Chronic Gestational Inflammation: Comparisons of Obesity-Mediated and Preeclampsia-Mediated Placental Inflammation using Transcriptomic and Histological Methods

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Statement of Authorship

The principal investigator for the following study was Dr. Shannon Bainbridge, who was responsible for the conceptualization, funding, and oversight of the study. Hannah Poisson and Fahmida Jahan were responsible for the development and implementation of the animal models, conducting all experiments related to the animal model. The Louise Pelletier Imaging Core in the Department of Pathology and Laboratory Medicine at the University of Ottawa was responsible for all placental paraffinization/deparaffinization, sectioning, and staining. The Centre for Applied Genomics at the Hospital for Sick Children (SickKids), affiliated with the University of Toronto, was responsible for all RNA Sequencing. Jasmine Tripathi, Andrés Gerardo Bautista Orozco, Diana Isabel López Valdés, and Shannon Blake were responsible for assisting with RNA extraction. Jasmine Tripathi was responsible for all analyses. The manuscript was prepared by Jasmine Tripathi, revised by Dr. Shannon Bainbridge and Dr. Jonathan Lee.

Statement of Ethics and Animal Care Approval

No human recruitment, samples, or data were used; thus ethics approval was not required for the current study. Animal care committees (ACC) protocols # HSe-2923-R3 A2 and #CMM-3937e were approved for this study, with a copy of the ACC protocols included in Appendix 1.

Keywords/Abbreviations:

Placenta, Preeclampsia, Gestational Obesity, Mitochondria, Chronic Inflammation, Nicotinamide Adenine Dinucleotide (NAD+), Sirtuins (SIRTs), Poly-ADP Ribose Polymerases (PARPs), Nicotinamide Riboside (NR), Tumor Necrosis Factor (TNF), Interleukins (ILs), Interferons (IFNs)

Abstract

Introduction: Several obstetrical complications, including maternal obesity (MO) and preeclampsia (PE), are associated with states of chronic gestational inflammation and poor pregnancy outcomes. However, commonalities and/or differences from these divergent chronic inflammatory insults on placental development and function remain elusive. Preliminary work by our group suggests placental NAD^+ depletion and subsequent mitochondrial dysfunction may be common pathophysiology features. Using rodent MO- and PE-mediated gestational inflammation models, our aim was exploring common and differing mechanisms through which placental function may become compromised, targeting NAD^+ signalling pathways and mitochondrial health.

Methods: Rodent models of immune-driven PE and MO were previously established in our laboratory. Dams were euthanized at embryonic day 18.5, with assessments of fetal and placental weights. Placentas were then collected and processed for detailed histomorphometry and transcriptomic analyses. Placenta transcriptomics data underwent differential gene expression analysis using DESeq2. Ranked gene set enrichment was conducted using fold change values through GO Annotation Database. Similarly, pathway enrichment was performed through KEGG database (FDR <= 0.10).

Results: Fetal growth restriction was observed in both chronic gestational inflammation models. Both MO- and PE-mediated inflammation was associated with reduced placental labyrinth exchange areas. MO-mediated inflammation additionally demonstrated increased junctional zone areas. Transcriptomic analysis revealed enriched inflammatory signalling pathways in both models; the PE model demonstrated profound TNF-Alpha signalling activation while the MO model demonstrated activated IL-1β, IFN-β and TNF signalling, and leukocyte chemotaxis. Likewise, both models demonstrated transcriptomic evidence of mitochondrial dysfunction including downregulated mitochondrial ATP synthesis, oxidative phosphorylation and NADH synthesis pathways.

Conclusion: Chronic gestational inflammation is associated with disrupted placental structure and gene expression, culminating in altered fetal growth. While our PE and MO models displayed clinically relevant inflammatory phenotypes, the pathological mechanisms underlying

the established placental dysfunction appear distinct. Further studies are needed to clarify the potential role of inflammation-mediated mitochondrial dysfunction in establishing inflammatory placental pathologies to inform design of better therapeutic management of these pregnant population.

Chapter 1 – Introduction

1.1 Pregnancy and the Placenta

Pregnancy is the process through which a fetus develops within a uterus. It is approximately 40 weeks long and is divided into 3 trimesters consisting of about 3 months each, with their individual developmental milestones. The first trimester is the embryonic period during which fertilization, implantation as well as placentation occurs. Organogenesis, the formation of major organs and systems, starts during this period, setting the groundwork for the fetus's future development. Continuing into the second trimester, the fetus experiences substantial growth, and its organs continue to mature. Notable advancements include the development of functional kidneys, the formation of facial features, and the refinement of sensory systems. The mother may begin to feel fetal movements, often referred to as quickening, which is a sign of the fetus's increasing activity and strength. The third and final trimester is a crucial period marked by the maturation of vital organ systems such as the brain, kidneys, and lungs. Fetal growth accelerates with the significantly increased fetal size and weighs as well as greater fetal fat deposition necessary for regulating body temperature after birth and providing energy reserves. Simultaneously, the gestational parent undergoes critical physical changes such as increased weight gain, increased uterine size, and hormonal shifts to facilitate childbirth. These changes ensure that both the mother and fetus are well-prepared for delivery (Levi & Lyons, n.d.).

The placenta is the vital organ of pregnancy. As the first fetal organ to develop, normal placental growth and development is crucial to create a vasculature network at the parental-fetal interface (Gude et al., 2004; Maltepe & Fisher, 2015). This unique organ is a key player in nutrient and gas transport, as well as metabolic, endocrine and protective processes, all of which is reflected structurally (Herrick & Bordoni, 2023). Functionally, the placenta must be able to facilitate an efficient exchange of nutrients, gas and waste products between the gestational parent and fetus, while maintaining selective control over what is able to pass through the numerous cellular layers that separate the parental and fetal circulations (known as the interhemal membrane) - namely the multinucleated syncytiotrophoblast (SynT) layer, the villous cytotrophoblast cells (CTB), and the fetal vascular endothelial cells (Huppertz, 2008). This protective aspect of the placenta is balanced by its numerous active and passive transport systems (Maltepe & Fisher, 2015). The high surface area of the placental interhaemal membrane allows for rapid respiratory gas exchange to occur between the parental and fetal circulations through passive diffusion

(Huppertz, 2008). Efficiency in this process is amplified by fetal hemoglobin having a greater affinity for oxygen compared to parental hemoglobin, ultimately favoring the transfer of oxygen from the gestational parent to the fetus (Gude et al., 2004). Transport of all other substances including nutrients, water, inorganic ions, minerals and hormones, is highly controlled and occurs through facilitated diffusion and protein-mediated active transport mechanisms seen on the placental interhemal membrane (Herrick & Bordoni, 2023). Additionally, the placenta also produces various endocrine, paracrine, and/or autocrine factors (i.e. progesterone, human chorionic gonadotrophin, lactogen, placental growth hormone, insulin-like growth factors I and II) that are released into both the parental and fetal circulations, permitting parental physiological adaptation to pregnancy and supporting optimal fetal development (Gude et al., 2004; Herrick & Bordoni, 2023).

The development of the placenta begins in the first trimester, initially forming from the outer cellular layer of the pre-implantation blastocyst, the trophectoderm (Turco & Moffett, 2019), which differentiates into an inner layer of CTBs and an outer multinucleated mass of SynTs. The SynT layer invades into the uterine endometrium, creating lacunar structures which serve as the initial nourishment for the embryo (Aplin, 2000; Gude et al., 2004). Meanwhile, the CTBs undergo proliferation and begin to push columns of cells into the SynT layer, creating the primary villous structures (CTB core with a SynT outer layer) (Benirschke, 1973; Turco & Moffett, 2019). Extraembryonic mesenchymal cells subsequently invade the primary villi core to form secondary villi. These structures undergo further expansion and branching, with accompanying fetal vasculogenesis (de novo formation of new blood vessels) creating a capillary network within the villi core, connecting the fetus to the placenta via the umbilical vasculature. These tertiary villi structures, referred to as the chorionic villi, will be bathed in parental blood, and serve as the functional site of all parental-fetal exchange across pregnancy (Burton & Jauniaux, 2015; Gauster et al., 2022). A subset of cytotrophoblast cells that sit at the tip of the CTB columns also undergo terminal differentiation into the extravillous cytotrophoblast (EVT) cell population, whose purpose is to further invade into the uterine decidua and actively remodel the uterine spiral arteries (Gude et al., 2004; Turco & Moffett, 2019). This process results in large diameter uterine blood vessels that are no longer responsive to parental vasopressors (Gude et al., 2004), ultimately increasing parental blood flow into the placenta.

By the end of the first trimester of pregnancy, the placenta has established two distinct circulatory systems (**Fig. 1.1.1**), bringing parental and fetal blood in very close proximity without permitting mixing of blood. The utero-placental circulation is formed from the initial remodelling of the decidual portion of the spiral arteries by the EVTs, through the secretion of proteolytic enzymes and cytokines, such as IL-6 and IL-8 (Aplin, 2000; S. D. Smith et al., 2009, 2016). This recruits a specialized population of immune cells – the uterine natural killer (NK) cells - to the vascular smooth muscle layers in the decidua basalis where, in conjunction with the invasive EVTs, they facilitate the breakdown of vascular extracellular matrix in the vessel wall, replacing it with fibrinoid which often holds residual trophoblasts (Choudhury et al., 2017; Harris et al., 2010; S. D. Smith et al., 2016). As a result, the spiral arteries are softened to render them passive expanded channels with no elastic recoil and a progressively decreasing resistance as the remodelling process continues. This transformation of the spiral arteries is completed by the final displacement of trophoblastic plugs in the terminal lumens of these arteries, enabling blood to enter the intervillous space at high volumes with low velocity, creating a haemochorial placental system, characterized by a highly regulated barrier at the parental-fetal exchange interface (Harris et al., 2010; V. H. J. Roberts et al., 2017; Soares et al., 2012, 2018). By the end of the first trimester, the uterine remodelling is completed, and the parental blood flow, rich in nutrients and oxygen, is now able to flow freely into the interstitial space. At this point, parental cardiac output is seen to increase with greater blood flow through the main uterine arteries, consistent with the increased artery diameter (Dickey & Hower, 1995).

In opposition, the feto-placental circulation is composed of highly branched capillary beds encased in the chorionic villous structures – the functional exchange units of the placenta. These tree-like structures are bathed in the oxygen and nutrient rich parental blood that has travelled into the intervillous space from the utero-placental circulation. The chorionic villous structures are connected directly to the fetus through a pair of umbilical arteries (UA) - which carry oxygen-deficient, carbon dioxide-rich fetal blood to the placenta – and one umbilical vein - responsible for returning oxygen-rich fetal blood from the placenta to the fetus (Aplin et al., 2020; Gude et al., 2004).

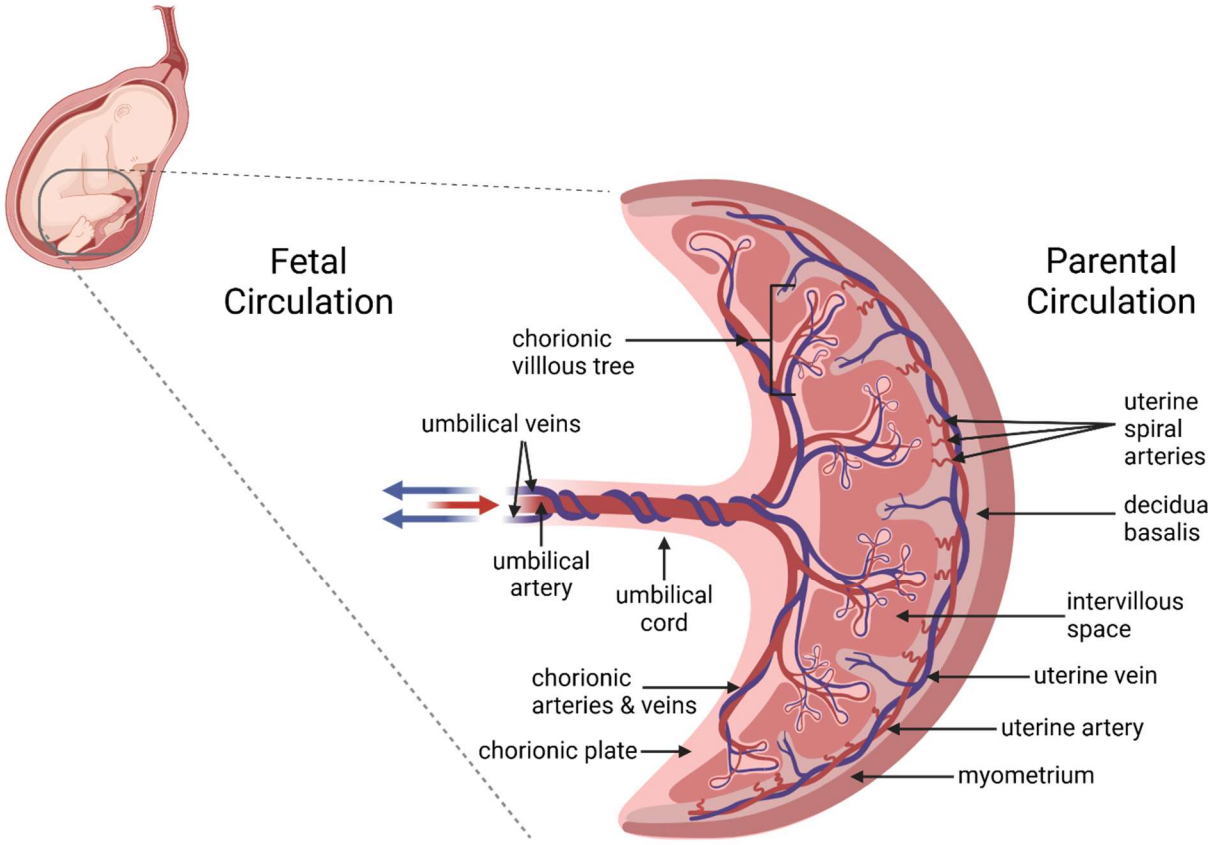


Figure 1.1.1 Anatomy of a human placenta. The diagram illustrates the two interconnected but distinct circulations in the placenta: parental blood flows through the intervillous space, while fetal blood circulates through the chorionic villi, enabling vital and efficient exchange of nutrients and gases. Figure generated from BioRender.com

Due to obvious ethical and sampling constraints, rodent models including the mouse and rat, are often used to study the development and function of the placenta across pregnancy (Aguilera et al., 2022; Carter, 2007). Although there are no perfect models to study human placentation, both murine models of placentation are quite like humans, with a few key structural differences (**Fig. 1.1.2**). The human placenta has two distinct functional zones: the villous region, the site of the exchange between parental and fetal blood at the chorionic villous tree structures, and the extravillous regions, where the placenta is attached to the uterine wall through the EVTs that also remodel the parental vasculature (Huppertz, 2008). In the rodent placenta similar structures exist, however the cellular composition and functions differ slightly; the labyrinth zone (human equivalent: villous trophoblast) and junctional zone (human equivalent: extravillous trophoblast) (Soares et al., 2012; Soncin et al., 2018).

Within the rodent ‘villous region’, called the labyrinth, the interhaemal membrane consists of an incomplete layer of trophoblast giant cells (in contact with parental blood), followed by two overlying layers of SynTs, the CTBs and finally the fetal endothelial cells (Aguilera et al., 2022; Carter, 2007). Within the rodent ‘extravillous region’, called the junctional zone, there exists, in addition to the original trophoblast progenitor cells, 3 additional trophoblast cell lineages separating the parental and fetal circulation: the trophoblast giant cells, the spongiotrophoblasts, and glycogen cells (De Rijk et al., 2002; Fonseca et al., 2012; Hemberger et al., 2020). This region of the rodent placenta plays important roles in nutrient storage and hormone production, in addition to the invasive and remodelling functions seen in the equivalent human placenta region. It is important to note that while trophoblast invasion of the uterine wall in rats resembles that of the human placenta, this is not the case for the mouse placenta, in which a much shallower intrauterine invasion and ultimately superficial placentation is observed (John & Hemberger, 2012; Soares et al., 2012). However, all three species undergo hemochorial placentation with vascular remodeling of the uterine spiral arteries occurring similarly, largely driven by invasive trophoblasts and uNK cells (Soares et al., 2012, 2018).

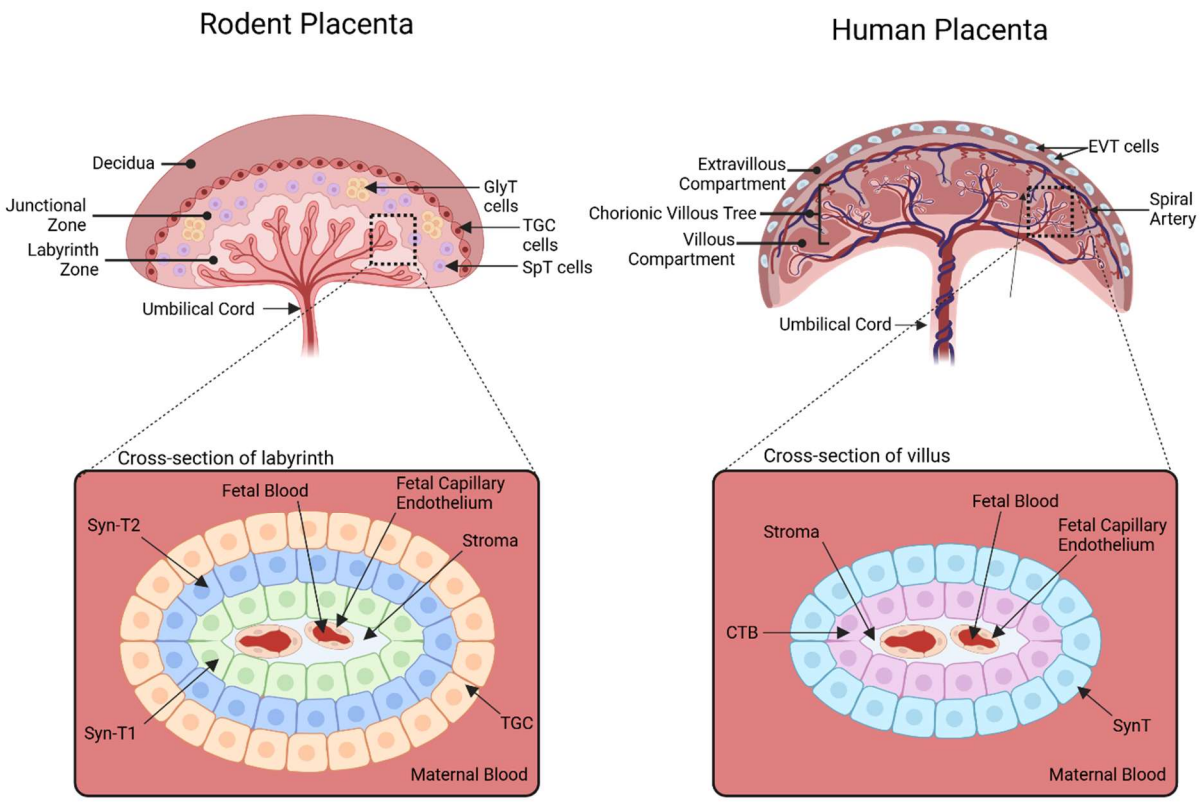


Figure 1.1.2. Comparison of Rodent and Human Placenta. In rodents, the placenta is organized into three zones: the decidua (an extravillous compartment containing uterine arteries), the junctional zone (comprising glycogen trophoblasts, spongiotrophoblasts, and trophoblast giant cells involved in remodeling uterine arteries), and the labyrinth zone (the villous compartment containing fetal trophoblasts). Rodent fetal trophoblasts are characterized by two syncytiotrophoblast layers: syncytiotrophoblast layer I and syncytiotrophoblast layer II, in addition to the trophoblast parietal giant cell. In humans, the placenta has two main compartments: the extravillous compartment (the decidua, which includes extravillous trophoblasts that remodel uterine arteries) and the villous compartment (featuring chorionic villi composed of fetal trophoblasts). Human fetal trophoblasts consist of a single syncytiotrophoblast layer. GlyT = glycogen trophoblast/glycogen cell, SpT = spongiotrophoblast, TGC = trophoblast parietal giant cell, SynT1= syncytiotrophoblast layer I, SynT2 = syncytiotrophoblast layer II, EVT = extravillous cytotrophoblast, CTB = cytotrophoblast, SynT = syncytiotrophoblast. Figure generated from BioRender.com

1.2 Inflammation in pregnancy

A successful pregnancy is inextricably intertwined with appropriate immune system adaptations and regulation. Inflammation is a key component of numerous reproductive processes, such as menstruation, ovulation and implantation, as well as an important contributing factor for a successful pregnancy (Romero et al., 2007). Pregnancy has a complex immunological profile through which a highly regulated, low grade inflammatory response creates the optimal conditions for key developmental processes across pregnancy (Kalagiri et al., 2016). There are 3 distinct immunological stages, corresponding to each stage of fetal development and its unique needs, that are described in pregnancy: 1) implantation and placentation inducing a dominant adaptive T-helper (Th)-1 cytokine pro-inflammatory phase; 2) a Th-2 cytokine dominant anti-inflammatory period associated with fetal growth; 3) the final Th-1 mediated pro-inflammatory response required for parturition (Mor et al., 2017).

Prior to pregnancy, ovarian steroid hormones 17β-estradiol and progesterone which are secreted during the ovulatory phase of the menstrual cycle initiate decidualization, the process of uterine endometrium differentiation into the functional decidua. During this time, marked changes are seen in all structures of the uterine mucosa (i.e. epithelial and blood vessels, stromal and immune cells), with a rapid accumulation of a specialized population of pregnancy-specific immune cells known as uterine natural killer (uNK) cells (Moffett & Shreeve, 2023; Mor et al., 2011). These uNK cells are vital for successful implantation, placentation and early spiral artery remodelling events.

Implantation and placentation are responsible for the first pro-inflammatory stage in pregnancy as a delicate balance is struck between the invading trophoblasts and the receptivity of the parental decidua - central for the success of the pregnancy. The initial blastocyst implantation to the uterine epithelial lining and its subsequent placentation in which the decidua is actively broken down and restructured, resembles an inflammatory response seen in tissue injury and repair processes (Moffett & Shreeve, 2023). Early implantation is characterized by a presence of proinflammatory Th-1 cells, as well as immune cells such as uNKs, dendritic cells (DCs) and M1-macrophages (MPs) recruited to the implantation site, triggering the secretion of proinflammatory cytokines such as IL-6, IL-15, IL-8, CC-chemokine ligand 4 (CCL4) and TNFα (Mor et al., 2011, 2017). Additionally, the uNKs further propagate this immune response through the release of angiogenic factors, required for regulating parental vascular growth within the decidua (Mor et al., 2011).

Following a successful implantation and placentation phase, comes a prolonged period of fetal growth and development, associated with an anti-inflammatory Th-2 mediated cytokine response, characterized by the presence of M2-MPs, regulatory T-cells (T-reg) and the release of pro-inflammatory factors such as IL-10 and transforming growth factor-beta (TGF-b) (M. B. Brown et al., 2014). The third, and final pro-inflammatory response, is seen at the end of pregnancy, promoting processes involved in parturition via the nuclear factor-κB (NF-κB) signalling pathway. The predominance of both Th-2 and M1-MPs is once again seen, as well as other pro-inflammatory cytokines, all of which promote uterine contractions and successful delivery of both fetus and placenta, as well as aid in the subsequent recovery of the uterus (Mor et al., 2017; Y.-H. Zhang et al., 2017).

Inflammatory pathologies in pregnancy are often due to a disruption of the balance between Th1 and Th2 cytokines, with a shift towards Th1 dominance during the fetal growth and development period, creating an intensified cascade of inflammatory factors which have been linked to numerous adverse health outcomes for both gestational parent and child, including spontaneous abortion, fetal growth abnormalities, preterm birth and preeclampsia (Challis et al., 2009). Furthermore, an abnormal placental microenvironment caused by bacterial or viral infections also creates excessive placental inflammation, compromising placenta and fetal growth, and

predisposing the child to future metabolic and cardiovascular diseases through adverse developmental programming events (Mor et al., 2017; Robineau-Charette et al., 2020).

1.3 Placenta Mediated Diseases

Placental-mediated diseases play a key role in virtually every major obstetrical complication of pregnancy seen in North America, including but not limited to preeclampsia, fetal growth restriction, preterm labor, abruptio placentae, spontaneous abortion, stillbirth and many more (Brosens et al., 2011). The placenta is pivotal to the success of the pregnancy, and as such, disruptions to normal placental development or compromised placental function are seen at the center of the pathophysiology of numerous gestational diseases, jeopardizing the viability and success of the pregnancy as well as posing serious health concerns for both gestational parent and fetus (Thornburg & Marshall, 2015). Placental-mediated diseases are often a result of key mechanisms such as structural abnormalities caused by poor trophoblast differentiation or placental malperfusion and ischemia-reperfusion injury resulting from poor trophoblast invasion and abnormal spiral arteries remodelling, compromising nutrient and oxygen transport to the fetus. Both etiologies have been well described in gestational diseases such as preeclampsia (Choudhury et al., 2017; V. H. J. Roberts et al., 2017; S. D. Smith et al., 2009). Increasingly, excessive inflammation at the parental-fetal interface has also been shown to be a potential causative pathway leading to the establishment of placental dysfunction, with numerous histological studies showing a link between villous inflammatory lesions and pathologies with adverse pregnancy outcomes including fetal growth restriction, preterm labor, fetal death and preeclampsia (Katzman, 2021; C. J. Kim et al., 2015; Redline, 2002; Sato, 2022).

1.4 Preeclampsia

Preeclampsia (PE) is a complex hypertensive disorder of pregnancy. As per WHO, the three leading causes of parental death worldwide are severe bleeding, infection and PE, with a global incidence rate of 4.6% (Abalos et al., 2013; Parental Mortality, 2024). PE is defined as a severe multisystemic syndrome in pregnancy, diagnosed by the new-onset of hypertension after the 20th week of gestation, accompanied by other serious symptoms indicating parental end organ

damage, such as: renal insufficiency often seen as a sudden onset of proteinuria (>300 mg/day), hematological conditions such as thrombocytopenia, liver dysfunction, neurological issues including visual disturbances, along with uteroplacental dysfunction often seen in the form of fetal growth restriction (M. A. Brown et al., 2001).

Historically, the pathophysiology of PE was thought to be driven by placental ischemia and hypoxic injury, secondary to abnormal EVT invasion into the uterine wall and insufficient uterine spiral artery remodelling (J. M. Roberts & Gammill, 2005). As previously described, in a normal uncomplicated pregnancy, the parental vascular luminal diameter increases, accompanied by a loss of smooth muscle and inner elastic lamina in the vessel wall, providing low resistance blood flow to the intervillous space. PE pregnancies, however, demonstrate a disruption in the EVT invasion process, leading to shallow placentation and placental oxidative stress, ultimately creating an amplified parental inflammatory response as well as endothelial dysfunction (Cindrova-Davies, 2009).

Overall, inadequate modeling of the uterine spiral arteries leads to hypoxic conditions in the placenta and inconsistent blood flow, resulting in placental ischemia-reperfusion injury. Oxygen concentrations are tightly controlled in the placenta, playing a key role in the regulation of not only EVT invasion but also in the development of the villous vascular trees and the subsequent proliferation of the villous trophoblasts (Nelson & Myatt, 2020). Early placental development requires hypoxic conditions for the initiation of placental vasculogenesis, stimulating the transcription factor HIF-1β, inducing hypoxia-related genes necessary for normal placentation as well as regulating the production of vascular endothelial growth factor (VEGF), which aids in EVT invasion. This occurs through the simulation of adhesion factors such as intercellular adhesion molecule-1 (ICAM-1) (Matsubara, 2017; Zhou et al., 2014). As placental angiogenesis progresses with the capillarization of the chorionic villous structures, as well as the remodelling of the spiral arteries, the placenta is increasingly oxygenated, reducing the proliferation of the trophoblast cells in villi and cell columns (Nelson & Myatt, 2020). In PE, the placental ischemia-reperfusion injury, due to the prolonged and heightened hypoxia, disrupts further placental development by promoting the production of excessive anti-angiogenic factors such as soluble vascular endothelial growth receptor (sFlt-1) and soluble endoglin (sEng), which impair the development of fetal vasculature such as the chorionic villi (Matsubara, 2017). Further, these

anti-angiogenic factors can enter the parental circulation altering vascular tone and permeability, which may contribute to endothelial dysfunction in the gestational parent (Cindrova-Davies, 2009).

The continued ischemia-prefusion placental injury seen in many cases of PE induces oxidative stress and the excessive generation of free radicals within the placenta (Cindrova-Davies, 2009; Germain et al., 2007; Tjoa et al., 2006). Oxidative stress is highly involved in the mediation of inflammation, through the activation of pathways such as nuclear factor-κB (NF-κB) and activating protein-1 (AP-1) signal transduction pathways, which promote the expression of genes related to processes such as inflammation, immunity, cell growth, stress respones and apoptosis. PE is associated with an amplified parental inflammatory response, with previous studies reporting an activation of NF- κB pathways linked with significant activation of neutrophils, monocytes, T cell as well as increased recruitment of circulating proinflammatory agents such as IL-6, TNF-α and IL-1β (Cindrova-Davies et al., 2007; Luppi et al., 2006). Additionally, PE placentae have been documented to have higher rates of trophoblast apoptosis as well elevated secretion of other circulating syncytial debris markers such as fetal DNA, cytokeratin and SynT microfragments (STBM) (Knight et al., 1998; Leung et al., 2001; Lo et al., 1999; Schrocksnadel et al., 1993). With shedding of these components into the parental circulation, a further exacerbation of systemic parental endothelial dysfunction and inflammatory response is observed, ultimately contributing to the pathophysiology observed as the classical clinical hallmarks of PE, including hypertension and glomerular endotheliosis (and ensuing proteinuria), along with end organ dysfunction of other highly vascularized paternal organs (i.e. liver, brain) (Cindrova-Davies, 2009; Germain et al., 2007; Tjoa et al., 2006).

Despite this well describe pathophysiology paradigm, there unfortunately exists a high degree of heterogeneity in the clinical presentation and placenta pathology findings across all patients with PE – likely indicative of divergent underlying disease processes. Clinically, a distinction between patients with an early-onset (<34 weeks of gestation) vs a late-onset of PE disease (≥34 weeks of gestation) has been described, with more severe parental and fetal health outcomes linked to the earlier manifestation (Ramos et al., 2017). However, even within these clinical subgroups there exists tremendous heterogeneity, leading many groups to investigate whether additional subclasses of PE disease might exist with distinct underlying pathophysiology. To this end, our

group has characterized the presence of at least three clinically relevant subclasses of PE disease using comprehensive molecular profiling of the placenta (Leavey et al., 2015, 2016a). These subclasses include: 1) a canonical subclass that displays the hypoxic placental injury classically described, 2) a parentally-driven subclass with only mild placental disease involvement, likely driven by inappropriate parental adaptation to pregnancy, and 3) a unique and poorly described subclass of patients with significant inflammation at the parental-fetal interface – termed the inflammatory-driven subclass of PE. (Benton et al., 2018; Leavey et al., 2015, 2016a; Than et al., 2022). Interestingly, this last subclass demonstrates activation of placental genes related to the immune response, which, compounded with an upregulation of CXCL10, marker of organ rejection, suggests a potential incompatibility between gestational parent and fetus, compounded by a poor parental response (Leavey et al., 2016a; Than et al., 2022).

Currently there is no available cure for PE. Symptom management is currently the standard clinical practice, including the use anti-hypertensive medications and magnesium sulfate to prevent seizures at the time of delivery (Parental Mortality, 2024; Ramos et al., 2017). However, this symptom management does not address the underlying cause of the PE, in most cases a dysfunctional and damaged placenta. In fact, the only 'cure' for PE is removal of the placenta, which can have severe adverse health outcomes for gestational parent and child (Ramos et al., 2017). To move this field of study forward, in depth studies of distinct subclass-specific PE pathophysiology and the development of subclass-specific interventions is needed. In the current thesis, the potential of therapeutically targeting the NAD^+ signalling pathways specifically within the subpopulation of inflammation-mediated PE is explored.

1.5 Gestational obesity

Obesity, defined as a body mass index (BMI) of > 30 kg/m^2, has been a growing public health crisis over the past decade, with Statistics Canada reporting that obesity impacted 26.8% of Canadian adults, with an additional 36.3% considered overweight (Overweight and Obese Adults, 2018, 2018). With the rising prevalence of obesity comes the increasing incidence of obesity seen in pregnant people. Indeed, the obesity rate seen among individuals of reproductive age in Canada is reported to be 21% (Overweight and Obese Adults, 2018, 2018).

Gestational obesity is a growing concern due to its associations with numerous serious obstetrical complications, such as PE, gestational obesity mellitus, thromboembolism as well as various cardiovascular and metabolic disorders post-partum (Pantham et al., 2015; "Pregnancy Complications and Outcomes among Overweight and Obese Nulliparous Women," 2001; Sebire et al., 2001). Obese individuals also demonstrate slower labor progression, with a studied correlation between increasing BMI and higher rates of labor duration times, oxytocin requirements and rates of cesarian deliveries (Vahratian et al., 2004; Weiss et al., 2004). Furthermore, obesity in the gestational parent is associated with adverse neonatal outcomes, including fetal growth extremes - both macrosomia (increased fetal weight) and fetal growth restriction ((Radulescu et al., 2013) - and stillbirth (Crane et al., 2009; Flenady et al., 2011; Rasmussen et al., 2008; Siega-Riz et al., 2009). As per the Developmental Origins of Health (DOHaD) paradigm, children of obese individuals are predisposed themselves to have increased adiposity, obesity, insulin resistance, and cardiometabolic disorders in later life (Pantham et al., 2015).

Obesity is clearly associated with a chronic state of systemic inflammation, with increased measures of circulating proinflammatory markers (i.e. tumor necrosis factor-alpha (TNF-α), C-reactive protein (CRP), interleukins (IL-6, IL-18), resistin and visfatin), often associated with insulin resistance, atherosclerosis, hypertension and some cancers (Bastard et al., 2006; Bulló et al., 2003; Ellulu et al., 2017; Festa et al., 2001; Hotamisligil, 2006; Mabrouk et al., 2013; Park et al., 2005). Not only is the direct pro-inflammatory signalling stimulated by excess adiposity a concern for parental/fetal health, but excess adiposity can also lead to heightened circulating free fatty acids, which can further stimulate the pro-inflammatory state through the c-Jun N-terminal kinase (JNK)), protein kinase R (PKR), and nuclear factor kappa B (NF-kB) signalling pathways (Pantham et al., 2015). It is important to note, that inflammation induced by obesity is distinct from the classic inflammatory response in the following ways: 1) it has metabolic origins chronically induced by the excessive consumption of nutrients, whereas classic inflammation is an acute targeted response to a specific event such as infections or trauma; 2) it is a low-grade and chronic systemic response, constantly maintained by adipocytes and other metabolic cells without resolution, while the acute inflammatory response is seen to subside quickly after neutralization of its trigger; 3) altered immune cell profiles in obese individuals are often seen to heavily promote the pro-inflammatory environment seen in tissues such as adipose, pancreas and

liver; 4) the metabolic rate is substantially diminished, which is not a feature of the acute inflammatory response (Gregor & Hotamisligil, 2011; Pantham et al., 2015). The increased consumption of calories without corresponding calorie expenditure, leads to the establishment of obesity through both hyperplasia and hypertrophy of the white adipose tissue within the body, greatly reducing their blood supply, resulting in an adipocyte hypoxic environment (Ellulu et al., 2017). The presence of the ensuing oxidative stress triggers the recruitment of proinflammatory macrophages (MPs), which in turn locally release tumor necrosis factor-alpha (TNF-a), interleukin (IL)-6 and adiponectin for the purpose of resolving the hypoxia (Bastard et al., 2006; Gregor & Hotamisligil, 2011). Hepatocytes are stimulated by the secretion of IL-6 to release C-reactive protein (CRP) which further exacerbates the inflammatory response mounted (Ellulu et al., 2017). The unique chronic inflammation seen in obese pregnant individuals interferes with the highly regulated immune response in pregnancy creating a distinct and aberrant immunological response. This interruption to the normal immunological processes in pregnancy significantly increases the risk of developing gestational complications, which can have adverse health outcomes for both the pregnant individual and their child (Gregor & Hotamisligil, 2011; Pantham et al., 2015).

Although the exact mechanisms through which gestational obesity may contribute to the development of adverse obstetrical health outcomes remain unclear, the establishment of a chronic systemic inflammation, along with oxidative stress, are most likely key players. Studies have demonstrated strong associations between maternal obesity and increased placental TNF-α levels, lower fetal capillary IL-6 expression as well as morphological defects in the human placenta (Musa et al., 2023). Indeed, histopathological studies have shown that gestational obesity is associated with greater placental vascular lesions and greater susceptibility for the development of high-grade chronic villitis, an inflammatory condition known to lead to poor fetal growth, preterm delivery and other gestational complications (Brouwers et al., 2019; Layden et al., 2023). Placental markers of oxidative stress were also seen to be elevated in mouse studies of gestational obesity, with placental tissue showing an activation of 8-OHdG, 4HNE, malondialdehyde (MDA), and protein carbonyl, suggesting increased DNA, lipid, and protein oxidative damage (Hu et al., 2019, 2021; Napso et al., 2022; Tao et al., 2023). Additionally, the altered metabolic process induced by obesity also leads to altered placental metabolism. Studies have shown that obesity-mediated alterations to placental lipid metabolism is associated with

dysregulated expression of genes responsible for lipid transport (i.e. lipoprotein lipase), increased lipid synthesis, and suppression of lipid transport to the fetus - all of which contributes to placental lipotoxicity and heightened inflammation (Fattuoni et al., 2018; Saben et al., 2013; Segura et al., 2017; Strakovsky & Pan, 2012).

1.6 NAD^+ signalling and inflammation

When looking at the pathophysiology of chronic inflammatory disorders in non-pregnant populations (i.e. aging-related diseases, cancer, neurodegeneration, cardiovascular disease, metabolic diseases and many more), a clear causal role for depleted cellular nicotinamide adenine dinucleotide (NAD+) stores, and subsequent dysregulated energy metabolism, has been established (Navas & Carnero, 2021; Reiten et al., 2021; Xie et al., 2020). NAD^+ is a vitamin B_3-derived metabolite that serves as a critical cofactor required for essential metabolic processes such as glycolysis, the tricarboxylic acid (TCA) cycle, alcohol metabolism, β-oxidation, lactate oxidation and many more (Katsyuba et al., 2020). As such, the bioavailability of NAD^+ (including both its biosynthesis and recycling) are critical for maintain cellular metabolic homeostasis and survival (Houtkooper et al., 2010).

Due to its indispensable role in critical constitutive biological processes, NAD^+ is constantly produced through two major biosynthetic pathways: either de novo formation from the amino acid tryptophan or through salvage pathways, which is more commonly seen in mammals (Haigis & Sinclair, 2010). The salvage pathway regenerates NAD^+ through the recycling of niacin byproducts, such as nicotinic acid, nicotinamide and nicotinamide ribose, produced through intracellular NAD catabolic pathways or through dietary sources in the form of vitamin B3 (Braidy et al., 2019). Nicotinamide, the principal precursor of the salvage pathways, is catalyzed by the enzyme nicotinamide phosphoribosyltransferase (NAMPT) to revert back to nicotinamide mononucleotide which is further converted to form NAD^+ by the nicotinamide mononucleotide adenylyltransferase (NMNAT) enzyme family (Dölle et al., n.d.). Although it is not entirely understood, it is believed that NAD^+ biosynthesis is localized to subcellular compartments, affecting its cellular bioavailability (Dölle et al., n.d.; Wong et al., 2019).

Several key protein families rely on NAD^+ stores for their vital functions, with the largest consumers being the poly(ADP-ribose) polymerase (PARP) and the sirtuin (SIRT) enzyme families. The PARP family of enzymes, consisting of 17 unique members, contribute substantially to the maintenance of genomic stability and modulation of energy metabolism (Bai & Cantó, 2012; Smith, 2001). PARPs are nuclear enzymes which catalyze the posttranslational modification reactions known as PARylation, in which ADP-ribose is transferred onto the glutamic acid residues of proteins, with NAD^+ as a required substrate (Houtkooper et al., 2010; Smith, 2001). The most studied PARP members, PARP-1 and PARP-2 are key players in the DNA damage response to single-stranded breaks, estimated to be responsible for ~ 90% of the PARP family NAD^+ consumption in a cell (Bai & Cantó, 2012). In fact, PARP-1 activity constitutes ~1/3 of the total cellular NAD^+ consumption under basal conditions, however stressful conditions induce hyperactivation of PARylation, so much so that it becomes the primary NAD^+ consumers in the cell (L. Liu et al., 2018). This overconsumption of NAD^+ by the PARP enzymes can deplete cellular stores of NAD^+, detrimental for all NAD-dependent pathways, most of which are crucial for maintaining mitochondrial and metabolic cellular health (Strømland et al., 2019; Imai & Guarente, 2014).

The second largest consumer of cellular NAD^+ stores is the SIRT family of enzymes, consisting of 7 NAD^+-dependent histone acetylase proteins, whose function is essential for mitochondrial biogenesis and function, along with cell survival, senescence, apoptosis, proliferation, DNA repair and metabolism (Carafa et al., 2016; L. Liu et al., 2018). Although NAD^+ is believed to be a substrate in the activity of all members of the SIRT family, it is SIRT-1, SIRT-3 and SIRT-4 which are the most dependent on the cofactor, with roles involved in regulation of metabolic and energy related pathways (Haigis & Sinclair, 2010). SIRTs are deeply involved in restoration of cell homeostasis after a stress response, with the functional NAD^+ requirement allowing them to not only act as a bioenergy and redox sensor for the cell, but also to modify the activities of immune, metabolic and bioenergy pathways accordingly (L. Li et al., 2024; T. F. Liu et al., 2012, 2015). To elaborate, SIRT1 plays key regulatory roles in the secretion of insulin in pancreatic β-cells, gluconeogenesic processes in hepatocytes, and fatty acid oxidation and lipolysis pathways in macrophages (Moynihan et al., 2005; Odegaard et al., 2008; Schug & Li, 2011). However, under conditions of chronic stress, created by severe DNA damage, ageing processes or chronic inflammation, hyperactivation of PARP-1 causing depletion of intracellular NAD^+ stores, can

limit the activity of SIRTs and other NAD-dependent catabolic pathways involved in critical processes such as mitochondrial respiration and adenosine triphosphate (ATP) synthesis (Bai & Cantó, 2012; Smith, 2001)

This exhaustion of intracellular NAD^+ stores, without a corresponding increase in its biosynthesis, can be quite detrimental for maintaining mitochondrial integrity, health and function. Adenosine triphosphate (ATP) is the main source of energy for the cell and is primarily produced in the mitochondria through the electron transport chain (ETC) and oxidative phosphorylation, with the generation of reactive oxygen species (ROS) byproducts (Napolitano et al., 2021). The term ROS is used to describe oxygen radicals with at least one unpaired electron, consisting of both highly reactive members such as hydroxyl radicals ($^{\bullet}OH$) as well as lower reactive members such as the superoxide anion radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) (Napolitano et al., 2021). Under normoxic conditions with minimal cellular stress, the rate of ROS generation is low, with adequate mitochondrial antioxidant defense systems, comprising of both enzymatic (phospholipid hydroperoxide glutathione peroxidase, mitochondrial manganese-containing superoxide dismutase, catalase, glutathione-S-transferase, glutathione reductase, Glutathione peroxidase, peroxiredoxins, NAD^+ glycohydrolase) and non-enzymatic systems (i.e. membrane lipid peroxide removal systems, cytochrome c) to maintain a balance between the generation and removal of ROS (Andreyev et al., 2005). A study on resting myoblasts demonstrated that the mitochondrial ETC is responsible for ~45% of the extracellular ROS production, with an additional ~40% generated from the activity of nicotinamide adenine dinucleotide phosphate oxidases (NOX), the only enzyme family dedicated to the active generation of ROS (Wong et al., 2019). Under normal physiological conditions, low level ROS production is important for biological redox (reduction–oxidation) reactions, and is a key signalling factor for innate immunity, host defense immune responses, proliferation, cell differentiation and angiogenesis (Sies et al., 2022; Sies & Jones, 2020). Under hypoxic or cellular stress conditions, such as PARP-mediated NAD^+ depletion, ROS production surges to levels beyond the capacity of antioxidant detoxification systems, creating an accumulation of ROS-mediated oxidative stress which can not only deactivate components of the ETC but can cause biomolecular damage to all types of macromolecules, even leading to cell death (Napolitano et al., 2021; Sies & Jones, 2020). The NAD^+ depletion also suppresses the activity of mitochondrial sirtuins, especially SIRT 3, as well as pathways such as the mitochondrial

unfolded protein response and the NRF2 antioxidant response, all crucial for maintaining mitochondrial homeostasis and protecting the mitochondria against ROS-induced mitochondrial damage and cell death (Rottenberg & Hoek, 2017).

1.7 NAD^+ signalling in the context of inflammatory disorders of pregnancy

Adequate NAD^+ levels during pregnancy are crucial for normal embryonic development. Studies in mice models have identified NAD^+ deficiencies as the cause of congenital heart defects, neural tube defects, fetal organ maldevelopment as well as recurrent spontaneous abortions (Zhao et al., 2021). Although the exact mechanisms through maternal NAD^+ deficiency disrupts embryogenesis are yet to be elucidated, however it is believed that early embryos are dependent on maternal NAD^+ to support the significant demands of the biosynthetic processes during organogenesis. In the case of insufficient NAD^+ levels, it is speculated that the decline of anabolism, catabolism as well as the antioxidant defense systems would disrupt cellular homeostasis leading to cell cycle arrest and cell death (Dunwoodie et al., 2023; Jiang et al., 2022). In this case, it is expected that the timing of the arrest of the cell cycle as well as the cell death would impact the severity of the developmental outcomes, with early and/or severe cellular interruptions resulting in embryo loss, whereas milder cellular disruptions would result in morphological and/or congenital defects (Dunwoodie et al., 2023).

The depletion of NAD+ due to prolonged cellular stress not only impairs crucial developmental processes but may also contribute to other adverse pregnancy complications, such as PE, fetal growth restriction, gestational obesity and gestational diabetes (Gupta et al., 2005; Holland et al., 2017). As previously discussed, extended cellular stress depletes NAD+ levels, which in turn disrupts the function of NAD^+-dependent enzymes such as sirtuins. These sirtuins play a crucial role in embryonic development by regulating NANOG, a key transcription factor essential for maintaining pluripotency in the developing embryo (Palawaththa et al., 2022). PE, as previously described, is a hypertensive disorder with several distinct etiologies, one of which is aberrant inflammation at the maternal-fetal interface. Not unexpectedly, PE pathophysiology has also been characterized by placental mitochondrial dysfunction, seen in the form of increased oxidative stress-induced damage, reduced oxidative phosphorylation as well as structural damage

to the mitochondria (Holland et al., 2017; Jahan, Vasam, Green, et al., 2023). This suggests that NAD^+ depletion may play a role in the development of its clinical symptoms and could be a key component of its pathophysiology. Similarly, the disruption of the balance between PARP/SIRT activity creating a subsequent deficit in intracellular NAD^+ is also characteristic of obesity, originating from an overall dysfunction of the excessive adipose tissue (Ruskovska & Bernlohr, 2023). Further compounding the potential NAD^+ deficit is the likely downregulation of NAMPT-mediated NAD^+ biosynthesis pathway, observed in obese non-pregnant rodent models (Yamaguchi & Yoshino, 2017). While there have yet to be studies conducted in the context of pregnancy, similar mechanisms affecting NAD^+ bioavailability are believed to be occurring in an obese pregnant person. Overall, the exact role of NAD^+ signalling pathways in the pathophysiology of chronic inflammatory gestational pathologies is yet to be determined, however mounting evidence from non-pregnant populations suggests that the depletion of NAD^+ cellular stores may be an important piece of the puzzle.

1.8 NAD^+ supplementation as potential therapeutic avenue

Given the role NAD^+ depletion plays in ROS-mediated mitochondrial dysfunction, the restorative potential of NAD^+ boosting strategies is clear to see, with a significant foundation of preclinical research demonstrating its therapeutic ability in the context of diseases such as Alzheimer's, diabetes, dementia and more (Campbell, 2022). Replenishment of cellular NAD^+ is often done through one of two pharmacological strategies: either boosting NAD^+ biosynthesis pathways through the administration of NAD^+ precursors such as nicotinamide (NAM), nicotinamide mononucleotide (NMN), nicotinic acid (NA) and nicotinamide riboside (NR); or through inhibition of NAD^+ consumers such as PARP, SIRT or CD38 (Arenas-Jal et al., 2020). The mitochondrial favored NAD^+ precursor is NR, with significant pharmacological potential due its unique oral bioavailability as well as an ability to mitigate severe side effects seen through use of other precursors (Nikiforov et al., 2011; Reiten et al., 2021; Trammell et al., 2016). To elaborate, both pre-clinical and clinical studies have demonstrated that large single doses of up to 1,000 mg/kg body weight/day or 1,000 mg/day of NR to non-pregnant rodents and humans respectively, resulted in an increase in steady-state whole-blood NAD^+ levels, up to 2.7-fold higher than baseline. Further, these treatments were able to effectively stimulate NAD^+

metabolism and demonstrated no adverse effects on the recipients (Trammell et al., 2016). In contrast, similarly high concentrations of oral NAM supplements elicited dose-dependent increases of peripheral blood mononuclear cells (PBMC), even inhibiting the activity of SIRTs at high doses (Bitterman et al., 2002; Trammell et al., 2016). The effective and measurable effect of chronic NR supplementation through oral routes of administration on NAD^+ metabolism, boosting SIRT activity as well as overall improvement of mitochondrial health and function has also been validated through numerous clinical trials. One such study demonstrated that chronic oral supplementation with 1,000 mg NR per day was found to be a well-tolerated and determined to be an effective and safe strategy for boosting NAD^+ levels in healthy humans (D. Conze et al., 2019; Martens et al., 2018).

NAD^+ supplementation has also been therapeutically tested in a number of inflammatory disorders in non-pregnant populations. One clinical trial investigating the effect of NAD^+ replenishment therapy for Parkinson's disease is particularly notable. The researchers found that NR treatment, administered at a dose of 3,000mg daily, increased NAD^+ levels in the brain with a ~3.7-fold increase seen in the blood NAD^+ levels. The treated patients showed mild clinical improvement when compared to the placebo group. Importantly, this study also demonstrated that prolonged treatment with NR with doses up to 3,000mg is well tolerated in humans with no moderate or severe adverse effects seen (Berven et al., 2023; Brakedal et al., 2022). NAD^+ supplementation has also been explored for the treatment of obesity in a handful of clinical trials in non-pregnant individuals, where it has shown clear therapeutic promise. For example, administration of 1,000 mg/day of NR over a 6-week period raised skeletal muscle NAD^+ metabolite levels, as well as increasing skeletal muscle acetylcarnitine metabolism, and induced minor changes in body composition such as increased body fat-free mass and sleeping metabolic rate in healthy overweight or obese men and women (Remie et.al, 2020). Although the safety of NR has been confirmed clinically with a generally recognized as safe (GRAS) status by the FDA, its safety for use in pregnant human populations is yet to be established (D. Conze et al., 2019).

Promising evidence has, however, been collected in support of therapeutic NAD+ supplementation in animal models of gestational pathologies. While the exact role(s) of placental NAD+ depletion have yet to be clarified in the context of PE, a mouse model of the

hypoxia-driven PE subclass determined that NAM treatment throughout gestation, at a dose of 500 mg/kg body weight/day (equivalent to 2.5 g/day in a 60 kg human), resulted in an amelioration of PE-like symptoms, including lowering of parental blood pressure, prolonged gestational period and improved fetal growth profiles (Fushima et al., 2017; F. Li et al., 2016). In line with this work, our group has recently demonstrated a similar rescue of the clinical phenotype in a rat model of immune-driven PE with NR supplementation across pregnancy (daily dose of 200 mg/kg/day) (Jahan, Vasam, Cariaco, et al., 2023b; Jahan, Vasam, Green, et al., 2023). As such, there is certainly mounting evidence that the replenishment of placental NAD^+ stores may prove to be an effective and safe therapeutic intervention in the context of inflammatory conditions of pregnancy, including PE and gestational obesity.

Chapter 2 – Hypothesis and Research Aims

2.1 Rationale

Maternal obesity and PE are chronic inflammatory disorders of pregnancy linked to placental dysfunction as well as altered metabolic and immunological profiles (F. M. Von Versen-Hoeynck & Powers, 2007). The overarching aim of this thesis is to explore the potential mechanisms through which different pro-inflammatory states of pregnancy can lead to the establishment of placental dysfunction and adverse pregnancy outcomes, with particular interest focused on the potential role of NAD^+-mediated energy signalling pathways and mitochondrial (dys)function in the placenta.

Preliminary work carried out by our research group has discovered that replenishment of NAD^+ placental stores during pregnancy prevents the establishment of placental dysfunction and improves fetal growth profiles in an LPS-induced rat model of chronic inflammation in pregnancy (Jahan, Vasam, Cariaco, et al., 2023b). Should disrupted NAD^+-mediated energy signalling be central to the pathophysiology of different inflammatory conditions of pregnancy, including maternal obesity, the therapeutic potential of NAD^+ supplementation during pregnancy would be very widespread and would have tremendous potential to improve the health of millions of mothers and infants (Chen et al., 2018; Sullivan et al., 2000).

2.2 Hypothesis

It is hypothesized that placental NAD^+-depletion and subsequent dysregulation of mitochondrial function are central to the establishment of placental dysfunction and adverse pregnancy outcomes in several chronic inflammatory disorders of pregnancy, including immune-driven PE and maternal obesity. Further, it is proposed that therapeutically targeting placental NAD^+-mediated signalling pathways during pregnancy may improve placental health and function and promote adequate fetal growth under various states of chronic inflammation.

2.3 Research Aims:

To test the above mentioned hypotheses, the overarching aim of this study was to compare two rodent models of chronic inflammatory pregnancy pathologies that have been previously

established in the Bainbridge laboratory: 1) An LPS-induced Sprague-Dawley rat model of immune-driven PE, and 2) a high-fat high-sugar (HFHS) diet-induced C57BL/6N mouse model of gestational obesity. These two models were employed in the execution of the following research aims:

Aim 1: Compare placental development, fetal growth, NAD^+-signaling pathways and mitochondrial function across two distinct rodent models of chronic inflammation in pregnancy, both with and without NAD^+ supplementation.

Aim 2: Explore alternative and/or complementary mechanistic pathways that may contribute to the establishment of placental dysfunction and poor fetal growth, specifically identifying similarly or differentially regulated pathways across two distinct rodent models of chronic inflammation in pregnancy.

Chapter 3 - Methods

3.1. Aim 1: Compare placental development, fetal growth, NAD^+-signaling pathways and mitochondrial function across two distinct rodent models of chronic inflammation in pregnancy, both with and without NAD^+ supplementation.

3.1.1. Establishment of the Immune-Driven Preeclamptic Rodent Model

This animal study was performed in accordance with University of Ottawa animal care ethics and guidelines (protocol# HS2923). Sprague Dawley rats, acquired from Charles River Laboratories, were housed at a stable temperature of 23°C with 12:12-hr light-dark cycle. Mating was performed in the evening with vaginal plugs used to confirm pregnancy, denoted as gestational day (E)0.5. Body weights of dams were recorded daily across pregnancy.

A rat model of inflammation-mediated PE was established in the laboratory via daily LPS injections, as previously described (Fricke et al., 2018; Jahan, Vasam, Cariaco, et al., 2023a; Jeong et al., 2023; Mazgaeen & Gurung, 2020). The experimental groups examined (**Fig. 3.1.1.)** included:

1) **CTRL + saline** (N=10 dams; ~ 10 pups/placentas per litter): Dams received daily saline injections between gestational day (GD) 13 to 18.
2) **LPS + saline** (N=10 dams; ~ 10 pups/placentas per litter): Dams were administered daily IP injections of LPS derived from Escherichia coli O55:B5 (L2880-10MG, Sigma-Aldrich) solubilized in saline, at 20-70ug/kg body weight (with a 10ug incremental increase per day) between GD 13 to 18.
3) **CTRL + NR** (N=10 dams; ~ 10 pups/placentas per litter): Dams received nicotinamide riboside (NR) at 200mg/kg/day by oral gavage daily throughout pregnancy (GD 0.5- GD 18) in addition to the daily saline injections between GD 13 to 18.
4) **LPS + NR** (N=10 dams; ~ 10 pups/placentas per litter): Dams received nicotinamide riboside (NR) at 200mg/kg/day by oral gavage daily throughout pregnancy (GD 0.5- GD 18) in addition to the daily LPS injections (20-70ug/kg body weight, 10 ug incremental increases per day) between GD 13 to 18.

At GD19 (term pregnancy in rats), all dams were sacrificed, the uterine horns were dissected out, implantation and resorption sites were recorded, and placenta and fetal weights were collected.

From each viable implantation site, placentas were collected and cut at midline, with ½ placed in paraformaldehyde and processed for histological/morphological analysis, and ½ flash frozen and processed for protein and RNA expression by Western Blot and RNA-Seq analysis, respectively.

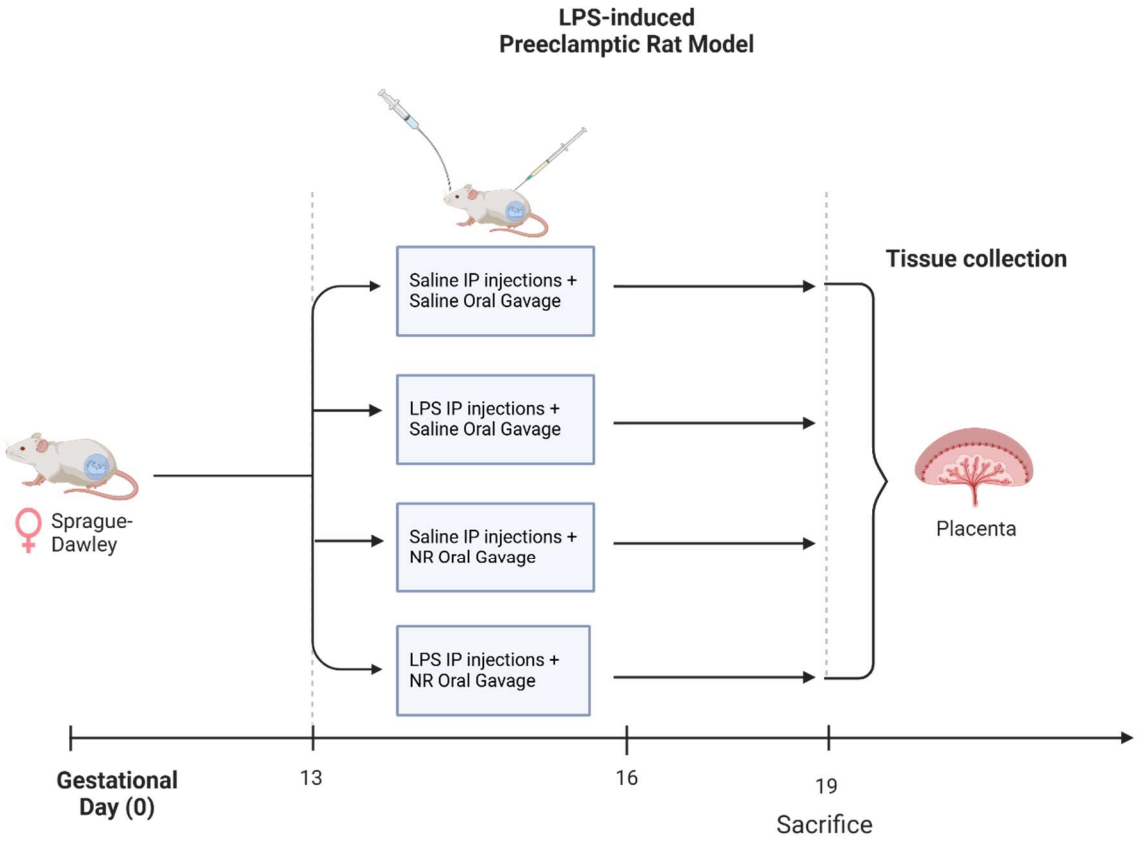


Figure 3.1.1. Experimental timeline for the LPS-induced preeclamptic rat model. This diagram outlines the experimental timeline for the LPS-induced PE model. After mating and confirmation of pregnancy, the dams were administered IP injections containing either saline or LPS in addition to an oral gavage with either a saline or an NR solution, for the entire gestational period. At term pregnancy, gestational day 19, the dams were sacrificed with their placenta collected for further analysis.
PE = preeclampsia, LPS = Lipopolysaccharide; IP = Intraperitoneal injection; NR = Nicotinamide riboside. Figure was created using BioRender.com

3.1.2. Establishment of the Gestational Obesity Rodent Model

This animal study was performed in accordance with University of Ottawa animal care ethics and guidelines (protocol# HS3937). C57BL/6 mice, acquired from Charles River Laboratories, were housed at a stable temperature of 23°C with 12:12-hr light-dark cycle. Pre-gestational

obesity was established in female mice through the administration of a high-fat high sugar (HFHS) diet beginning at 6 weeks of age. The HFHS diet contains 21.2% fat by weight and 34.5% sucrose by weight (Diet no.88137 Harlan-Teklad) and was chosen as it closely mimics the common Western diet typically consumed by obese mothers in Canada (Gibson, 1996; *Overweight and Obese Adults, 2018*, 2018). The original protocol for the pre-gestational HFHS diet exposure was to be limited to 12 weeks, however this exposure resulted in a severe obesity phenotype that was coupled to diminished fertility, measured through the number of false plugs and increased time to pregnancy. As such, the HFHS pre-gestational exposure ranged between 12-15 weeks.

Control mice were maintained on a standard RM1 chow diet (7% simple sugars, 3% fat by weight) during this same pre-gestational timeframe. Mating was performed in the evening with vaginal plugs used to confirm pregnancy. Dams were maintained on their respective diets throughout gestation and body weights were recorded daily across pregnancy (GD 0.5-GD 18.5). The experimental groups examined (**Fig. 3.1.2.)** included:

1) **CTRL + Saline** (N=10 dams; ~ 8 pups/placentas per litter): Dams were maintained on standard RM1 chow diet and received oral gavage of sterile drinking water between GD 0.5-18.5.
2) **HFHS + Saline** (N=8 dams; ~ 8 pups/placentas per litter): Dams were maintained on HFHS diet and received oral gavage of sterile drinking water between GD 0.5-18.5.
3) **CTRL + NR** (N=10 dam; ~ 8 pups/placentas per litter): Dams were maintained on standard RM1 chow diet and received oral gavage of 400mg/kg/day nicotinamide riboside (NR) between GD 0.5-18.5.
4) **HFHS + NR** (N=10 dams; ~ 5 pups/placentas per litter): Dams were maintained on HFHS diet and received oral gavage of 400mg/kg/day nicotinamide riboside (NR) between GD 0.5-18.5.

At E18.5 (term pregnancy in mice), dams were sacrificed, with the uterine horns removed, implantation and resorption sites recorded, and placenta and fetal weights collected. From each viable implantation site, fetal and placenta samples were collected as per the protocol described in 3.1.1.

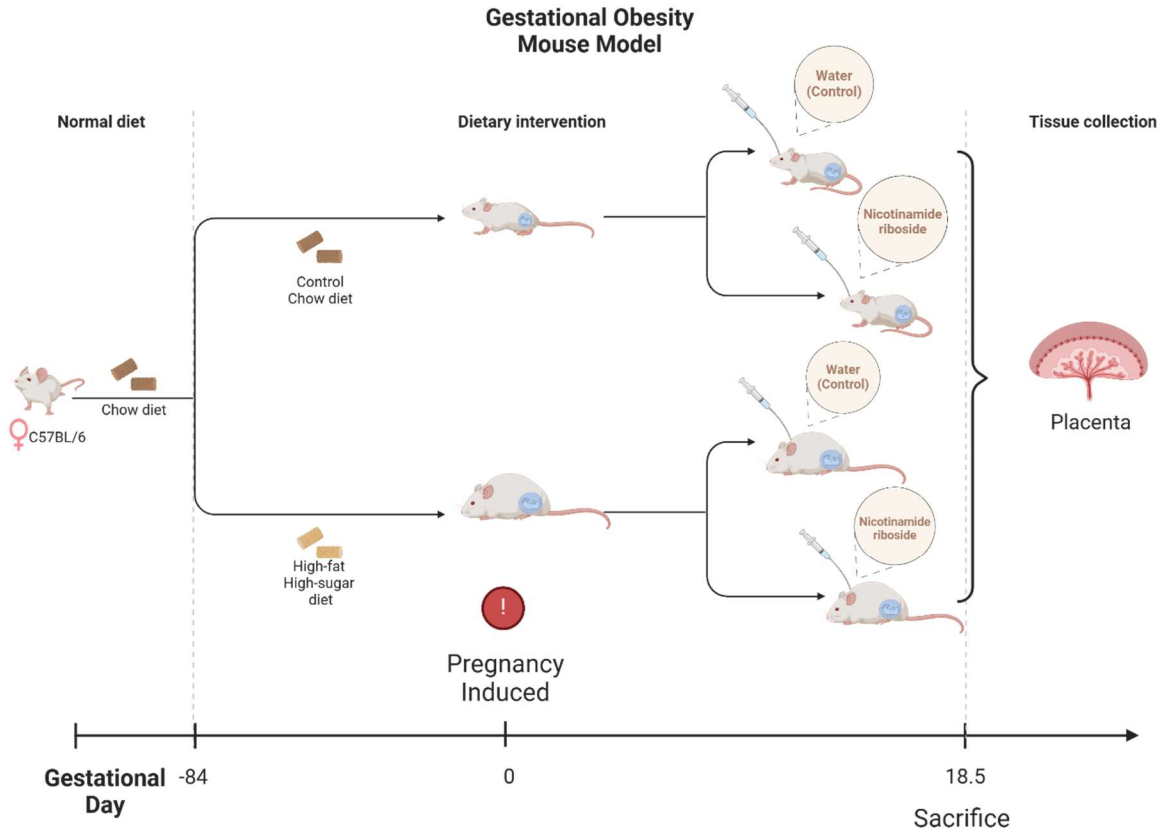


Figure 3.1.2. Experimental timeline for the gestational obesity mouse model. This diagram outlines the experimental timeline for the gestational obesity mouse model. The mice were placed on a high-fat and high-sugar diet or a control chow diet 12 weeks prior to mating, which was maintained for the gestational period. Additionally, after pregnancy was confirmed, the dams were also administered either a saline or nicotinamide riboside (NR) treatment for the gestational period. At gestational day 18.5, term pregnancy in mice, the dams were sacrificed with their placenta collected for further analysis. Figure was created using BioRender.com

3.1.3. Model comparison for metrics of placental and fetal health

Mean placental and fetal weights for each litter, as well as mean placental efficiency calculations (the ratio of fetal weight: placental weight) per litter, were calculated. Histomorphometry analysis of placentas was carried out on H&E-stained midline histological sections. Specifically, mean morphometric measurements of midline placental width and depth, and width/depth measurements of the distinct functional regions of the placenta (chorionic plate, labyrinth, junctional zone) were analyzed using ImageJ software.

3.1.4 Assessment and model comparison for metrics of placental protein PARylation

Flash frozen placenta (n=3-10 samples/experimental group; 15-35 mg) was crushed and homogenized completely with RIPA buffer. The homogenate was centrifuged at 16,000-20,000 relative centrifugal force (rcf) for 15-20 minutes at 4°C. Protein concentration of the supernatant was quantified through the DC assay (Bio-Rad Laboratories, Hercules, California, USA, Cat. #5000113) using Bovine Serum Albumin (BSA) standards mixed in RIPA buffer. Samples were diluted in 4x Laemmli Buffer (Bio-Rad Laboratories, Hercules, California, USA, Cat. #1610747) to a final stock concentration of 2 µg protein/µL. Western blotting was performed on 12% SDS-PAGE gels, made using the TGX Stain-free FastCast Acrylamide Kit (Bio-Rad Laboratories, Hercules, California, USA Cat# 1610183) with 10% Ammonium persulfate (APS), after which the gels were activated and transferred to TransBlot Turbo Mini size 0.2µm nitrocellulose or PVDF membrane (Bio-Rad) using the Trans-Blot TurboTransfer System (Bio-Rad Laboratories, Hercules, California, USA Cat# 1704150EDU). The blot was first imaged under the stain-free blot setting with automatic exposure time to estimate total loaded protein content. Then it was blocked again for one hour in blocking buffer (5% w/v bovine serum albumin (Sigma, SKU A7906) in TBS-T buffer (50mM Tris-HCl, pH 7.6; 150mM NaCl; 0.1% Tween)) while rocking gently at room temperature. Membranes were incubated with primary anti-PAR antibody (Millipore Sigma, AM80-100UG) diluted in TBS-T buffer 10X overnight at 4°C, washed 3X in TBS-T buffer for 10 minutes each, after which, the membrane was incubated for one hour with horseradish peroxidase (HRP)-conjugated secondary IgG antibody, rocking at room temperature. Detection was then performed on a ChemiDoc system (Bio-Rad Laboratories, Hercules, California, USA) using 1 ml Clarity (Bio-Rad Laboratories, Hercules, California, USA) or Clarity Max (Bio-Rad Laboratories, Hercules, California, USA) enhanced chemiluminescent solutions. After retrieval of the images, quantification of blots was carried out on either ImageLab or FIJI software.

3.1.5. Assessment and model comparison of placental NAD^+ content

Pulverized frozen placental tissue (pooled by litter per experimental group; 20-40mg) was homogenized in 300 µL of NAD^+/NADH extraction buffer (AB65348), with NAD^+ content

measured using either the Biovision NAD^+ /NADH kit (K337; LPS PE model) or the abcam NAD^+/NADH kit (AB65348; gestational obesity model), as per the manufacturer's instructions.

3.1.6. Hypothesis-driven assessment and model comparison of placental gene expression profiles

For RNA extraction, 10mg of placental tissues were crushed (N = 5-6 placentas per experimental group) and underwent a standard lysis procedure in a bead mill using Trizol Reagent. The resulting flowthrough was then purified through the QiaGen RNA isolation kit, after which quantification and quality assessment was conducted using NanoDrop® ND-1000 UV-Vis Spectrophotometer. The purified RNA was then sent for bulk sequencing at the Center of Applied Genomics, at the Hospital for Sick Children (SickKids), affiliated with University of Toronto. All samples had an RNA Integrity Number (RIN) above 7.0 as well as a DV200, used to assess % of fragment size above 200 nucleotides, above 70%.

Raw read quality was assessed using FASTQC (Srinivasan et al., 2020), with low quality nucleotide scores or reads containing sequencing adaptors and primer seqs removed. The HISAT software (Srinivasan et al., 2020) was used to align suitable reads to a reference genome of the respective dataset; rat for the PE inflammation model, and mouse for the obesity inflammation model. The aligned reads were assembled using featureCounts (Liao, 2004) software. The package DESeq2 was used to determine differentially expressed genes (DEGs) (Love et al., 2014), which were annotated functionally using Gene Ontology (GO). GO terms relevant to NAD^+ signalling and mitochondrial function (**Table 1**) were used filter and display the expression of genes of interest to investigate the overarching project hypothesis, visualized through heatmaps through the R package 'pheatmap'.

Table 1. GO terms examined in hypothesis-driven analysis of RNA-Seq data

Identifier	Label	Definition
GO:0006119	Oxidative Phosphorylation	The phosphorylation of ADP to ATP that accompanies the oxidation of a metabolite through the operation of the

		respiratory chain. Oxidation of compounds establishes a proton gradient across the membrane, providing the energy for ATP synthesis.
GO:0003954	NADH Dehydrogenase activity	Catalysis of the reaction: NADH + H+ + acceptor = NAD+ + reduced acceptor.
GO:0009435	NAD+ Biosynthetic Processes	The chemical reactions and pathways resulting in the formation of nicotinamide adenine dinucleotide, a coenzyme present in most living cells and derived from the B vitamin nicotinic acid; biosynthesis may be of either the oxidized form, NAD, or the reduced form, NADH.
GO:0070403	NAD+ Binding	Binding to the oxidized form, NAD, of nicotinamide adenine dinucleotide, a coenzyme involved in many redox and biosynthetic reactions.

3.1.6. Data Analysis

All data were presented as mean ± standard deviation (STD). All data was analyzed by one-way ANOVA with Brown-Forsythe multiple comparisons test was used to determine the statistical significance of intergroup differences. Error bars in data visualizations represent mean ± standard deviation (SD). A final value of $p \leq 0.05$ was considered significant for all analyses.

3.2. Aim 2: Explore alternative and/or complementary mechanistic pathways that may contribute to the establishment of placental dysfunction and poor fetal growth, specifically identifying similarly or differentially regulated pathways across two distinct rodent models of chronic inflammation in pregnancy.

3.2.1. Data-driven assessment and comparison of placental gene expression profiles.

For each rodent model, all significant DEGs, identified through DESeq2, were used to create a genome-wide gene expression signature for the inflammatory pathology they represent. Gene set enrichment analysis (GSEA) was performed using the ClusterProfiler package to identify high priority biological/mechanistic pathways that may be differentially regulated in states of chronic inflammation, using the both the Pathview databases as well as the Kyoto Encyclopedia of Genes and Genomes (KEGG) gene sets. To visualize the comparative expression profiles created, the packages EnhancedVolcano, VennDiagram, and ClusterProfilr were used to create volcano plots, Venn diagrams and dotplots respectively.

3.2.2. Generate and assess a gene expression profile for orthologous genes shared between the rodent models

Interspecies comparison to identify shared enriched pathways between the rodent models was also conducted, in which all differentially expressed genes were aligned to their respective human ortholog using ENSEMBL and OrthoDB databases. Originally, the plan was to use 1:1 orthologs, however due to the scarcity of DEGs in both models, all identified orthologs (including one-to-many relationships) were included. The genes found to be common between the two models underwent GSEA as well as pathway enrichment through GO, KEGG and Pathview databases to understand the potential contributing roles in inflammatory placental pathologies.

3.2.3. Data Analysis

For all transcriptomic analysis, the false discovery rate (FDR) was set at 10% with the Benjamini-Hochberg adjusted q-value ≤ 0.10 considered significant, using DESeq2. For gene set enrichment as well as pathway enrichment, genes were ranked based on their fold changes, through the ClusterProfilr package, using GO, KEGG and Pathview databases. Z-scores were

calculated through normalization of the enriched genes within the relevant genes to generate the heatmaps, through the pheatmap package.

Chapter 4 – Results

4.1. Aim 1: Compare placental development, fetal growth, NAD^+-signaling pathways and mitochondrial function across two distinct rodent models of chronic inflammation in pregnancy, both with and without NAD^+ supplementation.

4.1.1. Both rodent models of chronic inflammation in pregnancy demonstrate evidence of altered placentation and poor fetal growth

Previous work done by our group has demonstrated that in the rat model of LPS-induced PE there was a significant reduction in both fetal and placental weight, as well as an overall reduction in placental efficiency (**Fig. 4.1 A-C**) (Jahan et al., 2023). Likewise, in the mouse model of gestational obesity, a reduction in fetal weight was observed. While no significant change in placental weight was observed, an overall decrease in placental efficiency was noted in this model (**Fig. 4.1, D-F**). Importantly, NR treatment was capable of rescuing these adverse placental and fetal health outcomes in the LPS-model of PE, whereas no effects of NR treatment on fetal or placental growth profiles were observed in the gestational obesity model.

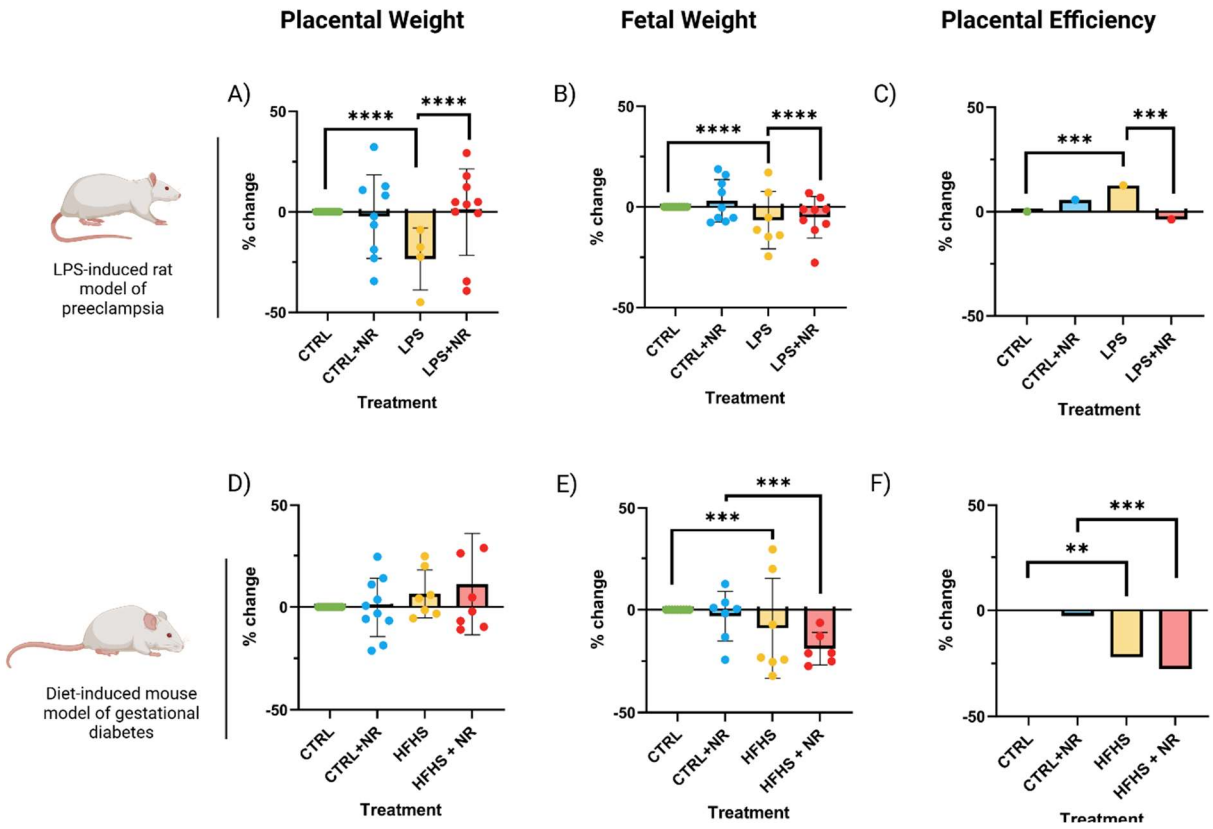


Figure 4.1. Comparison of fetal and placental health metrics across two models of gestational inflammation. Percent change in placental weights (A), fetal weights (B) and placental efficiency (C) in the LPS-model of PE when compared to the control group, with and without NR supplementation. Percent change in placental weights (D), fetal weights (E) and placental efficiency (F) in the HFHS diet-induced model of gestational obesity when compared to the control group, with and without NR supplementation. Differences were calculated through the multiple comparisons Brown-Forsythe ANOVA with p-values < 0.05. CTRL = control; LPS = Lipopolysaccharide; HFHS = High-fat high-sugar diet; NR = nicotinamide riboside.

Detailed morphometric measurements of the placenta allow for insight into potential defects in placenta function. Placentas from the LPS-treated PE model (**Fig 4.2 A-C**) demonstrated decreased depth of the labyrinth – the site of parental-fetal exchange of gases and nutrients. While a similar trend was observed in the HFHS-induced gestational obesity model (**Fig. 4.2 D-F**) this did not reach significance. Again, NR supplementation exerted a protective effect on placental labyrinth development in the LPS-treated PE model, with no significant impact on the structure of the placenta in the diet-induced obesity model (**Fig. 4.2**).

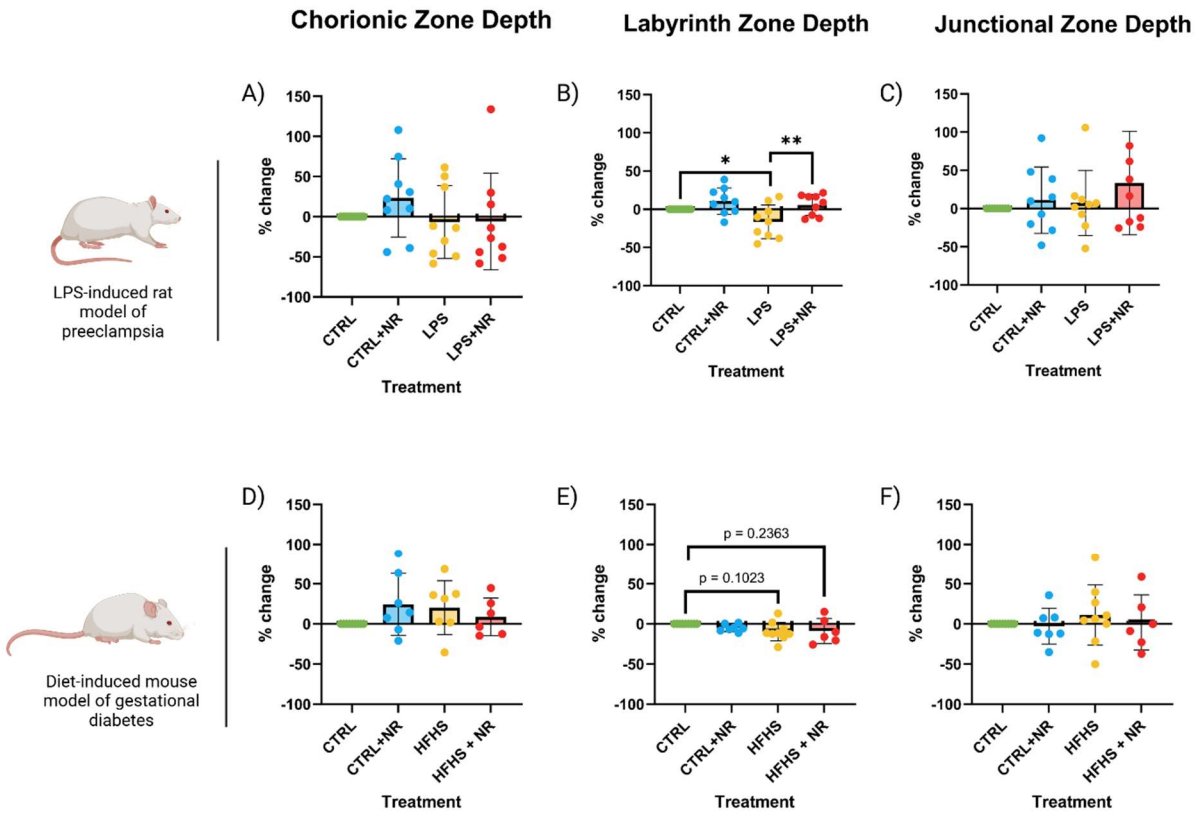


Figure 4.2. Comparison of placental morphometry across two models of gestational inflammation: Percent change in the depth of the chorionic plate (A), labyrinth zone (B), and junctional zone (C) of the placenta in the LPS-model of PE when compared to the control group, with and without NR supplementation. Percent change in the depth of the chorionic plate (D), labyrinth zone (E), and junctional zone (F) of the placenta in the HFHS-model of gestational obesity when compared to the control group, with and without NR supplementation. Differences were calculated through the multiple comparisons Brown-Forsythe ANOVA with p-values < 0.05. CTRL = control; LPS = Lipopolysaccharide; HFHS = High-fat high-sugar diet; NR = nicotinamide riboside. *P＜0.05,**P＜0.01,***P＜0.001.

4.1.2. Both rodent models of chronic inflammation in pregnancy demonstrate increased protein PARylation and depleted NAD$^+$ content in the placenta

Increased protein PARylation leading to mitochondrial dysfunction, as previously described, is hypothesized to be a central component in the establishment of chronic inflammatory disorders seen in pregnancy. In the current study, only the LPS-induced PE model demonstrated an increase in total placental protein PARylation (**Fig. 4.3 A-B**) whereas both models demonstrated a decrease in placental NAD$^+$ content (**Fig. 4.3 C, F**). NR supplementation exerted a protective

effect against excessive protein PARylation and NAD^+ depletion in the LPS-treated PE model (**Fig 4.3, A-C**). In the gestational obesity model, a non-significant trend of reduced protein PARylation was observed (p=0.0717, **Fig. 4.3, D-E**), however, surprisingly, no effect was observed on the placental NAD^+ content with NR treatment (**Fig. 4.3, F**).

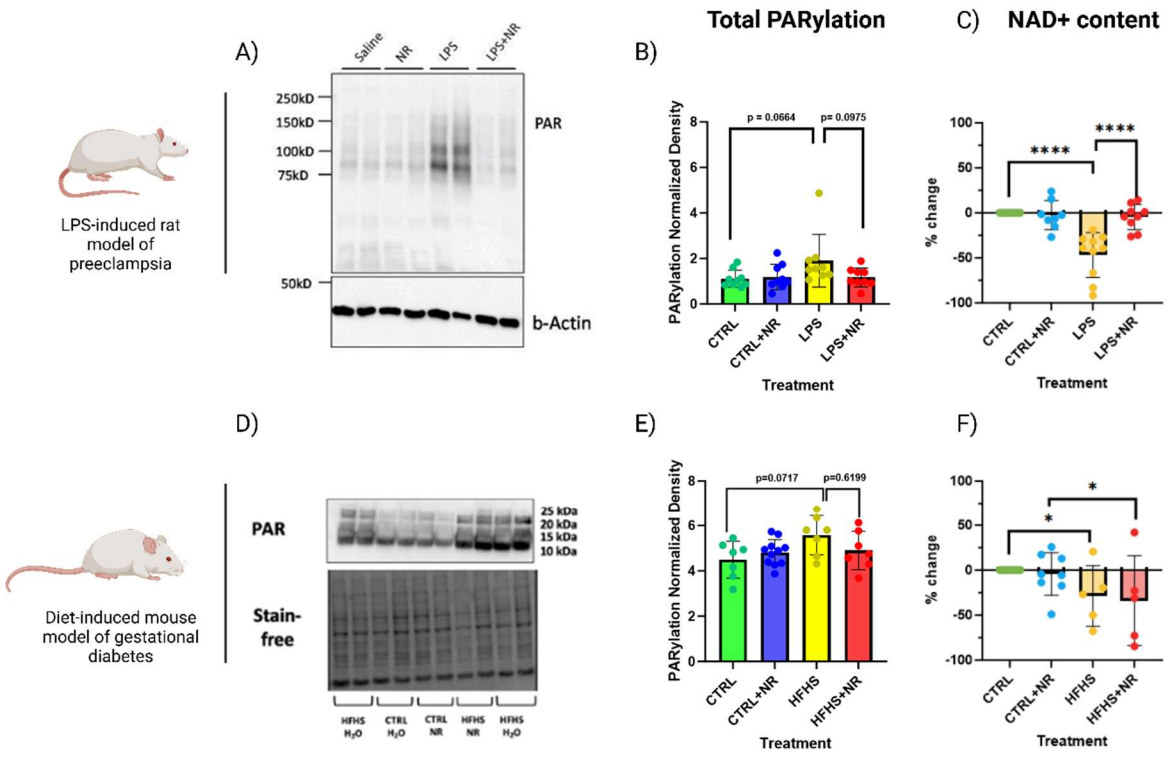


Figure 4.3. Comparisons placental protein PARylation and NAD^+ content across two models of gestational inflammation. Total protein PARylation (A-B) and NAD^+ content (C) in placenta tissues from the LPS-model of PE, with and without NR supplementation. Total protein PARylation (D-E) and NAD^+ content (F) in placenta tissues from the HFHS-model of gestational obesity, with and without NR supplementation. Differences were calculated through the multiple comparisons Brown-Forsythe ANOVA with p-values < 0.05. CTRL = control; LPS = Lipopolysaccharide; HFHS = High-fat high-sugar diet; NR = nicotinamide riboside. *P＜0.05,**P＜0.01,***P＜0.001.

4.1.3. Hypothesis-driven gene expression analysis confirmed evidence of mitochondrial dysfunction only in the LPS-treated PE model

Hypothesis-driven placental gene set enrichment was conducted to specifically investigate the role of genes involved in NAD^+ signalling pathways and mitochondrial health and function

pathways. In the LPS-treated PE model, an upregulation of genes involved in mitochondrial oxidative phosphorylation was observed in this model (**Fig. 4.4, A**), a finding that was reversed with concurrent NR treatment (**Fig. 4.4, B**). Further, a trend of increased expression of genes involved in NADH dehydrogenase activity (**Fig. 4.4, C**) was observed in some animals compared to healthy controls, and this effect was also seen to be reversed with NR treatment (**Fig. 4.4, D**). In the LPS-treated model, NAD biosynthetic pathways are show signs of downregulation (**Fig. 4.4, E)** with the NR treatment suggesting a trend towards activating NAD biosynthesis (**Fig. 4.4, F**). Similarly, the NAD^+ binding also suggests an increase in activity in the LPS-treated groups (**Fig. 4.4, G**) whereas the the effect of NR supplementation on NAD+ binding is less consistent, with mixed results observed (**Fig. 4.4, H**).

In the gestational obesity model, no discernable effect of HFHS diet or NR treatment was observed for genes involved in NADH dehydrogenase activity (**Fig. 4.5, C-D**), and in contrast to the PE model of inflammation, the HFHS model demonstrated a trend of downregulation of genes involved in mitochondrial oxidative phosphorylation (**Fig 4.5, A**) that was reversed with concurrent NR treatment (**Fig. 4.5, B**). Similarly, the genes related to NAD biosynthetic processes showed no significant changes in response to the HFHS diet or NR treatment (**Fig. 4.5, E-F**). However, NAD+ binding shows a trend toward increased activity in the HFHS-treated groups **(Fig. 4.5, G)**, while a downward trend in activity is observed in the NR-treated groups (**Fig. 4.5, H**).

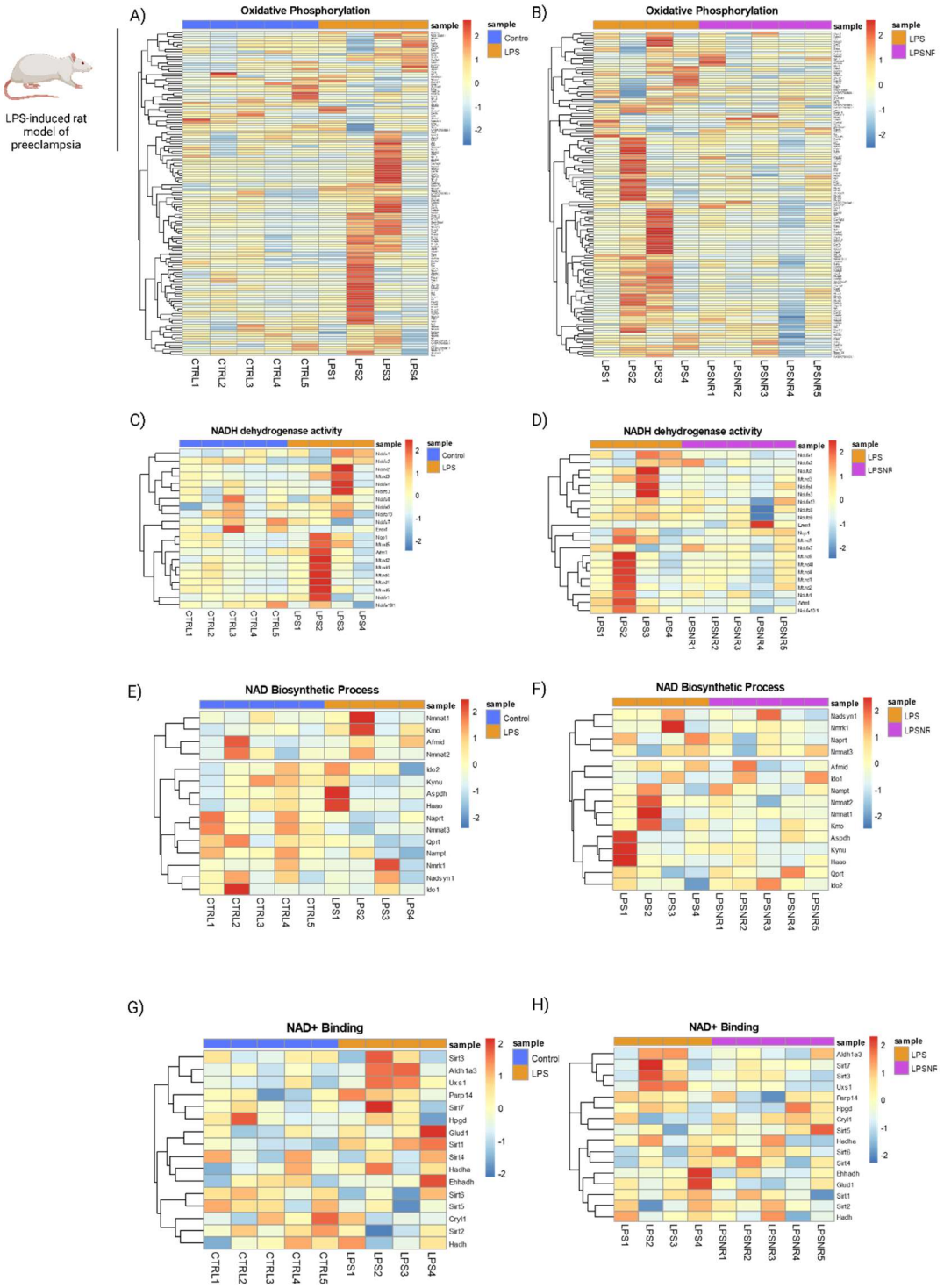


Figure 4.4. Hypothesis-driven comparisons of expression profiles for genes related to NAD$^+$ signalling and mitochondrial health and function across LPS-model of PE.
Differential gene expression signature in placentas from LPS-model of PE, both with and without NR supplementation for genes involved in: oxidative phosphorylation (A-B), NADH dehydrogenase activity (C-D), NAD Biosynthetic Processes (E-F) and NAD$^+$ binding (G-H). Heatmaps were created using ranked log fold change

gene enrichment analysis with an adjusted p-value <=0.10. CTRL = control; LPS = Lipopolysaccharide; HFHS = High-fat high-sugar diet; NR = nicotinamide riboside.

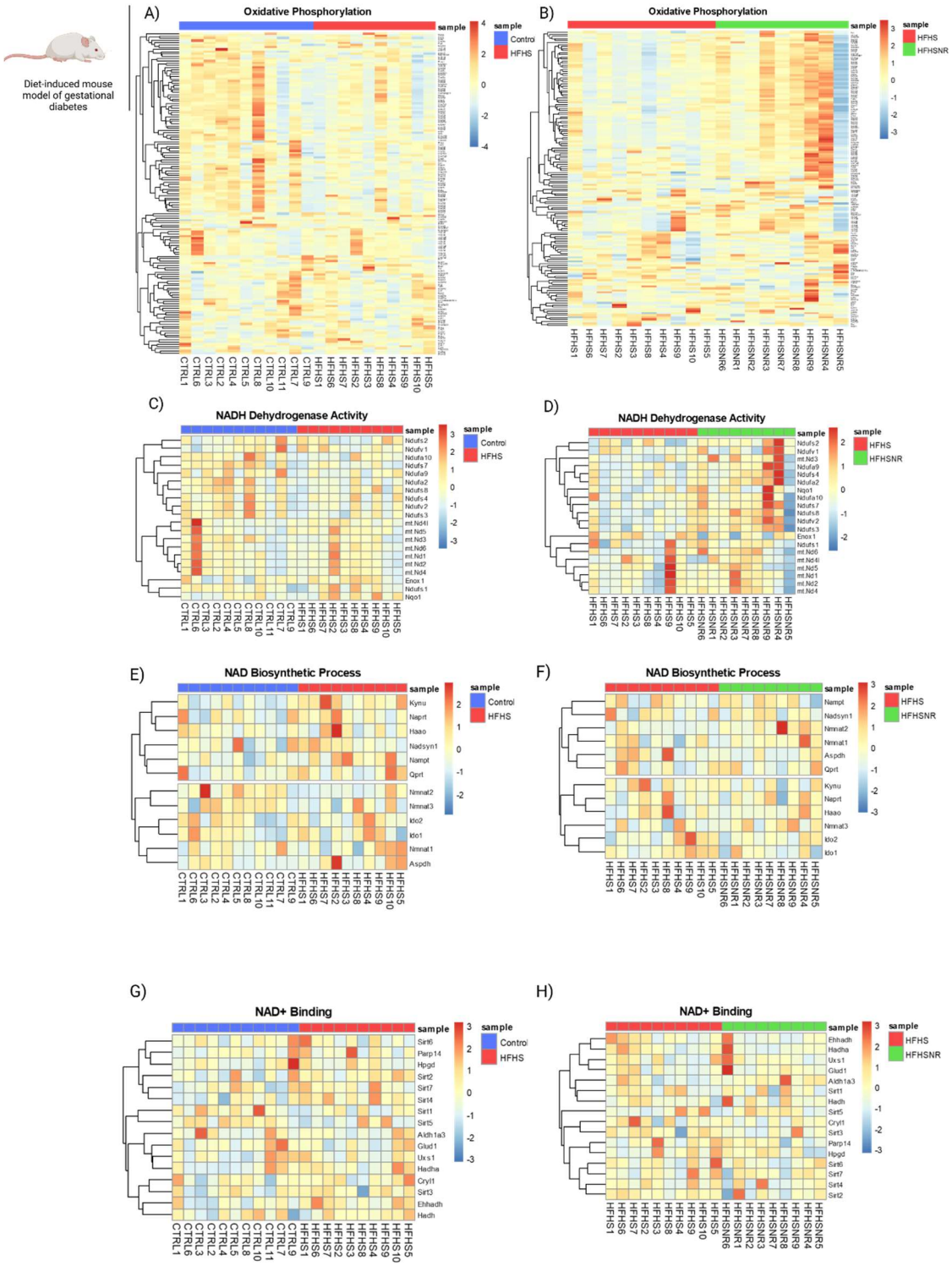


Figure 4.5. Hypothesis-driven comparisons of expression profiles for genes related to NAD+ signalling and mitochondrial health and function across HFHS-model of gestational obesity.
Differential gene expression signature in placentas from HFHS-model of gestational obesity, both with and without NR supplementation for genes involved in: oxidative phosphorylation (A-B), NADH dehydrogenase activity (C-D), NAD Biosynthetic Processes (E-F) and NAD^+ binding (G-H). Heatmaps were created using ranked log fold change gene enrichment analysis with an adjusted p-value <=0.10. CTRL = control; LPS = Lipopolysaccharide; HFHS = High-fat high-sugar diet; NR = nicotinamide riboside.

4.2. Aim 2: Compare placental development, fetal growth, NAD^+-signaling pathways and mitochondrial function across two distinct rodent models of chronic inflammation in pregnancy, both with and without NAD^+ supplementation.

4.2.1. Both rodent models of chronic inflammation in pregnancy demonstrate upregulated immune pathways with some evidence of mitochondrial dysfunction in the gestational obesity model

As our hypothesis-driven data analysis demonstrated a divergence in gene expression patterns across our two gestational inflammation models for the pre-identified pathways of interest, related to NAD^+ signalling and mitochondrial health/function, we next sought to carry out an unsupervised genome-wide gene expression comparison of the two gestational inflammation models. Unfortunately, this analysis was limited by the low number DEGs identified in both inflammation models, with 585 DEGs identified with LPS treatment compared to control (**Fig 4.6, A**) and only 4 DEGs identified with HFHS diet compared to control (**Fig 4.7, A**). Interestingly, despite a rescue of placental and fetal phenotype in the LPS-treated PE model, only 54 DEGs were identified when comparing the LPS-treated groups with and without NR (**Fig 4.6, B**). This number was even smaller, accounting for only 20 DEGs, in the HFHS diet groups with and without NR (**Fig 4.7, B**). However, despite a small number of DEGs identified, a distinct separation between the groups was observed, particularly in the LPS-treated PE model (**Fig 4.6, C** and **Fig 4.7, C**).

Pathway enrichment analysis was conducted on the datasets to identify biological pathways that were dysregulated in the different models of gestational inflammation (LPS or HFHS), with and without NAD^+ supplementation. Placentas from the LPS-treated PE model primarily

demonstrated an activation of pathways related to inflammatory exposures and signalling pathways, including cytokine and chemokine expression (**Fig. 4.6, E**). With concurrent NAD^+ supplementation, activation of pathways involved in glucose handling (i.e. insulin and glucagon signalling), cancer and oncogenic processes were observed, in addition to Wnt-signalling which has been associated with NAD^+ metabolic reprogramming (Lee et al., 2016). NAD^+ supplementation in this model was also associated with a suppression of pathways associated with proteosome and DNA replication (**Fig. 4.6, F**).

Similarly, placentas from the HFHS-diet gestational obesity model also demonstrated an activation of pathways related to inflammatory processes, including complement and coagulation cascades and chemical carcinogenesis – reactive oxygen species, in addition to suppression of essential metabolic pathways such as thermogenesis, cholesterol metabolism and PPAR signalling pathway. Interestingly, these placentas also demonstrate a suppression in oxidative phosphorylation pathways, indicative of mitochondrial dysfunction (**Fig. 4.7, E**).

NAD^+ supplementation in this HFHS model was associated with an activation of pathways primarily related to oxidative phosphorylation, as well as diseases with a central inflammatory component in their pathology such as Parkinson disease, non-alcoholic fatty liver disease (de la Pompa, 2023; Karmi et al., 2010; van Horssen et al., 2019), and a suppression of pathways related to cell motility (**Fig. 4.7, F**).

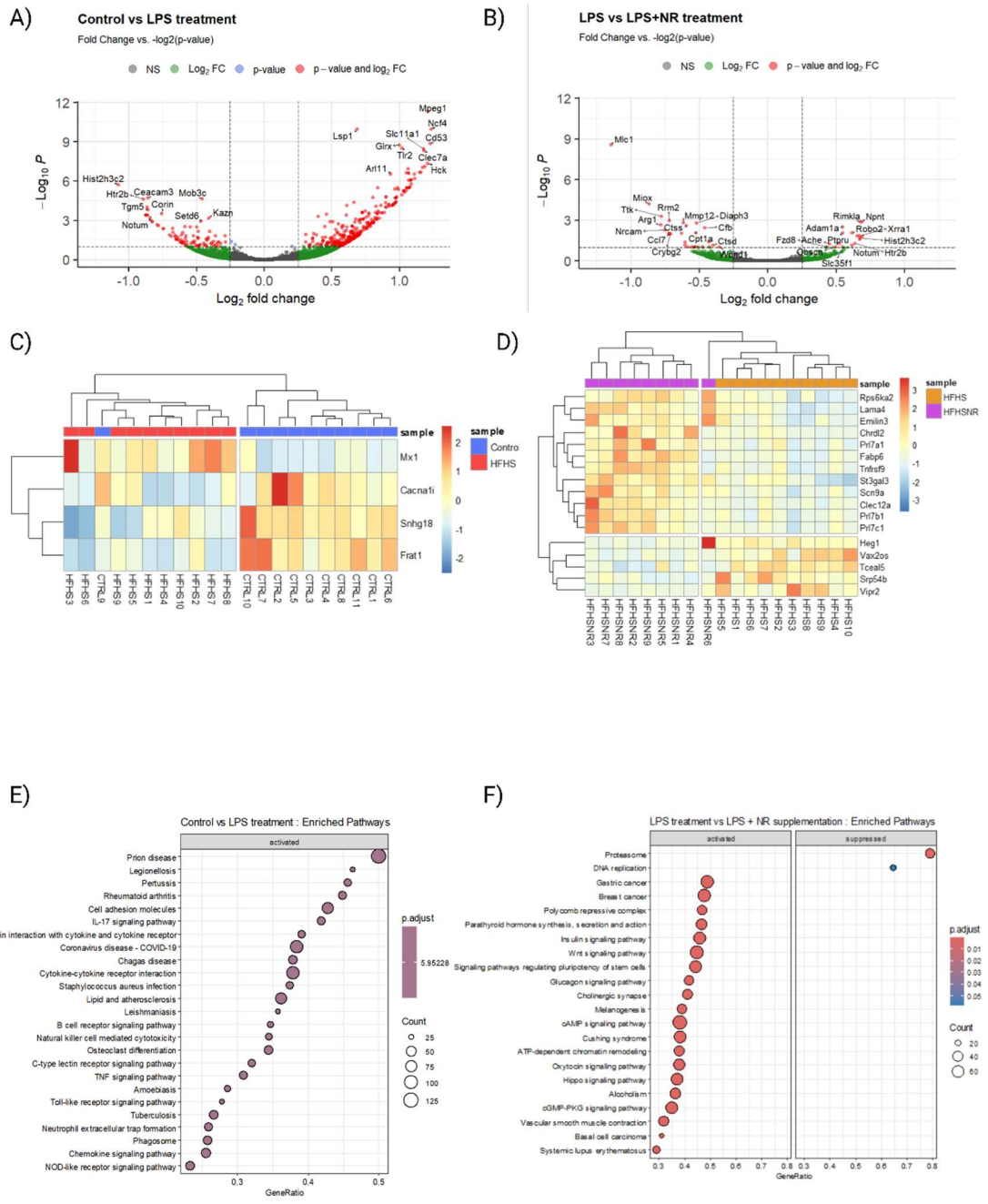


Figure 4.6. Gene expression signatures and fast-ranked pathway enrichment from placenta tissues of the LPS-treated PE model.

Volcano plots (A-B) and heatmaps (C-D) of all DEGs in LPS-treatment vs. control (A, C), with and without NR supplementation (D). Annotated by KEGG pathway databases, the top biologically pathways that were differentially activated or supressed in placental tissue from LPS-treated vs. control (E), with and without NR supplementation (F). Identification of DEGs and pathways used p-value cutoff = 0.10, log2FC = 0.25. CTRL = control; LPS = LPS treatment, NR = nicotinamide riboside

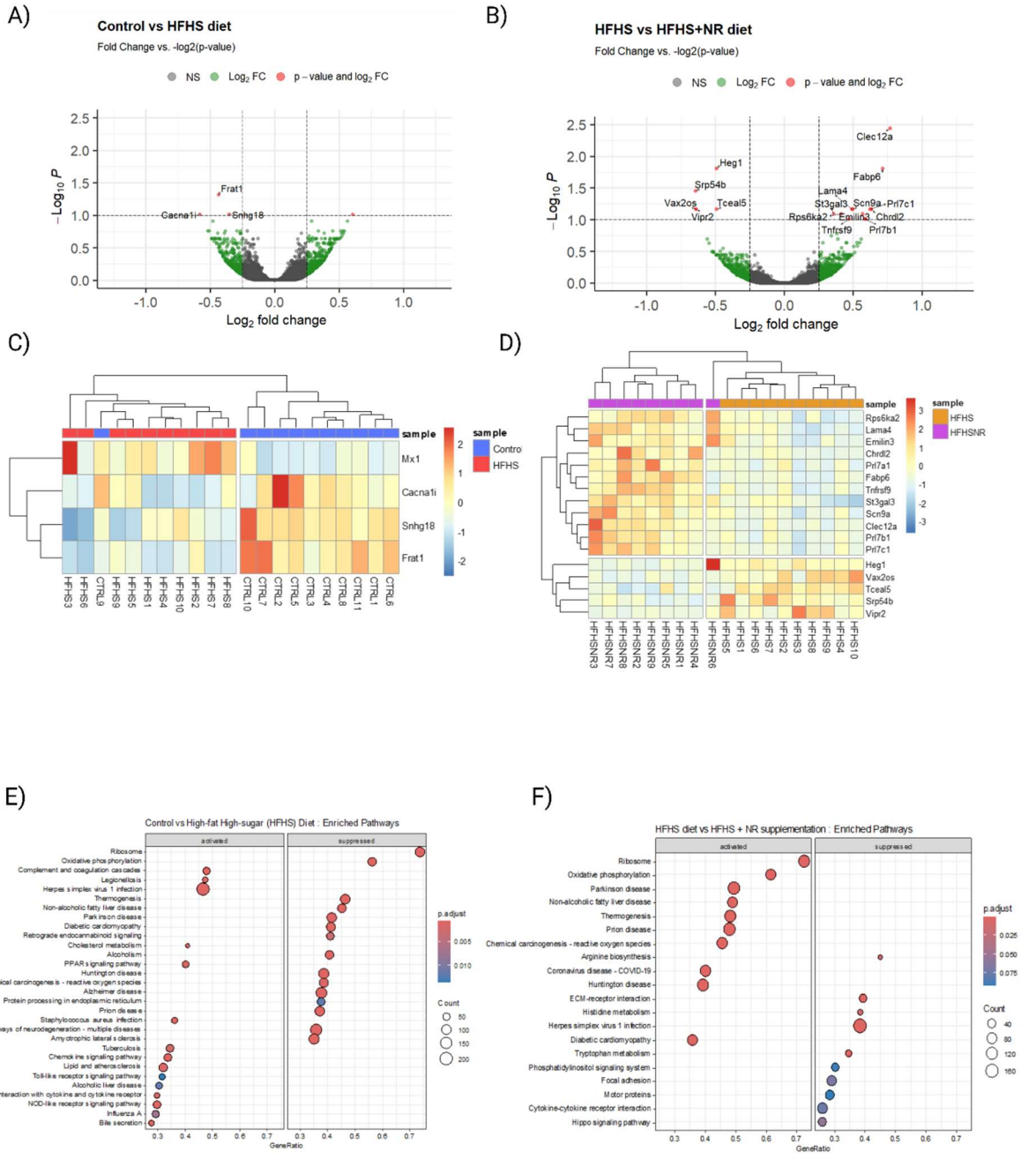
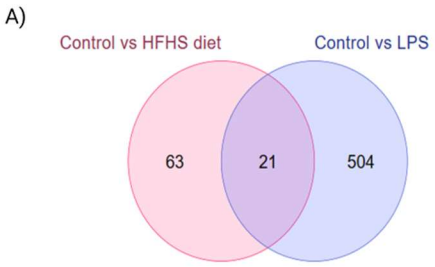
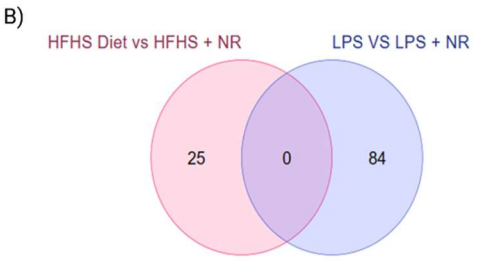


Figure 4.7. Gene expression signatures and fast-ranked pathway enrichment from placenta tissues of the HFHS-treated model of gestational obesity.
Volcano plots (A-B) and heatmaps (C-D) of all DEGs in HFHS diet vs. control (A, C), with and without NR supplementation (D). Annotated by KEGG pathway databases, the top biologically pathways that were differentially activated or supressed in placental tissue from HFHS diet vs. control (E), with and without NR supplementation (F). Identification of DEGs and pathways used p-value cutoff = 0.10, log2FC = 2.5. CTRL = control; HFHS = High fat high sugar diet, NR = nicotinamide riboside

4.2.2. Dysregulated immune response pathways were the most commonly shared features between the two rodent models of chronic inflammatory disorders in pregnancy.

For each of the rodent models of gestational inflammation, the ENSEMBL and OrthoDB databases were used to find a human ortholog for each of the statistically significant DEGs. Originally, the goal was to focus on the 1:1 orthologs, however due to the scarcity of DEGs in both models, all orthologs (including one-to-many relationships) were included. Surprisingly, no common DEGs were identified between the two NR-treated models of gestational inflammation (**Fig. 4.8, B**). In contrast, only 21 common DEGs were identified in the placentas of both LPS and HFHS-diet induced gestational inflammation models, when compared to controls (**Fig. 4.8, A**). These common DEGs included C-C motif chemokine ligands and zinc finger proteins, (**Fig. 4.8, C**), with the most enriched GO term being inflammatory processes such as monocyte chemotaxis and eosinophil migration. It should be noted, however, that not all placentas in each of these groups showed altered expression of these DEGs (**Fig 4.8, D-E**).





C)

Gene Symbol (HGNC)	Official Gene Name
CACNA1I	Calcium voltage-gated channel subunit alpha1
CCL18	C-C motif chemokine ligand 18
CCL3	C-C motif chemokine ligand 3
CCL3L1	C-C motif chemokine ligand 3 like 1
CCL3L3	C-C motif chemokine ligand 3 like 3
IL1RN	interleukin 1 receptor antagonist
ITGAM	integrin subunit alpha M
PRL	prolactin
PTPRC	protein tyrosine phosphatase receptor type C
SLFN12	schlafen family member 12
SLFN12L	schlafen family member 12 like
ZNF678	zinc finger protein 678
ZNF681	zinc finger protein 681
ZNF682	zinc finger protein 682
ZNF695	zinc finger protein 695
ZNF724	zinc finger protein 724
ZNF727	zinc finger protein 727
ZNF736	zinc finger protein 736
ZNF738	zinc finger protein 738
ZNF85	zinc finger protein 85

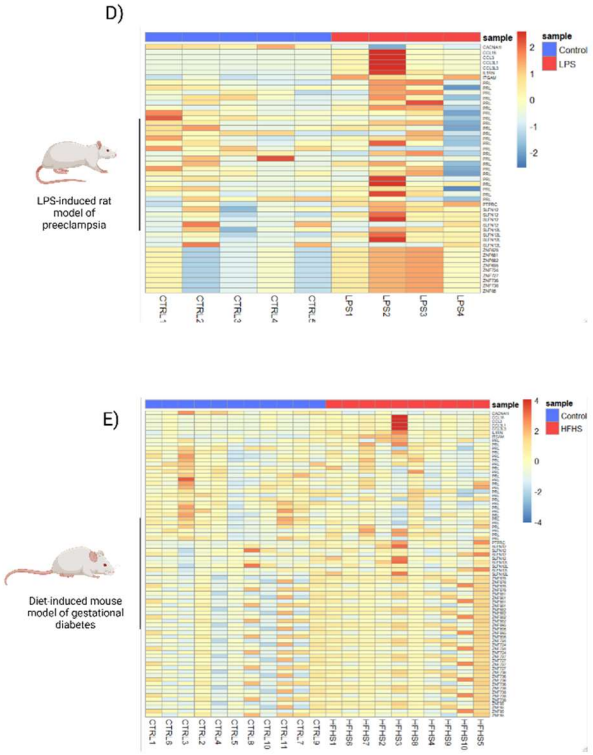


Figure 4.8. Comparison of shared genes across both models of gestational inflammation.
Venn diagrams showing the human ortholog of the differentially expressed genes compared between LPS and HFHS diet exposures (A), and both exposures with and without NAD^+ supplementation (B). A complete list of the 21 genes found to be commonly differentially expressed in both chronic inflammation gestational models (C), along with heatmaps displaying the expression patterns of all copies of these 21 genes across all animal profiles for both the LPS-treated (D) and HFHS diet exposed € models. LPS = LPS treatment, NR = nicotinamide riboside, HFHS = high-fat high-sugar diet.

Chapter 5 – Discussion

This study was the first to compare and contrast the underlying placental pathophysiology of different two rodent models of chronic inflammation in pregnancy, mimicking human cases of inflammation-driven PE and gestational obesity. More specifically, an integration of clinical phenotype, histological and transcriptomic profiles to examine a potential role for dysregulated placenta NAD^+ signalling and mitochondrial dysfunction in the establishment of placental disease in these two models was completed. Both gestational inflammation models shared similar pregnancy and fetal health outcomes, including decreased placental efficiency and fetal growth restriction. Further, evidence of placental inflammation, PARP hyperactivation, decreased NAD^+ content, and dysregulated mitochondrial function were in fact observed in both models, albeit to varying degrees. However, only a very small number of DEGs (21) in the placentas were found in common between these two models, suggesting the presence of alternate and divergent underlying placenta pathophysiology. Even more importantly, while NAD^+ replenishment demonstrated important therapeutic potential in the LPS-induced PE model, ultimately rescuing the clinical phenotype, this same therapeutic effect was not observed in the gestational obesity model. Although unexpected, these results serve as an important starting point for better understanding inflammation-mediated placental disease and identifying therapeutic targets that may be common or unique for the different inflammatory conditions.

5.1 Preeclampsia: Use of the LPS model and Establishment of Gestational Inflammation

Historically, the etiology of PE has been attributed to poorly remodelled spiral arteries and subsequent placental ischemic damage (Jung et al., 2022). However, emerging literature, including previous work done by our group, suggests there are in fact distinct subclasses of PE, one of which is driven by a heightened inflammatory response at the parental-fetal interface (Leavey et al., 2015, 2016b). Human placenta samples collected from this unique PE patient subclass uniquely demonstrates an overexpression of both pro-inflammatory genes and a number of the PARP family enzymes (Leavey et al., 2015, 2016b), as well as evidence of heightened PARP activation (excessive protein PARylation), NAD^+ depletion and mitochondrial dysfunction (Jahan, Vasam, Cariaco, et al., 2023b). These observational findings led to the establishment of a rodent model of this inflammation-mediated PE subclass, induced by daily LPS injections across pregnancy (Jahan, Vasam, Cariaco, et al., 2023a, 2023b). Likewise, this model demonstrated a

profound inflammatory response at the parental-fetal interface, PARP hyperactivation, depleted NAD^+ stores and mitochondrial dysfunction, ultimately leading to metrics of decreased placental efficiency and fetal growth restriction. Importantly, as observed in the literature in non-pregnant populations, therapeutic supplementation of NAD^+ with daily NR dosing was capable of boosting NAD^+ content in this model and rescuing the placental and fetal health outcomes. (Jahan, Vasam, Cariaco, et al., 2023a).

Despite decades of intensive research, no effective interventions have been identified for PE, likely the result of searching for a one-size fits all treatment, rather than subclass-specific therapies. Further compounding these endeavours, most animals do not naturally get PE, and as such we must rely of animal models that recapitulate some or most of the clinical findings of PE following hypothesis-driven genetic engineering, surgical interventions or infusion of PE-related factors (Bakrania et al., 2022; Chau et al., 2021; Gatford et al., 2020). In rodents, the most popular and widely used models recapitulate the placental ischemia and angiogenic imbalance facets of the human disease and are likely not completely appropriate for the study of the inflammation-mediated subclass of PE. One of the first inflammation-driven rodent models described was generated via injections of endotoxins into the pregnant dams to induce inflammation, resulting in parental hypertension and glomerular fibrinogen deposits (Faas et al., 1994)- clinical findings similar to what is observed in humans. More recently, the use of LPS as the inflammatory insult has become a standard protocol initiating the inflammatory insults observed in human cases of PE (Faas et al., 1994; Fricke et al., 2018). In preparation for the collection of the presented data set, our research group carried out a large scale model phenotyping exercise in order to identify a rodent model that best recapitulates the inflammation-driven subclass of PE in humans, specifically comparing rodent models infused with tumor necrosis factor-α (TNF-α) (LaMarca et al., 2005), polyinosinic:polycytidylic acid (Poly I:C) (Tinsley et al., 2009) or LPS (Hu et al., 2019). Of the three models profiled, the LPS model was the only one capable producing parental hypertension, decreased placental weight and efficiency, fetal growth restriction, along with compromised placental metabolic and mitochondrial function (Jahan, Vasam, Cariaco, et al., 2023a) – findings that were completely aligned to our profiling of human cases of inflammation-mediated PE.

In the current study, considerable transcriptomic evidence of placental inflammatory response induce by LPS was observed, including the upregulation of numerous immune-response related pathways such as the IL-7 signalling pathways, B-cell receptor signalling pathway as well as the Toll-like receptor signalling pathway, all of which have previously been identified as important contributors to placental pathology in PE (Laresgoiti-Servitje, 2013; Tinsley et al., 2009). Interestingly, a number of inflammatory disease-related pathways were also identified. These identified disease processes included those with chronic inflammatory pathologies (i.e. prion disease, rheumatoid arthritis, tuberculosis, atherosclerosis) and acute inflammatory pathologies (i.e. legionellosis, pertussis, COVID-19, Chagas disease, Staphylococcus aureus infection, leishmaniasis, amoebiasis) (Chen et al., 2018; Sullivan et al., 2000). Central to the pathophysiology of these inflammatory disease conditions, and to the inflammatory pathways specifically seen in our LPS-induced model of PE, are toll-like receptor and tumor necrosis factor (TNF) signalling pathways (Chu, 2013; Duan et al., 2022a; Sameer & Nissar, 2021; van Loo & Bertrand, 2022).

The TLRs, belonging to a family of pattern recognition receptors (PPRs) responsible for pathogen recognition and their subsequent removal, can identify pathogen-associated molecular patterns (PAMPs) and regulate downstream inflammatory mediators required for removal (Duan et al., 2022a; Sameer & Nissar, 2021). TLR activation in our model confirms that LPS, a potent PAMP, was recognized and was able to create the desired inflammatory environment (Mazgaeen & Gurung, 2020). Members of the PPR can identify distinct PAMP classes and are able to elicit their own downstream transcriptional activation of numerous inflammatory mediators as well as processes related to autophagy, cytokine processing, cell death and phagocytosis (Sameer & Nissar, 2021). Further, our model demonstrated an upregulation of C-type lectin receptors (CLRs) and NOD-like receptors (NLRs), both members of the PPR family, following LPS treatment, further confirming a strong signalling component of the TLR pathway in this placental pathophysiology (Sameer & Nissar, 2021). In human placentas, transcripts for TLR1 to TLR10 are expressed, with numerous of their protein products triggering immune responses at the parental and fetal tissue interfaces. It is speculated that an upregulation of these TLR expression profiles following an inflammatory insult, couple to their associated cytokine activation profiles, contribute to the establishment of oxidative stress and inflammation in a number of inflammatory gestational diseases (Duan et al., 2022b; Riley & Nelson, 2010; Tinsley et al., 2009).

Similarly, the TNF signalling pathway is a crucial component in the activation and regulation of PAMP-mediated inflammatory cascades (Chu, 2013; van Loo & Bertrand, 2022). It does this by activating the MAPK and NF-κB signalling pathways that promote TNF-mediated cell death, through either apoptosis, necroptosis or pyroptosis, and ultimately mount an inflammatory response against the pathogenic threat to the host cell. The dying cells exacerbate the pro-inflammatory gene expression in neighbouring cells, further recruiting immune cells to the site. This process can certainly be beneficial in clearing pathogens in the case of infection; however, in the absence of pathogens/infection, this same signalling pathways serves to generate profound cellular stress and tissue damage with no physiological benefit – as is the case at the parental-fetal interface in the context of PE. This pathological contribution of TNF signalling in the context of non-pregnant chronic inflammatory diseases has been well established in both humans and rodent models (Duan et al., 2022a; Sameer & Nissar, 2021; van Loo & Bertrand, 2022). Importantly, the TNF signalling pathway is likewise implicated in the pathophysiology of PE (Kumar et al., 2013), and in particular the pathophysiology of inflammation-mediated PE (Leavey et al., 2016b), and has been explored as a potential biomarker for the early diagnosis of PE. Interestingly, elevated circulation concentration of TNF-α are also associated with other common placental pathologies, including spontaneous abortions, fetal growth restriction and preterm labor (Fakhr et al., 2022). The TNF signaling pathway's involvement in such a broad spectrum of placental pathologies underscores its critical role in pregnancy-related complications, making it a promising subclass specific target for therapeutic intervention and early diagnosis of PE.

5.2 Gestational Obesity: Use of the HFHS model and Establishment of Gestational Inflammation

Chronic overconsumption of a calorie-rich diet, high in both fats and sugars, is one of the main driving factors behind the establishment of obesity in humans (Gibson, 1996). In American/European lifestyles, an average diet is comprised of ~36-40% kcal fat, with an obesity generating high-fat diet instead consisting of 50-60% kcal fat (an ~ 24% increase in fat content) (Speakman, 2019). The most widely used rodent models in the study of obesity, in both non-pregnant and pregnant animals, involve the usage of a high-fat diet at the upper extremes of these values (~ 60% kcal fat). In these models, an extreme obese phenotype is observed very rapidly,

which is not entirely reflective of the onset of human obesity which develops at a much more gradual pace over a longer period of time, often taking years to reach an obese state (Katan & Ludwig, 2010). For the purposes of the current study, our gestational obesity model used a rodent diet with ~42% kcal fat, which represents a 30% increase in fat content compared to the standard chow diet (~10% kcal fat). This fat content was selected for study to generate an obesity that is less severe and more accurately captures the development of obesity in humans (Hall & Jordan, 2008).

In North America, excessive dietary sugars, often consumed in the form of added sugars such as sucrose and high fructose corn syrup, have also been proposed to play a key role in the establishment of obesity (Chung et al., 2021). The CDC recommends a maximum of 10% of an individual's daily caloric intake to come from added dietary sugars (*Get the Facts: Added Sugars | Nutrition | CDC*, n.d.; Rakhra et al., 2020), however in North America individuals with obesity on average consume an upwards of 13% added dietary sugars. This excessive sugar intake has been associated with increasing body adiposity, systemic inflammation, hyperglycemia and dyslipidemia (Agarwal et al., 2018; Gregor & Hotamisligil, 2011; P. Mancuso, 2016). In the context of human pregnancy, more recent data has likewise suggested an association between high simple sugar intake and excessive gestational weight gain (GWG), gestational diabetes, PE and preterm birth (Crane et al., 2009; Guelinckx et al., 2008; Segura et al., 2017). As such, in order to best recapitulation the diet-induced gestational obesity seen in human population in our rodent model, we chose to use a moderately high-fat diet that also included a moderately high-sugar component. With the use of this HFHS-diet model, we were successful at establishing parental obesity, with a 9.98 g +/- 1.13 g increase in pre-pregnancy body weight, 9.48% +/- 1.03% increase in body fat percentage, coupled with parental hyperglycemia when compared to the standard chow-fed dams (**Supplementary Fig.1**).

Although the etiology and underlying pathophysiology of metabolic syndrome (MetS) are not entirely clear, it is believed that excessive adiposity and insulin resistance are key factors (Cornier et al., 2008; Terauchi et al., 2007). Activation of pathways in our transcriptomic analysis such as thermogenesis, alcoholic liver disease, bile secretion as well as cholesterol metabolism also suggest metabolic dysfunction may be present in our model in the form of MetS in our model. Indeed, adipokines secreted from the large adipose tissue beds help to establish a chronic

low-grade pro-inflammatory environment (P. Mancuso, 2016), and have been associated with the development of metabolic syndromes such as type 2 diabetes mellitus, cardiovascular disease and atherosclerosis, fatty liver disease and several cancers. Interestingly some of these same disease pathology pathways were identified in the gene set enrichment analysis conducted on placental tissues from our HFHS model, suggesting common inflammation-mediated pathophysiology elements (Cornier et al., 2008; M. Mancuso et al., 2006; Neto et al., 2023). An imbalance in pro- and anti-inflammatory adipocytokine (i.e. leptin and adiponectin, respectively) levels favoring a pro-inflammatory state, is known to dysregulate energy, glucose and lipid metabolic pathways in individuals with obesity (P. Mancuso, 2016). Adipose-derived leptin concentrations are considerably higher in the context of obesity, often leading to a systemic state of leptin-resistance and hindering lipid oxidation, ultimately leading to lipotoxicity, insulin resistance and increased inflammation (Lionetti et al., 2009). Furthermore, an increase in circulating fatty acids can further exacerbate the inflammatory cascade via activation of TLR receptors on numerous cell types, propagating further the observed insulin resistance, lipolysis and the inflammatory response (Rull et al., 2010). Importantly, our collected placental transcriptomic data supported the presence of many of these inflammation-mediated metabolic disturbance in our gestational obesity model, including gene set enrichment for chemokine signalling pathways, TLR signalling and NOD-like receptor signalling. Further, this analysis demonstrated enrichment of disease-specific pathways with a central PAMP-driven inflammatory component (i.e. legionellosis, herpes simplex virus 1 infection, tuberculosis, influenza) – similar to what we observed in our LPS-PE model (Chenet al., 2018).

Interestingly, and unique to the HFHS model, was the observation of suppressed protein processing in the endoplasmic reticulum (ER). Within the ER, under normal physiological conditions, the process of protein homeostasis (i.e. protestasis) is responsible for identifying misfolded proteins and initiating targeted degradation processes (Balch et al., 2008; Kaushik & Cuervo, 2015). However, under pathological conditions, possibly present in the placentas of our HFHS diet model, the accumulation of misfolding proteins and the initiation of the unfolded protein response (UPR) is observed. The activation of the UPR is meant to mediate the stress being placed on the ER by facilitating protein folding mechanisms, decreasing protein synthesis processes and enhancing degradation proteasomal pathways. However, UPR activation also promotes autophagy processes and amplifies the expression of cytokines and inflammatory

factors such as NF-κB and IRF3, exacerbating the inflammatory response present (Chipurupalli et al., 2021; Hetz & Saxena, 2017). In this vein, it comes as no surprise that ER-induced stress and UPR pathways have been associated with several chronic inflammatory diseases such as irritable bowel syndrome, rheumatoid arthritis and obesity in non-pregnant humans and animal models (Chipurupalli et al., 2021). Further, these results align with previous observations of increased placental ER stress, UPR activation, inflammation and altered placental function in the context of gestational obesity (Bastida-Ruiz et al., 2017; Capatina et al., 2021; Shen et al., 2023).

5.3 Consequences of chronic gestational inflammation on fetal growth profiles

Infant birth weights are a useful marker of in utero fetal growth and development profiles and can identify infants that may have undergone adverse in utero programming events. The term small-for-gestational age (SGA) is applied to infants with birth weights less than the 10th percentile, adjusted for length of gestation, fetal sex and race (Lewandowska, 2021). While many infants found in the lowest 10th centile are simply genetically and constitutionally pre-disposed to be smaller than their peers, a portion of these infants have instead experienced pathological growth restriction in utero – termed fetal growth restriction (FGR). A clinical diagnosis of FGR therefore requires the presence of SGA in addition to evidence of compromised in utero growth, such as abnormal uterine or umbilical artery doppler recordings or placenta pathology findings. Fetuses experiencing placenta-mediated growth restriction are at high risk of poor short term health outcomes, such as premature birth and stillbirth, and also have increased lifetime risks for the development of chronic diseases, including cardiovascular and metabolic disturbances (Bligard & Odibo, 2020).

In both the LPS-induced PE model and the HFHS-induced obesity model, a marked reduction in fetal weights were observed. It is postulated that the chronic systemic inflammation led to the observed placental insufficiency in both models, ultimately compromising fetal growth. This hypothesis is supported by a large body of literature that links a pro-inflammatory in utero environment with poor fetal growth profiles. Specifically, a strong inverse relationship has been reported between umbilical cord blood concentrations of interleukin 6 (IL-6), TNF-α and C-reactive protein (CRP) and infant birth weights (Lausten-Thomsen et al., 2014). PE in human populations is strongly associated with FGR, with estimates that ~ 30% of all PE pregnancies

result in placenta-mediated FGR (Kovo et al., 2012; J. M. Roberts & Escudero, 2012). This same obersvation has been observed in a number of rodent models of PE as well (Sones & Davisson, 2016). Parental obesity, on the other hand, is associated with birthweight extremes in both directions, both cases of FGR and cases of large for gestational age infants (LGA; >90th percentile) (Lwin et al., 2023; Yu et al., 2011; Bligard & Odibo, 2020; Kalagiri et al., 2016; C. X. W. Zhang et al., 2024). It is thought that in instances of profound parental metabolic disturbance (i.e. severe hyperglycemia, gestational diabetes) a predominance of fetal overgrowth profiles are seen (Hong & Lee, 2021), attributed decreased parental insulin sensitivity, excessive glucose accumulation in utero and fetal hyperglycemia. This can promote the excessive release of growth factors such as insulin as well as insulin-like growth factors I (IGF-I) and -II (IGF-II), which can lead to the LGA phenotype (Catalano, 2010; Yu et al., 2011). Conversely, it is proposed cases of gestational obesity that instead demonstrate a more profound inflammation-mediated placental dysfunction lead to the predominance of an FGR phenotype (Bligard & Odibo, 2020; Kalagiri et al., 2016; C. X. W. Zhang et al., 2024),

Placental pathology findings associated with FGR are highly heterogenous, and include evidence of placental ischemia, abnormal placental development and structure and chronic inflammatory lesions (C. J. Kim et al., 2015; Beneventi et al., 2023). Importantly, in human cases of both PE and gestational obesity there is considerable overlap in the types of chronic inflammatory lesions described in the placenta, as detailed below. Chronic inflammatory lesions in the placenta are marked by the presence of lymphocytes, plasma cells, and/or macrophages. These lesions are most frequently observed in the villous tree (known as villitis), the extraplacental chorioamniotic membranes (referred to as chorioamnionitis), the chorionic plate, and the basal plate (termed deciduitis) (C. J. Kim et al., 2015). Such lesions can result from bacterial, viral, or parasitic infections, or may be immune-mediated, as seen in cases of maternal anti-fetal rejection. These profound inflammatory lesions are indicative of poor placental function compromising placental efficiency and ultimately leading to poor fetal growth outcomes, such as FGR. Chronic inflammatory lesions in the placenta have also been proposed to be indicative of parental anti-fetal rejection, which triggers a distinctive fetal systemic inflammatory response characterized by elevated levels of the fetal plasma T cell chemokine (CXCL10) (C. J. Kim et al., 2015). Notably, CXCL10 was also found to be increased in our analysis of the inflammatory subclass of PE, reinforcing the involvement of the immune response in the development of the clinical

symptoms associated with the disease. Importantly these chronic inflammatory lesions are commonly described not only in cases of PE, but also normotensive FGR and gestational obesity (C. J. Kim et al., 2015; M. J. Kim et al., 2009; Labarrere & Althabe, 1985; Lee et al., 2011; Romero et al., 2013).

5.4 Consequences of chronic gestational inflammation on placental development, structure and function

Normal fetal growth requires the following: 1) sufficient maternal delivery of nutrients, which is dependent on the anatomy and the physiology of the uteroplacental vasculature, 2) sufficient placental uptake of nutrients, which is dependent on appropriate villous trophoblast differentiation and nutrient transporter expression, 3) sufficient placental-fetal nutrient transfer, which is dependent on the architecture of the placental interhaemal membrane and appropriate development of the feto-placental vasculature (Salafia, 1997). Considering these requirements, placental-mediated diseases commonly present with one or more of the following features: abnormal uteroplacental perfusion leading to prolonged uteroplacental malperfusion, chronic inflammatory lesions or coagulation-related pathologies in the uteroplacental, intervillous and/or the fetoplacental vasculature (Salafia, 1997).

Structural abnormalities of the placenta, resulting from either defective placentation or damage by environmental/physiological insults, can disrupt placental function, adversely impacting fetal growth and development (D. W. Kim et al., 2014; Kovo et al., 2012; Opichka et al., 2021; Sones & Davisson, 2016). Reduced placental weights are associated with poorer pregnancy health outcomes, such as FGR, owing to the potential for reduced surface area for parental-fetal exchange of gas and nutrients. However, placental size alone is not always the best predictor of placental function. Rather, placental efficiency, which is calculated as the ratio of infant birth weight to placental weight, is a more informative metric, providing better insight into the placentas capacity to support fetal growth (Zhang, 2023). However, it should be noted that in cases of pre-term delivery with severe placental dysfunction, the immature placenta often demonstrates very low placental weights, resulting in an artificially inflated efficiency ratios (Christians et al., 2018; P. Zhang, 2023). While only the LPS-induced PE model demonstrated

reduced placental weight, both models studied in the current thesis did demonstrate significant reductions in the measured placental efficiency.

A reduction in placental efficiency can be the result of structural and/or molecular abnormalities that compromise parental-fetal exchange. Previous characterization of similar rodent models used in the current thesis have included descriptions of distinct and similar structural abnormalities (D. W. Kim et al., 2014; Opichka et al., 2021; Sones & Davisson, 2016), specifically a reduction in the size of the labyrinth zone – the site of parental-fetal exchange. We likewise noted a reduced labyrinth depth in the LPS-induced PE model, and a similar non-significant trend in the gestational obesity model. Maldevelopment of the labyrinth, often involves deficient remodelling of the uterine spiral arteries and/or reduced branching angiogenesis in the feto-placental vascular tree. The abnormal and persistent high placental vascular resistance caused by the inadequately transformed spiral arteries can lead to atherosis or fibrinoid necrosis of vessels as well as exacerbating the pre-existing hypoxia-reperfusion injury (Aplin et al., 2020). Of interest, incomplete maturation of labyrinth zone has previously been characterized in PPARγ knockout rodent models. Considering we observed reduced expression of gene sets related to PPAR signalling pathways in the placentas of our gestational obesity model, future investigations into the role of obesity-mediated PPAR signalling disruption and placental dysfunction may be warranted (Mahadevan et al., 2023).

Reduced placental efficiency can arise from improper development of the feto-placental vasculature. This includes insufficient thinning of the interhaemal membrane. In a normal pregnancy, this membrane gradually thins to optimize the exchange of nutrients and gases. When the membrane fails to thin appropriately, the increased distance for exchange impairs placental efficiency, which can reduce the oxygen and nutrient supply to the fetus and potentially contribute to conditions such as PE and FGR (Sehgal et al., 2018). Additionally, while neither model revealed changes in gene expression related to nutrient transport, FGR can still arise from compromised placental nutrient transport processes that may not be visible under a microscope. Inadequate nutrient transport, whether from decreased expression or impaired activity, limits the supply of essential nutrients such as amino acids, fatty acids and glucose, thereby restricting fetal growth (Gaccioli & Lager, 2016). This same problem is also seen in PE, where inadequate nutrient transport contributes to FGR and related complications.

5.5 Consequences of chronic gestational inflammation on placental NAD^+ signalling and mitochondrial health

Both rodent models examined in the current study showed a significant increase placental protein PARylation, indicative of increased PARP activity, accompanied to a decrease in placental NAD^+ content. These findings support our overarching hypothesis that PARP-mediated NAD^+ depletion may be a common contributing mechanism through which placental dysfunction is established in inflammatory conditions of pregnancies, such as PE and parental obesity. NAD^+ deficiency is detrimental to the health of a cell, as it is crucial cofactor for essential cellular processes such as immune regulation, energy metabolism, redox homeostasis, DNA repair, epigenetic modification, and stress resistance (Balsa et al., 2020). Further, cellular NAD^+ depletion compromises oxidative phosphorylation and other related ATP synthesis processes, leaving the mitochondria unable to regulate excessive inflammation-mediated oxidative stress (Balsa et al., 2020; Walker & Tian, 2018; Xie et al., 2020). Previous work carried out by our group demonstrated decreased respiration rates in isolated placental mitochondrial from the LPS-induced PE model, as well as evidence of increased oxidative stress in these placentas – findings that were reversed with NR treatment. Interestingly, when examining the gene expression profiles of these same placentas (**Fig. 4.4, A-B**), we surprisingly observed an upregulation of genes related to oxidative phosphorylation. There was considerable variation in the expression profiles of this gene set across placentas examined, however it is possible that these findings may be indicative of a protective feedback attempt by the placenta to improve the compromised mitochondrial function. It should be noted that compromised oxidative phosphorylation and heighted oxidative stress are commonly described placental features in human cases of PE. When looking at the gene set enrichment analysis of placenta tissue from our gestational obesity model, evidence of dysregulation of ATP synthesis was observed, with mild (and variable) suppression of oxidative phosphorylation pathways (**Fig 4.5, A-B**).

The discrepancy in the functional gene set enrichment findings between these two models is suspected to be connected to difference in the source and nature of the immune response seen between the two models of gestational disease. To elaborate, the immune-mediated PE subtype is distinguished by an overactive immune response at the maternal-fetal interface (Leavey et al.,

2015, 2016b; Than et al., 2022). This response is believed to result from maternal rejection of placental or fetal tissues, evidenced by the upregulation of CXCL10, leading to the activation of inflammatory pathways such as NF-kB and the recruitment of pro-inflammatory cytokines like TNFα and IL-6 (C. J. Kim et al., 2015b; Opichka et al., 2021; Roberts & Escudero, 2012). These processes intensify the inflammatory processes in the placenta, leading to placental damage and dysfunction (C. J. Kim et al., 2015a; Nelson & Myatt, 2020). While an initial localized placental inflammatory insult is proposed to initiate the disease process in cases of inflammation-mediated PE, in cases of gestational obesity, it is instead a widespread systemic pro-inflammatory environment in the parent that may underlie dysregulated placentation. The obesity-mediated inflammation is characterized by the release of pro-inflammatory factors by adipose tissue beds, such as leptin, IL-6 and TNF-α, creating a chronic, low-grade and systemic response which ultimately results in impaired insulin signalling and overall metabolic dysfunction, affecting multiple organs and systems, including those involved in pregnancy such as the placenta (Bastard et al., 2006; Ellulu et al., 2017; C. X. W. Zhang et al., 2024).

As such, while the inflammatory mediators might be similar in both models of gestational pathologies, the dysregulated pathways linked to immune responses or other disease states are likely distinct. Specifically, the PE model is proposed to demonstrate placental-specific inflammation, whereas gestational obesity is proposed to demonstrate systemic metabolic inflammation. As predicted, our transcriptomic analysis demonstrates that our LPS-mediated PE model was associated with activation of immune pathways that were directly implicated in placental dysfunction (i.e. signalling pathways for IL-17, B-cell receptor, TNF, TLR chemokines as well as cytokine-cytokine receptor interactions). Similarly, gestational obesity was connected to the dysregulation of pathways and disease states that represent metabolic dysfunction (activated: cholesterol metabolism, chemokine signalling pathway, lipid and atherosclerosis, TLR signalling pathway, bile secretion; suppressed: thermogenesis, non-alcoholic fatty liver disease, diabetic myopathy).

The link between inflammation and impaired mitochondrial function due to PARP hyperactivation and NAD^+ overconsumption is well established (Katsyuba et al., 2020; Navas & Carnero, 2021; Strømland et al., 2019). Overall, the collected results do demonstrate aspects of disrupted NAD+ signalling in both inflammatory models studied, however the degree to which

this mechanism contributes to the collective pathophysiology may certainly differ across models – a notion further supported by the NR intervention data discussed below.

5.6 Impact of therapeutic supplementation of placental NAD^+ stores in models of chronic inflammation

As placental NAD^+ depletion was observed in both rodent models of gestational inflammation, and sufficient NAD^+ content is required to support appropriate cellular energy metabolism, an investigation into the therapeutic intervention of NAD^+ supplementation was evaluated for its ability to improve placental and fetal health outcomes. Indeed, upon administration of a NAD^+ precursor, nicotinamide riboside (NR), the LPS-induced PE model demonstrated normalization of placental NAD^+ signalling, coupled to suppression of inflammatory pathways, and importantly, a rescue of the placental and fetal weight phenotype. Collectively, these data certainly support a critical role for NAD^+ depletion in the establishment of placental dysfunction in this model and highlight the therapeutic potential of targeting these pathways to improve pregnancy outcomes in the inflammation-driven subclass of PE. It is also interesting to note that NR supplementation in this PE model also led to the activation of insulin signalling pathways, a finding supported by previous rodent models (Revollo, Grimm, et al., 2007; Revollo, Körner, et al., 2007). In fact, mice with diminished concentrations of NAMPT, a critical cofactor regulating the biosynthesis of NAD^+ in the cell, demonstrated lowered glucose tolerance because of compromised insulin secretion. Supplementation of NMN, the product of NAMPT, was able to rescue both NAD^+ content as well as insulin production (Revollo, Grimm, et al., 2007; Revollo, Körner, et al., 2007). This is of particular interest for our PE model, as PE patients have a 2-fold increased risk of developing gestational diabetes (P. Wu et al., 2016). Up-regulated insulin signalling pathways suggests that NAD^+ supplementation may also exert a protective effect against insulin resistance and the subsequent development of metabolic syndrome – an avenue of importance for future research endeavours.

Unexpectedly, in our HFHS-diet induced gestational obesity model, we saw no therapeutic benefit whatsoever with NAD^+ supplementation, despite observing heightened PARP activation and depleted placenta NAD^+ content in the untreated animals. Most surprisingly, placental

NAD^+ content was *not* found to increase with NAD^+ supplementation (Fig 4.3F), in contrast to that observed in the LPS-induced PE model. One potential factor that could have led to these discrepant results could be that the same NR dosage was used in both models, while this dosage had only been previously validated in the LPS-induced PE model. As these are not only different rodent species (mouse vs rat), the type and timing of the inflammatory insult in both models is quite different, and as such a dose-response validation series in the HFHS mouse model is certainly warranted. It is certainly possible that the long length of the inflammatory exposure in the HFHS diet (~ 17 weeks) may have resulted in a much more profound and systemic NAD^+ deficiency, that could not be overcome in such a short therapeutic window (18 days). Additionally, the timing of the NR intervention, only commencing once the systemic inflammation was already established (after 12-15 weeks on the HFHS diet), coupled to the relatively short duration of the NR intervention (18 days), may not have been sufficient to see clinical benefits. In contrast, in the LPS-induced PE model, the NR intervention was initiated *prior* (GD 0.5) to the initiation of the LPS inflammatory insult (GD13-18), representing more of a preventative intervention. It is also possible that while NAD^+ signalling is dysregulated in the gestational obesity model, it is not in fact a key factor *contributing* to the placental dysfunction observed in this condition. Rather, could be a consequence of ongoing/pre-exisiting placental disease. This could also explain why NR supplementation was not successful as a therapeutic intervention. However, it is important to note that the NR supplementation was unable to increase NAD^+ content in our model, confirming that the insufficiency of the administered dose was likely partly responsible for the treatment's lack of success. This difference in treatment strategies, in conjunction with a potentially insufficient NR dosage, may have limited the success of NR intervention in the gestational obesity model. Indeed, there have been rodent model studies in which NR administration at a higher dose (up to 1,200 mg/kg/d) for a much longer time frame (90-day repeated dose) demonstrated therapeutic potential with boosting NAD^+ levels with minimal adverse side effects (D. B. Conze et al., 2016; Marinescu et al., 2020). Understanding at what point NAD^+ supplementation might be capable of protecting against inflammation-mediated metabolic placental dysfunction would be a keystone moment in understanding the mechanisms at play and therapeutic options for this class of inflammatory obstetrical disorders.

5.7 Comparing the two models of chronic inflammation for the purpose of identifying commonalities

Quite surprisingly, very few DEGs were shared in the placental gene expression profiles across the two inflammatory models of pregnancy. The primary commonality shared between models included members of the inflammatory response, such as various chemokine (C-C motif) ligands (CCL), interleukin-1 receptor antagonist (IL1RN) and integrin alpha M (ITGAM). Interestingly, a significant number of zinc finger (ZNF) protein family members (i.e. ZNF678, ZNF681, ZNF682, ZNF695, ZNF724, ZNF727, ZNF736, ZNF738, ZNF85) were also found to be commonly up- and down-regulated in both models. Interestingly, ZNF724, with its role in stress response pathways, has been associated with the mechanisms of gout development, particularly the crystal-induced inflammation stage that results in the establishment of gout (Kawamura et al., 2019).

Downregulation of the transcription factors ZNF724 and ZNF85 have been closely associated with the development of cancerous nodes and its metastatic stages in lung squamous cell carcinoma, which the authors attributed to activation of carbohydrate binding and oxidoreductase activity (Lian et al., 2020). Both ZNF724 and ZNF85 were upregulated in our models. The LPS-induced PE model exhibited a strong activation of these genes, while the gestational obesity model showed a more variable but generally increasing trend. These observations are likely attributed to the reduced mitochondrial activity in our models. Although specific disease associations are less well-characterized for the remaining ZNF members, many of them (i.e. ZNF678, ZNF681, ZNF682, ZNF695, ZNF727, ZNF736, ZNF738, ZNF85) are associated with transcriptional regulation pathways that, when dysregulated, contribute to diseases including cancer or developmental disorders, identifying them as a potential therapeutic avenue for inflammatory disease (S. Kim & Shendure, 2019).

5.8 Future Directions

This body of work importantly confirms that although both inflammatory-mediated conditions, parental obesity and PE, share aspects of a common phenotypic profile and dysregulated NAD^+ signaling in the placenta. However, the gene expression dataset certainly provides convincing evidence that the placental pathophysiologies of these two inflammatory conditions of pregnancy are in fact divergent in nature.

Further avenues of investigation into potential similarities between gestational obesity and immune-mediated PE includes exploring the role played by insulin signalling pathways in the pathology of the two inflammatory disease conditions. Both conditions exhibit disrupted insulin signaling, with many individuals with PE showing hyperinsulinemia similar to those with gestational obesity. Insulin resistance in these contexts is linked to elevated inflammation, oxidative stress, and endothelial dysfunction, which can further impair insulin signaling (Anton et al., 2014; de Barros Mucci et al., 2020; Lopez-Jaramillo et al., 2018). Exploring how these interconnected factors influence both conditions could provide deeper insights into their shared inflammatory processes and enhance our understanding of their underlying mechanisms.

Furthermore, due to the multifactorial nature of both diseases, it may be more applicable to analyze not only the transcriptome, but also the proteome and metabolome for a multidisciplinary multi-omics approach. (Manzoni et al., 2018). Transcriptomics allows for a deeper investigation of the dynamics of cellular and tissue gene expression profiles and how their changes pertain to health and disease, whereas proteomics or metabolomics have the ability to uncover potential diagnostic markers in disease as they can identify altered cellular processes and pathways. Future work would entail creating more thorough and integrated omics datasets for both targeted and untargeted approaches for each of the diseases, to thoroughly examine each of the dysregulated pathways that were seen in both diseases. Our study indicates that dysregulated NAD^+ signaling pathways are crucial to the pathology of immune-mediated PE in rodent models, as evidenced by the success of NAD^+ supplementation in reducing disease symptoms. Integrating data from various omics layers can help identify specific biomarkers for early sub-class specific detection, diagnosis, and monitoring of PE. For example, combining proteomics with metabolomics might identify NAD^+ and other specific related metabolites associated with the onset of this PE subtype. Although the NAD^+ supplementation therapy appears to be successful in restoring normal health outcomes in the PE model in the context of our study, its safety profile in pregnant human populations remains to be thoroughly investigated. A multi-omics approach enables comprehensive monitoring of unintended effects of drugs on pathways beyond their primary target. For instance, integrating proteomics and metabolomics data can uncover off-target effects, providing crucial insights into drug safety. This information can then guide adjustments to the drug or its dosage to minimize adverse effects and optimize therapeutic outcomes.

5.9 Conclusion

In conclusion, a detailed comparison of two rodent models of gestational inflammation, representing cases of inflammation-drive PE and gestational obesity in humans, demonstrated similar dysregulation of key components of the placental NAD^+ signaling pathway, suggesting that this mechanism may in part contribute to the establishment of placental disease in these conditions. Further, therapeutically targeting these pathways, specifically in the LPS-induced PE model, shows tremendous potential, and certainly warrants further investigation as this approach may have tremendous potential to improve the health of millions of pregnant people and their infants (Chen et al., 2018; Sullivan et al., 2000).

Appendix 1 – Animal Care Protocols

Friday, August 9, 2024 at 11:11:58 Eastern Daylight Time

Subject: Protocol request #2923 Approved
Date: Thursday, October 14, 2021 at 10:17:53 AM Eastern Daylight Saving Time
From: NoReply@UOttawa.ca
To: Courtney Reeks, Shannon Bainbridge-Whiteside, Fahmida Jahan, Kim Reid, Goutham Vasam, Nikita Larionov, Alex Green, Keir J. Menzies, ACVSREQ, Marie Bédard, Caterina Belle

The protocol request #2923, NAD+ depletion as a cause of placental dysfunction in preeclampsia has been approved by the Animal Care Committee and has been assigned the following protocol number HSe-2923-R3 A2.

To meet University and public health directives, all planned work in animal facilities needs to be approved by ACVS before you begin. Please reserve each room to be used through the ACVS Scheduler, https://acvsapps.med.uottawa.ca/acvs-portal/acvs-scheduler/ in advance of your work (5 days before please, and 2 hr maximum bookings unless pre-approved by ACVS). ACVS will get back to you about each request.

Please contact Holly Orlando (horlando@uottawa.ca) for questions related to work in the animal facilities, and Marie Bédard (mbedar7@uottawa.ca) for any protocol or training related questions.

Thank you and best wishes,

The uOttawa Animal Care Committee

Subject: Protocol request #3937 Approved
Date: Monday, September 19, 2022 at 9:33:13 AM Eastern Daylight Saving Time
From: acc@uottawa.ca.ca
To: Shannon Bainbridge-Whiteside, Keir J. Menzies, Yusmaris Cariaco, Hannah Poisson, Nikita Larionov, Alex Green, Fahmida Jahan, ACVSREQ, Marie Bédard, Courtney Reeks, Caterina Belle, Jennifer Andrusiak

Attention : courriel externe | external email

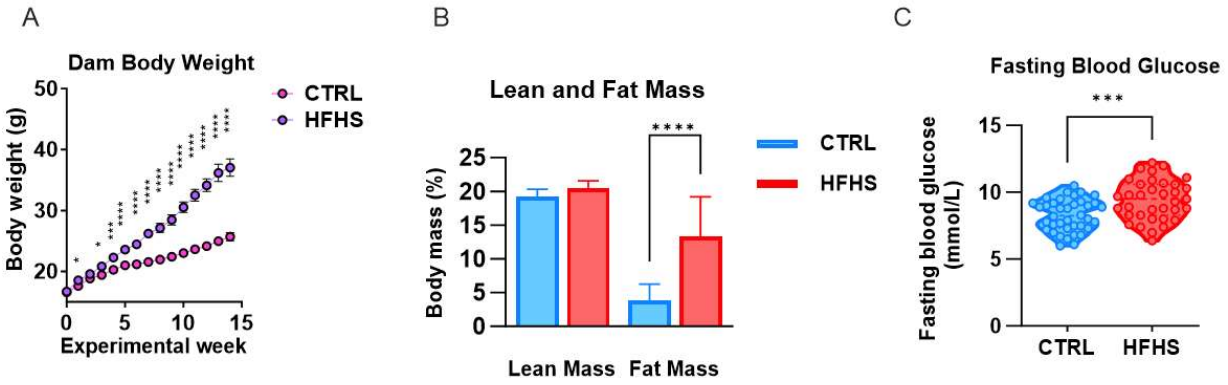
The protocol request #3937, Role of poly (ADP-ribose) polymerases in placental and fetal development during maternal obesity has been approved by the Animal Care Committee and has been assigned the following protocol number CMM-3937e.

For your animal work, please reserve the space you will be using through the ACVS Scheduler, https://acvsapps.med.uottawa.ca/acvs-portal/acvs-scheduler/ normally 5 days in advance of your work. Please specify what procedures you will be doing, including any invasive procedures or chemicals or biologicals to be used, so that ACVS can prepare what is needed, the work can be done safely and the animals can be followed-up on appropriately.

Thank you and best wishes,

The uOttawa Animal Care Committee

Appendix 2 – Supplemental Data



Supplemental Figure 3.1. Dam measurements in a HFHS diet-induced model of obesity.
Baseline body mass of 6-week-old female mice prior to diet administration (A) Body weights throughout the gestational period (B) Lean and fat mass obtained from Echo-MRI tesing(C) Fasting Blood Glucose. Differences in diet exposure assessed via unpaired t-test with Bonferroni post-hoc test (*p<0.05). Two-way ANOVA with Bonferroni post-hoc test assessed differences by diet and treatment (*p<0.05). CTRL= control chow diet, HFHS = high-fat high-sugar diet

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