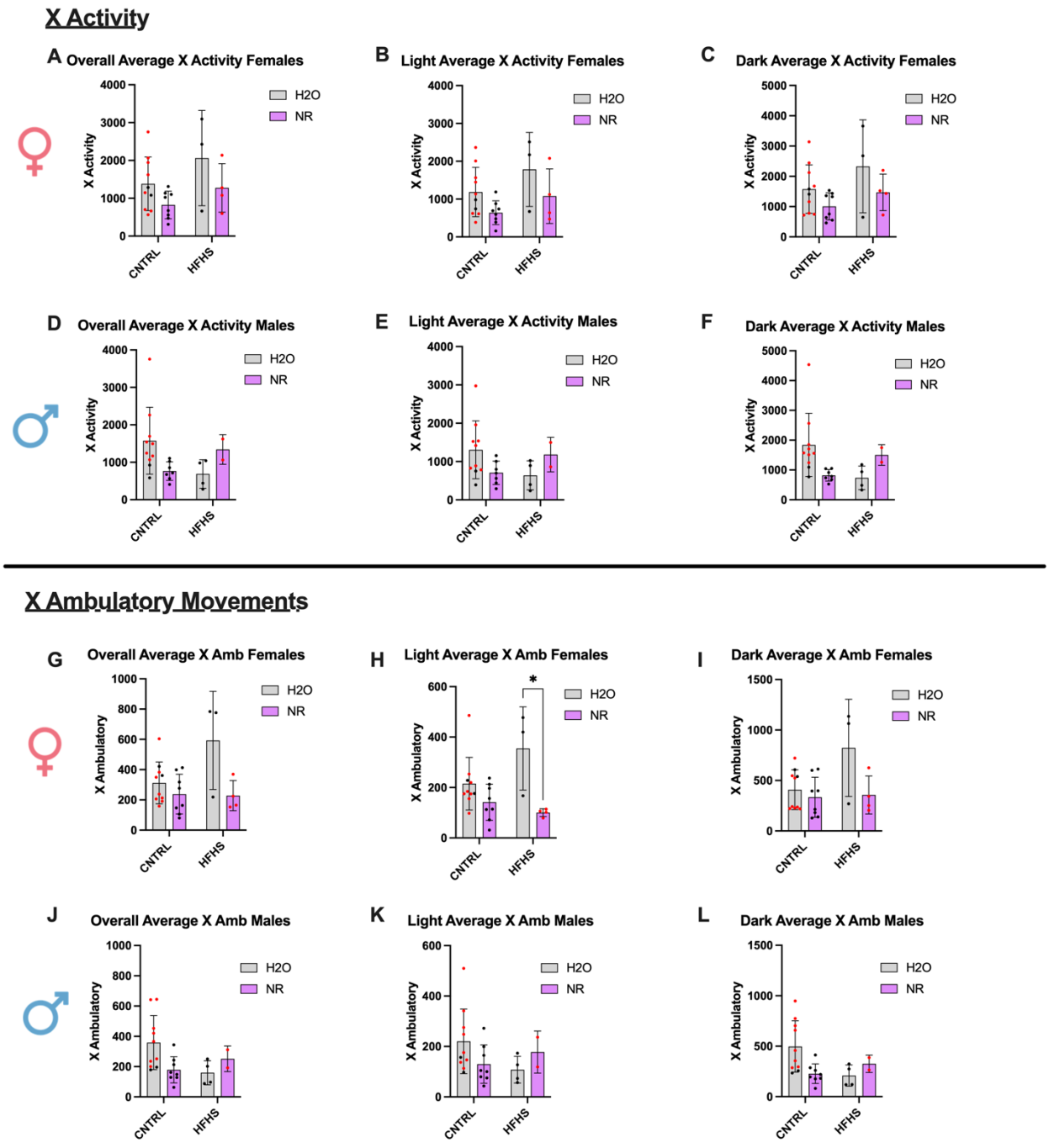

Figure 4.4.2 Effect of maternal obesity on offspring average X activity and X ambulatory movements. Average X activity movement female (A, B, C) and male (D, E, F) offspring, further broken down according to daytime ('light', 7am to 7pm; B, E) and nighttime ('dark', 7pm to 7am; C, F) measurements. Average X ambulatory (X amb) movement for female (G, H, I) and male (J, K, L) offspring, further broken down according to daytime ('light', 7am to 7pm; H, K) and nighttime ('dark', 7pm to 7am; I, L) measurements. Measurements

were taken using the CLAMS, with presented data collected during the final 24 hours of data collection. Black points denote offspring from cohort 1 while red points denote offspring from cohort 2. Two-way ANOVA with Bonferroni post-hoc test assessed differences by diet and treatment. *p<0.05. X acvitivy = amount of beam breaks; X ambulatory = amount of foot steps; CNTRL = chow-diet exposed offspring; HFHS= high fat high sugar diet exposed offspring; NR = nicotinamide riboside exposed offspring; H_2O = water vehicle exposed offspring.

4.5 Female offspring exposed to maternal obesity have more inguinal white adipose tissue

At 47 weeks of age, after euthanasia, key organs - the heart, pancreas, kidneys, spleen, brain, and testes (in males) - were collected and weighed. No significant differences in organ weights were observed across exposure groups in either sex (Fig 4.5A–B).

In adipose depots, female HFHS + H_2O offspring exhibited significantly increased inguinal white adipose tissue (IWAT) compared to controls, which maternal NR treatment was unable to rescue (Fig 4.5C). No differences were observed in gonadal white adipose tissue (GWAT) or brown adipose tissue (BAT) (Fig 4.5C). In males, no significant differences in IWAT, GWAT or BAT were detected across all groups (Fig 4.5D).

Skeletal muscle mass measurements - including quadriceps, gastrocnemius–plantaris–soleus complex (GPS), gastrocnemius–plantaris (GP), soleus, and transversus abdominis (TA) - showed no group differences in either sex (Fig 4.5E-F), with one exception: TA mass was significantly lower in female HFHS + NR offspring compared to their HFHS + H_2O counterparts (Fig 4.5E). However, TA muscle mass did not differ between HFHS + H_2O and control females, and no differences were observed in males (Fig 4.5F).

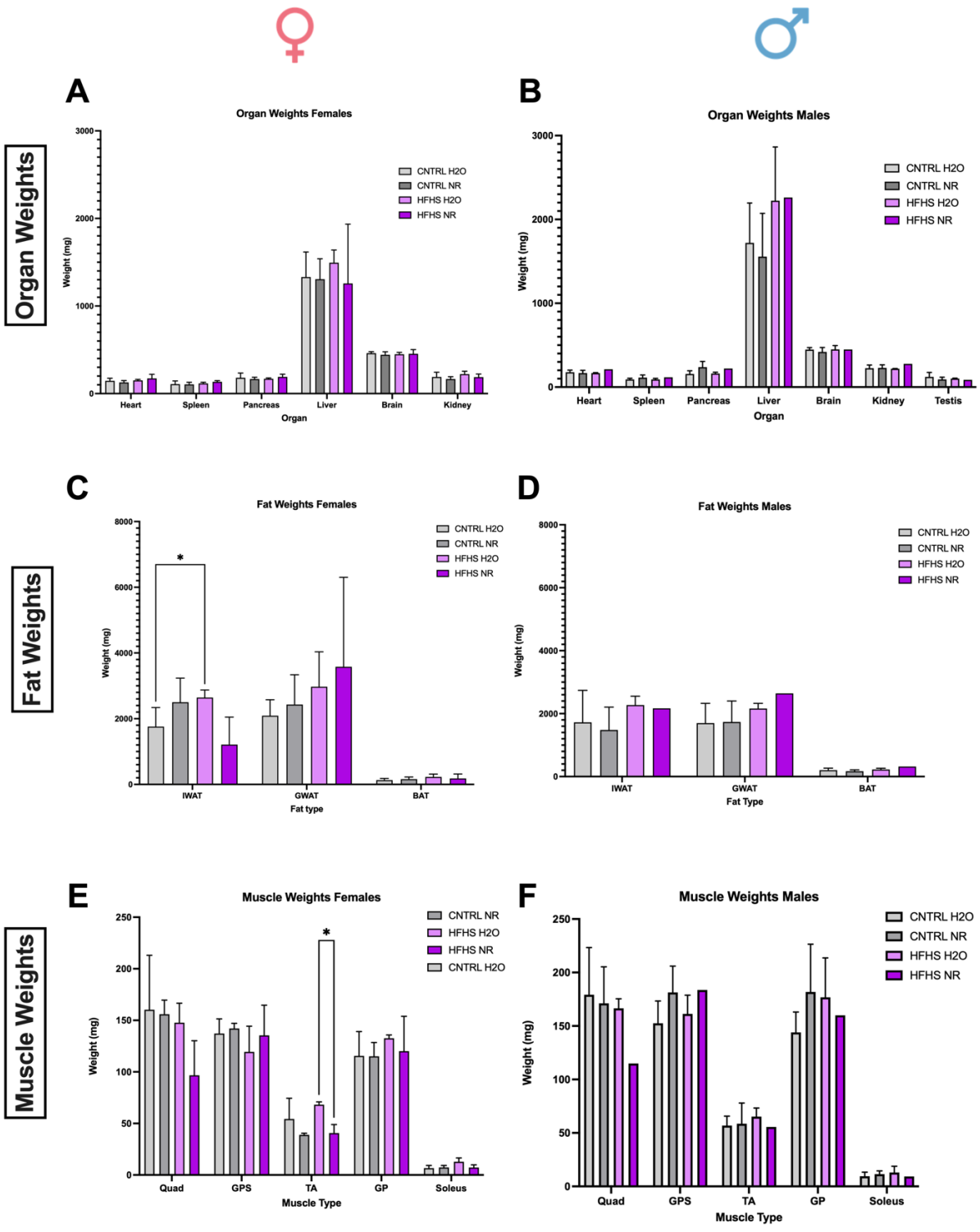

Figure 4.5 Effect of maternal obesity on offspring organ and muscle weights. At 47 weeks of age, weights of the offspring heart, spleen, pancreas, liver, brain, and kidney were collected for both females (A) and males (B). Fat depot mass was assessed, specifically the inguinal white adipose tissue (IWAT), gonadal white adipose tissue (GWAT), and brown adipose tissue (BAT) in females (C) and males (D). Skeletal muscle mass was also assessed for quadriceps, gastrocnemius–plantaris–soleus complex (GPS), gastrocnemius–plantaris

(GP), soleus, and transversus abdominis (TA) in females (E) and males (F). Two-way ANOVA with Bonferroni post-hoc test assessed effects by diet and treatment. *p<0.05. CNTRL = chow-diet; HFHS= high fat high sugar diet; NR = nicotinamide riboside; H_2O = water vehicle.

4.6 Male offspring exposed to maternal obesity have kidney fibrosis

Following kidney collection, the glomerular area, number of nuclei per glomerulus, and extent of fibrosis were quantified. Maternal obesity exposure did not significantly alter glomerular size or the number of nuclei per glomerulus (Fig 4.6A–F). In contrast, male HFHS + H_2O offspring exhibited significantly higher kidney fibrosis (Fig 4.6H), a finding that remained significant for all HFHS + H_2O offspring when sex was not considered (Fig 4.6I). NR treatment markedly reduced fibrosis levels when data were analyzed without stratifying by sex (Fig 4.6I). Unrelated to maternal obesity, both male and female CNTRL + NR offspring had significantly higher fibrosis levels compared to controls.

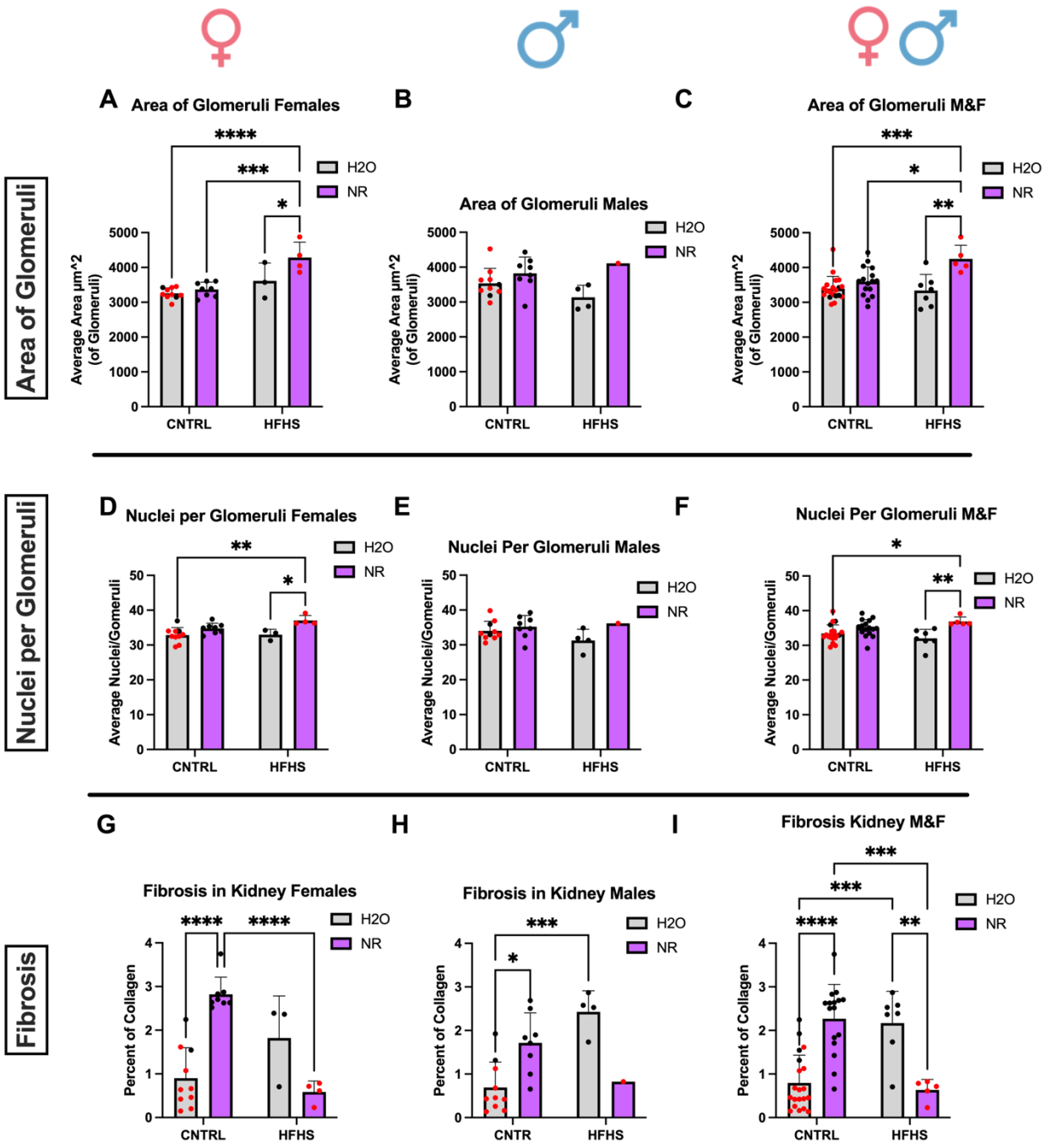

Figure 4.6 Effect of maternal obesity on kidney histology. At 47-weeks, kidneys were collected, and various histological analysis were performed. Average size of glomeruli was analyzed for females (A), males (B), and combined (C), following by number of nuclei per glomeruli for females (D), males (E), and combined (F). Kidney fibrosis was quantified to get an idea of kidney damage for females (G), males (H), and combined (I). Two-way ANOVA with Bonferroni post-hoc test assessed effects by diet and treatment. *p<0.05. CNTRL = chow-diet; HFHS= high fat high sugar; NR = nicotinamide riboside; H_2O = water vehicle.

4.7 Exposure to maternal obesity has no impact on liver histology

Following organ collection, liver histology was analyzed looking at glomeruli size, nuclei per glomeruli, and liver fibrosis. In all exposure groups, there were no significant differences in fibrosis levels and fat content (Fig 4.7A-F). However, unrelated to maternal obesity, CNTRL + NR treated females have significantly higher fat content in their livers (Fig 4.7D).

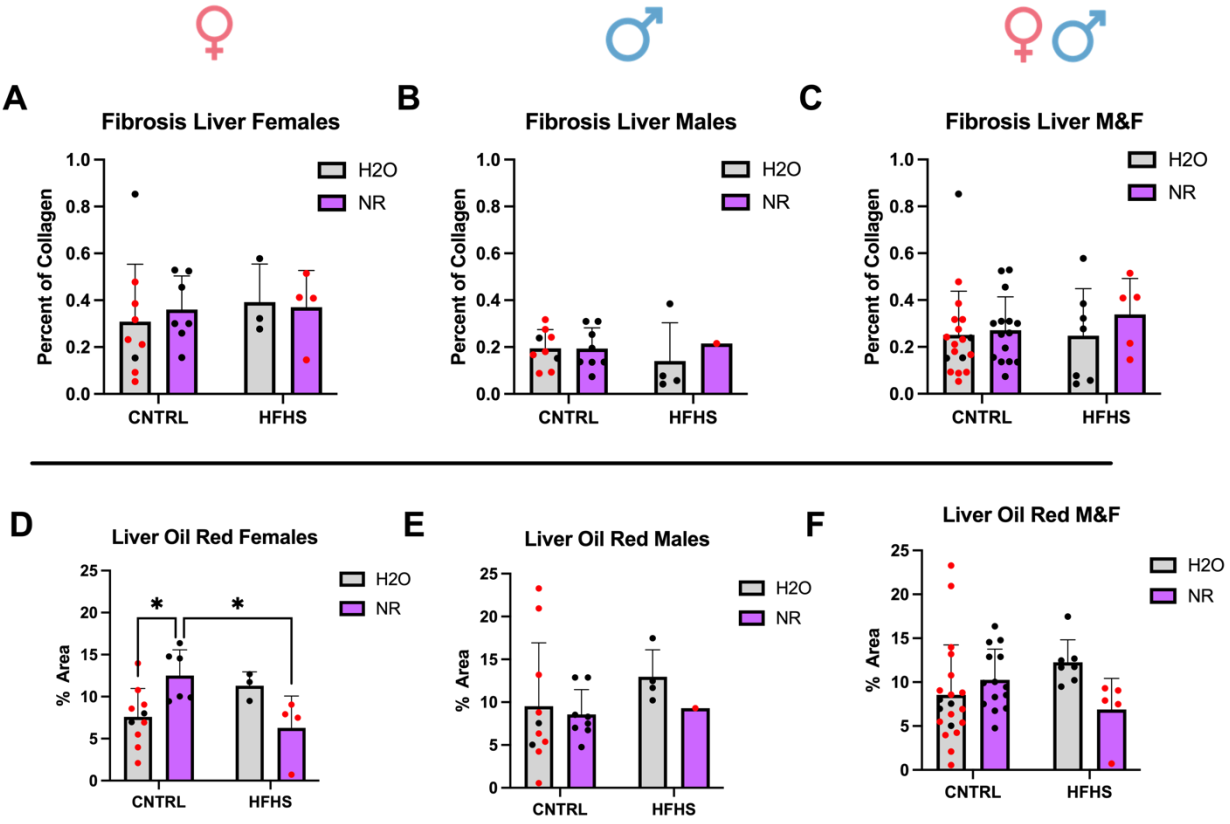

Figure 4.7 Effect of maternal obesity on liver histology. At 47-weeks, livers were collected, and various histological analysis were performed. To get an idea of liver damage, fibrosis was quantified for females (A), males (B), and combined (C). Fat content was quantified using oil red for females (D), males (E), and combined (F). Two-way ANOVA with Bonferroni post-hoc test assessed effects by diet and treatment. *p<0.05. CNTRL = chow-diet; HFHS= high fat high sugar; NR = nicotinamide riboside; H_2O = water vehicle.

4.8 Offspring exposed to maternal obesity have impaired lipolysis

Norepinephrine (NE)-induced glycerol release from adipose tissue was assessed as a measure of lipolytic function. Across all three fat depots assessed (IWAT, GWAT, and BAT) offspring exposed to a maternal HFHS diet in utero and during lactation exhibited a general

trend of reduced NE-induced glycerol release, in both sexes (Fig 4.8). This impairment reached statistical significance in female offspring for IWAT at 10 uM NE (Fig 4.8C) and in male offspring for BAT at 0.1 uM NE (Fig 4.8F). Notably, NR supplementation did not restore lipolytic responsiveness. In fact, offspring from the HFHS + NR group showed significant or trending in reduced glycerol release compared to controls across all fat depots, suggesting that NR failed to reverse, and may have exacerbated, impaired NE-induced lipolysis (Fig. 4.8C).

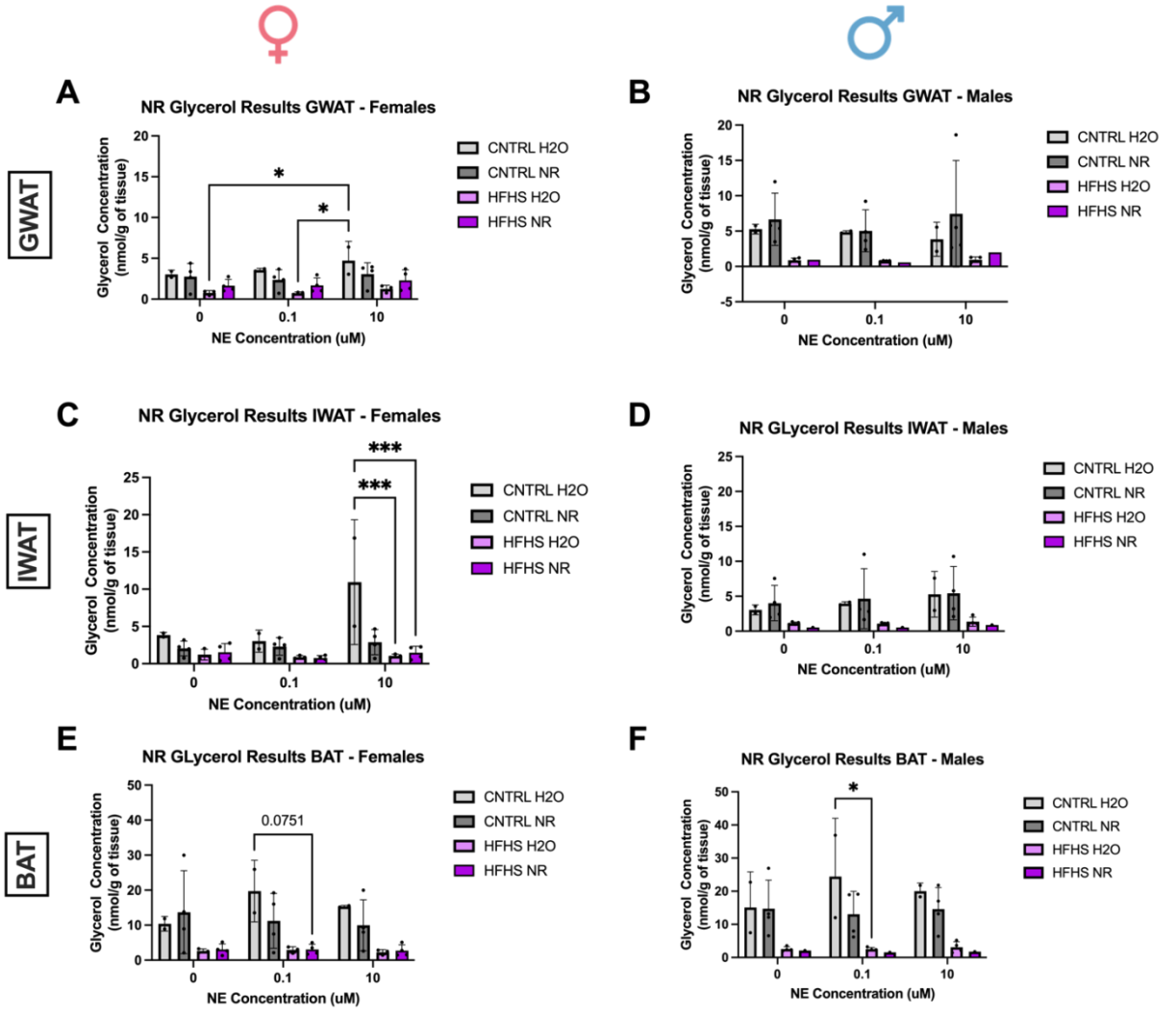

Figure 4.8 Effect of maternal obesity on norepinephrine-induced lipolysis in offspring. Norepinephrine (NE)-induced glycerol release was assessed for three adipose tissue depots in 47-week-old offspring. Gonadal white adipose tissue (GWAT; A-B), inguinal white adipose tissue (IWAT; C-D), and brown adipose tissue (BAT, E-F) were assessed across NE concentrations (0-10 uM) in females (A, C, E) and males (B, D, F), only for offspring in cohort 1. Glycerol concentration was quantified using a colorimetric glycerol assay. Two-

way ANOVA with Bonferroni post-hoc test assessed effects by diet and treatment. *p<0.05. CNTRL = chow-diet; HFHS= high fat high sugar; NR = nicotinamide riboside; H_2O = water vehicle.

Chapter 5 - Discussion

5.1 Discussion

This study builds on existing research to further elucidate long-term impact of gestational obesity on offspring cardiometabolic programming and health. Notably, it is the first to investigate the sustained effects of maternal NR supplementation during pregnancy in the context of in utero exposure to an obesogenic environment. Using a well-established HFHS diet-induced maternal obesity model, the association between gestational obesity and long-term metabolic impairments in offspring were reinforced. Sex-specific differences were observed in offspring weight gain and fasting glucose regulation, with distinct patterns emerging between male and female offspring. Surprisingly, early-life exposure to maternal obesity did not result in significant alterations in blood pressure or body composition later in life. These results underscore the importance of incorporating sex as a biological variable in studies of developmental origins of health and disease. Of note, NR supplementation during pregnancy conferred early protection against excessive weight gain and elevated fasting glucose, particularly in female offspring exposed to the HFHS conditions. Although some outcomes were unanticipated, these findings support further investigation into sex-specific interventions and the potential of NAD+ supplementation as a therapeutic strategy in pregnancies affected by obesity.

5.1.1 Long-term effects of gestational obesity on offspring growth profiles and the impact of NR supplementation

Offspring born to mothers exposed to a HFHS diet exhibited higher body weight at weaning (4 weeks of age) compared to offspring from control dams. Additionally, CNTRL NR offspring were also significantly heavier at weaning, whereas previous research reported these offspring to be of normal weight at this stage[77], indicating that they, too, experienced rapid postnatal growth. This rapid growth, though beneficial in terms of weight gain, may have long-term consequences, as postnatal growth without the typical SGA profile can also increase the risk of developing cardiometabolic health issues later in life. In contrast, HFHS H_2O offspring were significantly smaller at birth, consistent with the catch-up growth pattern typically seen in SGA infants. However, these differences did not persist into adolescence or adulthood, regardless of sex. These early postnatal weight patterns are

particularly notable given our previous findings with this same HFHS model – along with other rodent studies [91,92] [93–95], demonstrated fetal growth restriction at E18.5, including reduced fetal weight and crown rump length [96]. The rapid increase in postnatal body weight in HFHS exposed pups suggests a rapid—and potentially exaggerated—"catch-up growth" [97,98] phenotype, where offspring born SGA undergo accelerated early weight gain. While catch-up growth may promote short-term developmental benefits such as improved neurodevelopment and linear growth [99], it has also been associated with increased long-term cardiometabolic risk [100]. This pattern likely reflects compensatory activation of nutrient-sensitive growth pathways in response to earlier placental insufficiency or metabolic stress [101], highlighting the critical influence of the intrauterine environment on health trajectories.

Indeed, divergent fetal growth patterns are a hallmark of pregnancies complicated by obesity [4,102,103]. In Canada, approximately 14% of infants born to individuals with obesity are classified as SGA, and nearly 9% as LGA[4]. Birthweight serves as a convenient and widely used clinical proxy for assessing fetal exposure to adverse intrauterine conditions, and population-level data consistently link extremes in birthweight with elevated risk of long-term cardiometabolic diseases, including cardiovascular disease, type 2 diabetes, obesity, and chronic kidney disease [104] [105] [106]. However, it is important to emphasize that birthweight should be recognized as a proxy indicator of potential intrauterine stressors rather than a causal factor; it reflects underlying intrauterine conditions rather than directly causing later disease. In the context of gestational obesity, low or high birthweight may signal disruptions in placental development or function, altered nutrient transport (e.g., fatty acids, glucose, amino acids), heightened exposure to pro-inflammatory cytokines, oxidative stress, and/or endocrine imbalances [107,108] —all of which can contribute to maladaptive fetal programming and increase the risk of chronic disease in later life.

Interestingly, maternal supplementation with NR during pregnancy and lactation attenuated this early postnatal weight gain in HFHS-exposed offspring. Offspring from HFHS + NR pregnancies showed weight trajectories indistinguishable from control animals. This suggests that NR may buffer the offspring against adverse in utero

programming growth restriction followed by rapid early postnatal compensatory growth. Mechanistically, this aligns with the growing body of evidence implicating NAD^+ depletion as a driver of inflammatory and metabolic dysfunction in maternal obesity. NR, a well-tolerated NAD^+ precursors [79], has shown promise in improving placental function and fetal outcomes in other models of inflammation-mediated pregnancy complications,

For instance, NR has been demonstrated to restore placental NAD+ levels and enhance fetal growth in a rodent model of inflammation-driven preeclampsia, a condition often co-occurring with maternal obesity[76]. Additionally, a rodent study targeting maternal hypoglycemia induced FGR found that NR treatment during pregnancy enhances gluconeogenesis and mitochondrial respiration, improving fetal nutrient availability and growth[109]. While these differ from maternal obesity per se, they share core features such as systemic inflammation, placental dysfunction, and metabolic derangement. Taken together, these findings suggest that NR may mitigate the adverse effects of an obesogenic intrauterine environment by restoring placental bioenergetics and nutrient handling.

In the context of the current study, the normalization of postnatal growth in HFHS + NR offspring suggests that NR may prevent the maladaptive catch-up growth phase that often follows in utero restriction. This points to a broader therapeutic potential for NR in modulating fetal programming outcomes in pregnancies complicated by obesity—supporting not only immediate fetal growth but also longer-term metabolic stability in the offspring.

5.1.2 Long-term effects of gestational obesity on offspring glucose handling and the impact of NR supplementation

Maternal obesity is known to adversely affect fetal development, often resulting in offspring born either SGA or LGA. Both phenotypes have been linked to increased risk of metabolic dysfunction later in life. In particular, SGA offspring -despite undergoing postnatal catch-up growth and achieving normal body weight – frequently have features of metabolic syndrome, including elevated fasting glucose levels and insulin resistance[110,111]. This disconnect between body weight and metabolic status is a hallmark of maladaptive fetal programming.

In the present study, HFHS-exposed offspring demonstrated signs of impaired glucose handling, particularly among females, who showed a trend toward increased fasted glucose levels at both 12 and 19 weeks of age. Notably, even in the absence of sex-stratification, HFHS-exposed offspring overall had significantly higher fasting glucose levels at 19 weeks compared to controls. These metabolic alterations occurred despite no differences in body weight between groups, reinforcing the concept that early-life metabolic programming can manifest independently of overt changes in adiposity. The blood glucose values in the HFHS-exposed offspring- ranging from 10.4 to 11.5 mmol/L – approach or exceed the diabetic threshold established for C57BL/6 mice (>11.1 mmol/L)[112] suggesting meaningful impairments in glucose regulation. These findings also align with population-level studies in humans, where low birth weight is associated with increased risk for impaired glucose tolerance, type 2 diabetes, and broader features of the metabolic syndrome[113,114].

NR supplementation has been shown in non-pregnancy models to improve glucose metabolism, likely through restoration of intracellular NAD+ pools and activation of key metabolic regulators such as SIRT1 and SIRT3[115]. In high-fat diet-fed diabetic mice, NR administration improved fasting glucose levels, reduced hepatic steatosis, and mitigated weight gain[116,117] – effects attributed to enhanced mitochondrial function and insulin sensitivity. However, these studies typically administer NR directly by incorporating it into the diets of adult animals, whereas in the current study NR was provided exclusively during pregnancy and lactation. This difference in timing and route of exposure suggests that the mechanisms of action in the present model may be distinct and developmentally mediated.

Several plausible mechanisms may underlie the improved glucose regulation observed in HFHS + NR-exposed offspring. One possibility is that NR enhanced fetal growth trajectories via improved placental health, as previously demonstrated in our rodent model of inflammatory preeclampsia[76]. By preventing fetal growth restriction and supporting normal development, NR may reduce the offspring's susceptibility to later metabolic dysfunction commonly associated with SGA or LGA status[8,17,111,118]. Consistent with this interpretation,

HFHS + NR-exposed offspring in the present study did not exhibit altered growth patterns, which may explain their more favorable glucose responses. In addition to prenatal effects, NR may also confer postnatal benefits through lactational programming. Studies have shown that NR supplemented mothers have increased milk production and quality, coupled to elevated levels of NAD^+ and NADP+ in the mammary tissue[119]. These enhancements are thought to support improved energy and biosynthetic capacity of maternal milk, potentially contributing to healthier postnatal metabolic trajectories. Taken together, these findings suggest that NR may act through multiple developmental windows - improving placental health and enhancing neonatal nutrition during lactation – to buffer offspring against the long-term metabolic risks imposed by maternal obesity.

5.1.3 Cardiovascular effects of in utero exposure to gestational obesity and the potential for sex-specific vulnerability

This study assessed blood pressure as a key indicator of offspring cardiovascular health. While numerous human epidemiological studies have linked maternal obesity to evaluated systolic and diastolic blood pressure during childhood and adolescence[120,121 121], the evidence for a direct effect persisting into adulthood is less consistent. Some studies suggest that elevated blood pressure in offspring exposed to an obesogenic in utero environment, may be mediated by increased offspring body mass [120,122]. This interpretation aligns with the findings in the current study, where despite evidence of fetal growth restriction and early catch-up growth, no significant differences in adult blood pressure were observed between the HFHS-exposed and control offspring. The offspring in this study were weaned onto a standard chow-diet, possibly mitigating any sustained hypertensive effects that may have been exacerbated by continued postnatal metabolic stress. The absence of strong blood pressure effects here could also be interpreted through the lens of the "Multiple hits" hypothesis [123]: (1) genetic/epigenetic susceptibility, (2) adverse intrauterine exposures (e.g., maternal obesity), and (3) postnatal environmental stressors. In this study, the absence of a third hit—such as a post-weaning HFHS diet or additional stressor—may have attenuated the programming effects typically observed in offspring of obese pregnancies.

Interestingly, a modest trend toward increased systolic and diastolic blood pressure was observed in HFHS-exposed male offspring at 36 weeks of age. This may reflect a delayed manifestation of gestational programming, which has been reported in rodent studies showing late-onset hypertension [124,125], particularly in males [125]. The hypothesis that males 'live dangerously' in utero - prioritizing their own growth at the expense of placental reserve capacity - offers a potential explanation for this sex-specific vulnerability[126]. In other rodent models of maternal obesity, blood pressure elevations have only been observed in male offspring as early as 6 months, while in some cases effects only emerge in late adulthood [124,125]. Although this study's findings did not reach statistical significance, the observed trend may warrant further investigation in a more prolonged model or with additional metabolic stressors.

5.1.4 Body composition and metabolic flexibility following in utero exposure to gestational obesity and NR

Offspring body composition and whole-body metabolism were assessed using Echo-MRI and CLAMS, respectively. No significant differences in fat mass percentage were observed at 12 or 24 weeks; however, by 47 weeks of age, a significant increase in fat content - particularly in females – was evident in HFHS-exposed offspring. While many studies report increased adiposity in offspring of obese dams[124,127,128], variations in the maternal diet models may explain the discrepancy. For instance, this study employed a HFHS diet that closely reflects a "Western diet," compared to others that used high-fat-only regimens. Moreover, the lack of postnatal stressors may have limited the expression of a programmed adiposity phenotype, again aligning with the Multiple Hits hypothesis.

Whole-body metabolic assessments of offspring at 21 weeks of age using CLAMS revealed no significant differences in oxygen consumption (VO_2), respiratory exchange ratio (RER), or energy expenditure between groups. Offspring born to obese versus control dams showed no significant differences in most of these parameters. This contrasts with other rodent studies which demonstrated impaired metabolic flexibility in offspring exposed to maternal obesity, marked by reduced energy expenditure and RER responses, suggesting a shift

toward greater fat oxidation [129130]. These effects occurred independently of food intake and activity levels [129,130], suggesting potential alterations in metabolic programming. Notably, when these offspring were maintained on a HFD following weaning, the disturbances were significantly exacerbated[130]. These findings suggest that metabolic disturbances from gestational obesity may remain masked in adulthood unless compounded by additional postnatal insults.

One novel and unexpected finding in this study was a trend toward increased X ambulatory activity in HFHS-exposed female offspring, compared to controls, which was significantly reduced in those treated with NR during gestation and lactation. While previous gestational obesity studies have not reported increased physical activity per se, increased stress- or anxiety-like behaviours have been documented in offspring[131–133], particularly females. Some evidence suggests that these behavioural changes may be linked to altered neurodevelopment or reduced brain-derived neurotrophic factor (BDNF) expression [132], both of which are susceptible to modulation by maternal diet. Interestingly, and of high relevance to the current project, NR supplementation during pregnancy mitigated behavioural abnormalities and restored BDNF levels in offspring exposed to maternal obesity. Although CLAMS-derived X ambulatory activity has not traditionally been as a proxy for neuropsychiatric behaviour, it may reflect underlying differences in arousal or stress responsiveness programmed by in utero exposures.

Altogether, while this study did not identify overt changes in body composition or metabolic function at mid-life, subtle trends in fat accumulation and behavior suggest that the programming effects of gestational obesity may emerge later or under specific conditions. Importantly, the observation that NR normalized these trends highlights its potential for mitigating the long-term cardiometabolic and neurodevelopmental risks associated with maternal obesity.

5.1.5 Consequences of gestational obesity on offspring organ histology

Various histological analyses were conducted on the liver and kidneys of the offspring to evaluate organ function. In the liver, no significant differences were detected, fat content

remained unchanged, and there were no signs of fibrosis or structural damage in offspring exposed to maternal obesity. Similarly, kidney assessments revealed no changes in glomeruli size or nuclei per glomerulus, suggesting no hypertrophy or hypercellularity. However, in male offspring, as well as in the overall group when not stratified by sex, exposure to maternal obesity was associated with a significant increase in kidney fibrosis, potentially indicating early signs of kidney damage, and a potential indicator of chronic kidney disease (CKD). Although it has been established that maternal obesity has substantial impact of chronic disease risk in offspring, with evidence in both human and animal studies suggesting programming of offspring towards metabolic syndrome[134], the exact link between kidney damage and CKD is not yet well understood. Other animal studies have recently shown that exposure to maternal obesity has substantial effects on offspring renal function, such as changes in kidney function, increase in renal fibrotic markers, and increase in both oxidative stress and inflammation[135]. Although maternal obesity can affect offspring kidney health through multiple pathways, one key mechanism involves the downregulation of the Farnesoid X receptor (FXR), a nuclear hormone receptor highly expressed in the kidneys that plays a critical role in bile acid, glucose, and lipid metabolism. In the kidney, FXR activation suppresses lipogenic and fibrotic genes, mitigating diabetes, and obesity-related changes[136]. The observed fibrosis in this current study may stem from impaired FXR signaling, suggesting a potential link between maternal obesity and early kidney damage, though the exact mechanism remains unclear without further study.

In the absence of maternal obesity, CNTRL + NR offspring demonstrated significantly greater kidney fibrosis and hepatic fat content, raising concerns that NR supplementation during a healthy pregnancy could lead to unanticipated risks to long-term offspring organ health. Although the mechanisms underlying the impact of NR treatment during healthy pregnancies on offspring organ health remain unclear, one possibility is that the rapid postnatal growth observed in these offspring at weaning contributes to these effects. Rodent pregnancy studies in which healthy dams received NR supplementation reported effects on both birthweight and lactation. Specifically, NR treatment enhanced lactation

and improved milk quality, producing larger weanlings that were of normal weight at birth[137,138], a pattern consistent with the offspring observed in the present study. Although these pregnancy studies did not follow offspring long enough to assess changes in organ health, other non, NR-related research has shown that overnutrition, potentially driven by enhanced lactation, particularly in small litter sizes—can alter both kidney and liver health[139,140].

In the kidneys, rapid postnatal growth in the absence of SGA is frequently associated with elevated renal renin and angiotensin receptor expression during nephron maturation[139]. This elevation can drive localized angiotensin II overactivity, leading to efferent arteriole constriction, increased glomerular pressure, and activation of fibrotic signaling pathways[139]. Moreover, such accelerated growth often results in early-life obesity and subsequent leptin resistance, which is linked to upregulation of suppressor of cytokine signaling 3 (SOCS-3). SOCS-3 disrupts normal cellular signaling, thereby promoting inflammation and fibrosis[139]. In the liver, early-life overnutrition and rapid postnatal growth, particularly during critical developmental periods, may heighten the risk of developing non-alcoholic fatty liver disease (NAFLD). More specifically, postnatal overfeeding triggers the activation of tissue glucocorticoid (GC) activity by upregulating the enzyme 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1)[140]. This enzyme converts inactive cortisone into its active form, cortisol, within various tissues, including the liver. The increased cortisol levels promote hepatic insulin resistance, lipid accumulation, and inflammation, ultimately raising the risk of NAFLD[140]. Although CNTRL + NR offspring in this study exhibited both kidney fibrosis and fatty liver, further research is needed to determine the underlying mechanisms driving these alterations.

5.1.6 Consequences of gestational obesity on offspring fat metabolism

Norepinephrine (NE) stimulation and subsequent glycerol assays were performed on three adipose depots (IWAT, GWAT, BAT) to assess NE sensitivity and NE-induced lipolysis via glycerol release. Although limited research exists on how maternal obesity impacts offspring lipolytic function, several plausible mechanisms can be inferred from established metabolic pathways. In a non-pregnant obese state, NE-induced lipolysis is often impaired

due to disruptions in β-adrenergic receptor (β-AR) signaling[141]. These receptors play a critical role in initiating lipolysis by activating adenylate cyclase, which increases intracellular cyclic AMP (cAMP) and subsequently activates protein kinase A (PKA). This signaling cascade leads to phosphorylation of enzymes such as hormone-sensitive lipase (HSL), which promotes the breakdown of stored triglycerides into free fatty acids (FFAs) and glycerol[142]. FFAs are then oxidized for energy or converted into ketone bodies, while glycerol serves as a carbon source for gluconeogenesis in the liver[142]. Obesity-associated impairments in lipolysis may arise from reduced β3-ARs expression in adipocytes, leading to blunted responsiveness to NE[143]. Furthermore, obesity-induced inflammation increased pro- inflammatory cytokines such as TNF-α, which are known to interfere with β-AR signaling and contribute to catecholamine resistance[144].

Although the HFHS-exposed offspring in the current study did not exhibit overt obesity at 47 weeks - showing no significant differences in body weight or composition compared to controls - early-life exposure to maternal obesity may still have lasting metabolic effects on adipose tissue function. Supporting this, human studies have shown that early-life obesity or altered adiposity can have long-term consequences on fat metabolism, even if BMI normalizes in adulthood[143,145]. In this context, the observed reduction in NE-induced glycerol release in both male and female HFHS-exposed offspring may reflect long-term alterations in adipose tissue sensitivity. Given that these offspring also showed evidence of fetal growth restriction and rapid postnatal catch-up growth it is plausible that this growth trajectory contributed to impaired lipolytic capacity. Several studies have linked SGA status at birth with altered fat distribution and metabolism later in life[118,146], suggesting that early developmental exposures can permanently reprogram adipose tissue function. Notably, NR supplementation during pregnancy and lactation did not restore glycerol release in HFHS-exposed offspring, indicating that this intervention may not be sufficient to reverse alterations in fat metabolism established during critical developmental windows.

5.1.7 Clinical implications of NR supplementation in pregnancies affected by obesity

While the preceding sections have outlined the specific effects of NR supplementation on various offspring outcomes, it is also important to consider the broader clinical relevance

and potential applications of these findings. This study contributes to a growing body of research exploring the use of NR—a precursor to NAD^+—as a potential intervention to mitigate the developmental effects of gestational obesity. NR supplementation during pregnancy and lactation showed partial efficacy in protecting offspring from early-life metabolic disturbances, such as excessive early postnatal weight gain and impaired glucose regulation, particularly in females. These effects suggest that NR may influence critical windows of metabolic programming and could hold therapeutic potential for improving cardiometabolic trajectories in at-risk offspring.

However, NR did not uniformly protect against all outcomes. No improvements were observed in blood pressure regulation, lipolytic function, or late-life adiposity, underscoring the need to further refine dosing strategies and treatment timing. Importantly, gestational obesity may involve a more chronic and severe inflammatory profile than acute inflammatory models like LPS-induced preeclampsia, where NR has shown robust benefits[76]. In the current study, NR was administered following confirmation of pregnancy, a timing chosen to reflect a clinical scenario in which a woman with obesity is requesting therapeutic options to optimize fetal health after finding out she is pregnant. However, emerging data from our research group suggest that preconception NR supplementation may offer superior protection by shifting maternal metabolism toward a “metabolically healthy obesity” phenotype prior to pregnancy, resulting in improved fetal growth and development[147]. At the same time, we did observe seemingly negative side effects of NR supplementation during healthy pregnancies, including rapid postnatal growth and histological changes, highlighting the complexity of its effects across different maternal contexts.

These findings highlight the complexity of targeting metabolic programming and suggest that future translational efforts must carefully consider the timing, dosage, and maternal metabolic context when designing NAD^+-boosting interventions. While NR remains a promising candidate, we also observed seemingly negative effects in healthy pregnancies, including rapid postnatal growth and histological changes, underscoring the need for caution in non-obese contexts. More work is therefore required to define which patient

populations and exposure windows will derive the greatest benefit. Ultimately, this research supports the ongoing investigation of NAD^+ metabolism as a modifiable pathway with clinical relevance for improving pregnancy outcomes and reducing intergenerational transmission of metabolic disease.

5.2 Limitations

This study has generated several novel findings; however, there are important limitations that must be considered when interpreting the results. Most importantly, data were collected from two independent cohorts of mice, established at different times, and exposed to different environmental conditions during development. The use of two cohorts was necessitated by significant breeding challenges, particularly animals maintained on the HFHS-diet, which exhibited a 20-25% reduction in mating success. Even among successful pregnancies, high rates of maternal cannibalism further limited and unequally reduced offspring numbers across diet and treatment groups, prompting the need to establish a second cohort.

Unfortunately, during the time between cohorts, certain uncontrolled environmental variables changed. Specifically, due to space limitations in the animal care facility, cohort 2 (red) was housed in a different mouse room than cohort 1 (black) and was exposed to additional external stressors, including ongoing construction and persistent vibrations. These conditions likely imposed additional parental stress on pregnant dams, which is well-documented to alter fetal development through elevated maternal glucocorticoid levels and can independently induce cardiometabolic programming in offspring[148,149].

An added complexity arises from the fact that not all treatment groups were equally represented across both cohorts. For example, some groups—particularly CNTRL and NR-treated offspring—were more heavily enriched in cohort 2. This uneven distribution introduces potential cohort-related confounding, where environmental and temporal differences between cohorts may influence outcomes independently of experimental treatments. For instance, CNTRL H_2O offspring from cohort 2 tended to be heavier, larger, and exhibit higher blood pressure and fasting glucose levels than their cohort 1

counterparts - potentially reflecting the influence of greater in utero stress rather than diet or treatment effects alone. To provide transparency, all figures in Chapter 4 distinctly indicate individual offspring cohort membership using colour coding (Cohort 1 = black; Cohort 2 = red), allowing readers to consider cohort effects when evaluating the data. Additionally, some comparisons may have lacked sufficient statistical power due to the substantial imbalance in group sizes, particularly the reduced number of HFHS-exposed offspring caused by mating and litter survival issues. Another limitation is that stratifying by sex sometimes did not yield significant results, while not stratifying by sex did, likely due to a power issue arising from small sample sizes, which made it challenging to analyze sex differences.

Other limitations worth noting include the reliance on a single NR dosing regimen without dose titration; and the lack of mechanistic follow-up, such as molecular analysis of placental tissue or adipose signaling pathways, which could clarify the pathways involved in observed phenotypes.

5.3 Future Directions

This study further advanced our understanding of how gestational obesity influences offspring development and cardiometabolic programming, and how NR supplementation during pregnancy and lactation may mitigate some of these effects. While early-life disturbances in offspring weight gain, glucose regulation, and adipose tissue metabolism were observed, overall metabolic health remained relatively stable into adulthood, particularly in the absence of postnatal metabolic stressors. However, in a real-world context, offspring born to individuals with obesity are often exposed to similar lifestyle and dietary patterns after birth, including HFHS diets. Future work will explore how offspring exposed to gestational obesity throughout pregnancy and lactation respond metabolically when challenges with additional postnatal stressors, such as high-fat diet feeding or sedentary environments, and how this response compares to control offspring under the same conditions. This line of inquiry is critical for understanding how prenatal

programming interacts with postnatal exposures to influence long-term cardiometabolic health across the lifespan.

An exciting and emerging area of focus within our group involves the preconception window as a target for intervention. Preliminary data suggest that NR supplementation initiated prior to pregnancy not only improves fetal growth and development but also fully rescues the subfertility phenotype observed in HFHS-fed dams—restoring both mating success and live birth rates. These findings, independent of maternal weight loss, suggest a novel role for NAD^+ metabolism in obesity-related infertility. However, we also observed seemingly negative effects of NR supplementation during healthy pregnancies, including rapid postnatal growth and histological alterations, highlighting the importance of exploring the safety of NR on healthy pregnancies. Additionally, future work will explore how preconception NR supplementation alters maternal reproductive physiology, placental function, and offspring outcomes, and whether it represents a viable strategy to break the intergenerational cycle of metabolic dysfunction.

Together, these future directions aim to build a more comprehensive understanding of how NAD^+-boosting strategies can be leveraged across key developmental windows to improve maternal-fetal health and long-term offspring resilience.

5.4 Conclusions

This study explored the impact of gestational obesity on the long-term cardiometabolic health and programming of offspring, both with and without NR supplementation. The findings demonstrate that in utero exposure to an obesogenic environment results in SGA offspring and sex-specific cardiometabolic alterations. Offspring exposed to gestational obesity also showed increased risk of fat accumulation and disrupted fat metabolism, mechanisms that may contribute to obesity, insulin resistance, and other metabolic disorders later in life. Although NR supplementation offered early protection against some cardiometabolic disturbances, it was not sufficient to fully rescue alterations in fat accumulation and adipose tissue function. Moreover, we observed that NR may exert seemingly negative effects when administered during healthy pregnancies, including rapid

postnatal growth and histological changes, though further research is required to confirm these findings. However, these findings lay important groundwork for future studies targeting earlier intervention windows and offer promising insight into the potential of NAD^+-boosting strategies to improve long-term health outcomes in at-risk offspring.

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