The role of NAD^+ signalling in the establishment of placenta dysfunction in cases of inflammation-driven preeclampsia

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Abstract

Preeclampsia (PE), a hypertensive disease of pregnancy, occurring at or after gestational week 20. PE can have life threating consequences for both the mother and the baby. PE is a highly heterogenous disease which makes it challenging to identify any effective therapeutic interventions. We previously discovered three molecular subclasses of PE disease. One of these subclasses is characterized by heightened placental inflammation (inflammation-driven PE). Since it is a newly identified form of PE, we currently do not know about the molecular mechanisms driving this inflammation-driven form of PE. Interestingly, we have observed that placentas from this inflammatory PE subclass uniquely express higher levels of NAD^+ consuming enzymes-PARPs - and thus exhibit a decrease in NAD^+ content. NAD^+ is a regulator of cellular energy metabolism and mitochondrial function. Several studies in the non-pregnant populations suggested that pro-inflammatory disease conditions can trigger hyperactivation of NAD^+ consuming enzymes causing a depletion in total NAD^+ content, leading to mitochondrial dysfunction and organ failure. Thus, we tested the hypothesis that NAD^+ depletion causes placental mitochondrial dysfunction in the inflammatory subclass of PE and that boosting NAD^+ could prevent development of this form of placental disease. We aimed to profile PARP activity, NAD^+ availability, and mitochondrial health in human cases of all three PE subclasses. We examined the causal relationship between inflammation and dysregulated NAD^+ signalling in both an *in vitro* human trophoblast culture model and in a rodent model of inflammation-driven PE. We also evaluated the therapeutic potential of NAD^+ booster, nicotinamide riboside (NR) to improve placental health and function in the rodent model of inflammation-

driven PE. Our results suggest that along with increased activity of PARP enzymes and decreased NAD^+ levels, human inflammatory PE placentas also exhibit decreased levels of mitochondrial proteins and increased oxidative DNA damage. Using an *in vitro* human placental (HTR8 cell line) inflammation model we showed that increasing NAD^+ under an inflammatory condition improved trophoblast mitochondrial and cellular function. Using an *in vivo* LPS induced rat model of inflammation-driven PE, we demonstrated that NAD^+ boosting during pregnancy improved placental mitochondrial function, reduced inflammation and oxidative stress. This subsequently resulted in improved pregnancy outcomes demonstrated by reduced maternal blood pressure, increased placental/fetal weights and increased fetal survival in the LPS model. Overall, this study identifies targeting NAD^+ signaling as a promising intervention for PE. NAD^+ boosting through NR has been tested in non-pregnant human populations and found to be safe and effective in enhancing NAD^+ levels. Thus, findings of this thesis lay the ground to test NAD^+ boosting strategies in PE patients in near future.

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List of Abbreviations

ADP	Adenosinediphosphate
ADPR	ADP-ribose
ATP5J2	ATP synthase membrane subunit f
vATP5A	ATP synthase subunit alpha of Complex V
CLPP	Caseinolytic Mitochondrial Matrix Peptidase Proteolytic Subunit
CIII	Cytochrome c oxidoreductase
CTBs	Cytotrophoblast cells
CCC	Cytotrophoblast cell column
CCL2	Chemokine ligand 2
iEVTs	Interstitial extra-villous trophoblasts
eEVTs	Endovascular extra-villous trophoblasts
GPX	glutathione peroxidase
p-H2A.X	histone H2A.X
ICAM-1	Intercellular ddhesion molecule 1
IFN-γ	Interferon
iNOS or NOS2	Inducible nitric oxide synthase
IP	Intraperitoneally
LPS	Lipopolysaccharide
Mif	Macrophage migration inhibitory factor
MELAS	Mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes
MRP	Mitochondrial ribosomal proteins,
mnSOD2	Manganese-dependent superoxide dismutase
MTCO1	Mitochondrially Encoded Cytochrome C Oxidase I
MARylation	Mono-ADPribosylation
NAD+	Nicotinamide adenine dinucleotide
NDUFB8	NADH:Ubiquinone Oxidoreductase Subunit B8
NAM	Nicotinamide
OPA1	Optic atrophy type 1
OXPHOS	Oxidative phosphorylation
PARP	Poly(ADPribose) polymerases
PIGF	Placental growth factor
Poly I:C	Polyinosinic:polycytidylic acid
PE	Preeclampsia
ROS, RNS	Reactive oxygen and nitrogen species
Redox	Reduction–oxidation
STB	Syncytiotrophoblast
SIRT	Sirtuins

SARM1	Sterile alpha and toll/interleukin-1 receptor motif-containing protein 1,
TCA	Tricarboxylic acid
TNF-α	Tumour necrosis factor
TSFM	Mitochondrial translation elongation factor
UQCRC1	Ubiquinol-Cytochrome C Reductase Core Protein 1
UQCRC2	Ubiquinol-Cytochrome C Reductase Core Protein 2 (UQCRC2)
UQCRS1	UQCR Rieske Iron-Sulfur Polypeptide 1
YME1L1	YME1 like 1 ATPase
8-oxo-DG	8-Oxo-2'-deoxyguanosine

Chapter 1 - General Introduction

1.1 The Human Placenta

The human placenta is the vital organ of pregnancy, responsible for all maternal-fetal exchange. Placental development begins at the time of blastocyst implantation, when the placenta trophoblast cells rapidly expand and differentiate through the process of placentation, generating two functional compartments of the placenta: 1) The villous compartment - composed of chorionic villi which house the feto-placental vasculature and serve as the functional units of the placenta involved in gas and nutrient exchange, and 2) The extravillous compartment – composed of a subset of placenta trophoblast cells responsible for invading into the uterine wall and remodelling the uterine spiral arteries to establish a robust utero-placental circulation that can support the growth of a developing fetus [1, 2].

1.1.1 Placental Structure

Human placentation begins approximately one week after conception, when the outer cellular layer of the blastocyst – the trophectoderm – attaches to and begins to invade through the uterine epithelium [3]. The trophectoderm will give rise to the placenta, whereas the inner cell mass of the blastocyst will give rise to the embryo proper [4, 5]. Over the next 10-12 weeks a complex series of cellular expansion, differentiation and migration (comprehensively reviewed in Knöfler et. Al, 2019 [6]) transforms the primitive trophectoderm into an intricate network of tree-like structures, termed tertiary chorionic villi, which will serve as the functional materno-fetal exchange units across pregnancy. These highly branched structures are covered in a multinucleated layer of

terminally differentiated syncytiotrophoblast, overlying a layer of proliferative cytotrophoblast cells. These trophoblast cell layers encapsulate a mesenchymal villous core that contains the feto-placental vasculature. A number of these villi structures remain anchored to the underlying uterine decidua through columns of highly proliferative cytotrophoblast cells. The most apical cells in these columns undergo partial epithelial-mesenchymal transition, becoming a population of invasive extravillous cytotrophoblast cells. These cells invade further into the uterine wall, and actively remodel the uterine spiral arteries, replacing maternal endothelium and displacing smooth muscle cells and elastin with inert fibrinoid material [7]. Remodeling causes loss of response to the maternal endocrine and vasoactive stimuli, ensuring constant blood supply to the fetus. As pregnancy transitions into the second trimester, maternal blood flows through these remodelled vessels into the intervillous space of the placenta, where it bathes the chorionic villi permitting materno-fetal exchange. An overview of placental structure is summarized in Figure 1, with the different placental cell types and their respective functions summarized in Table 1.

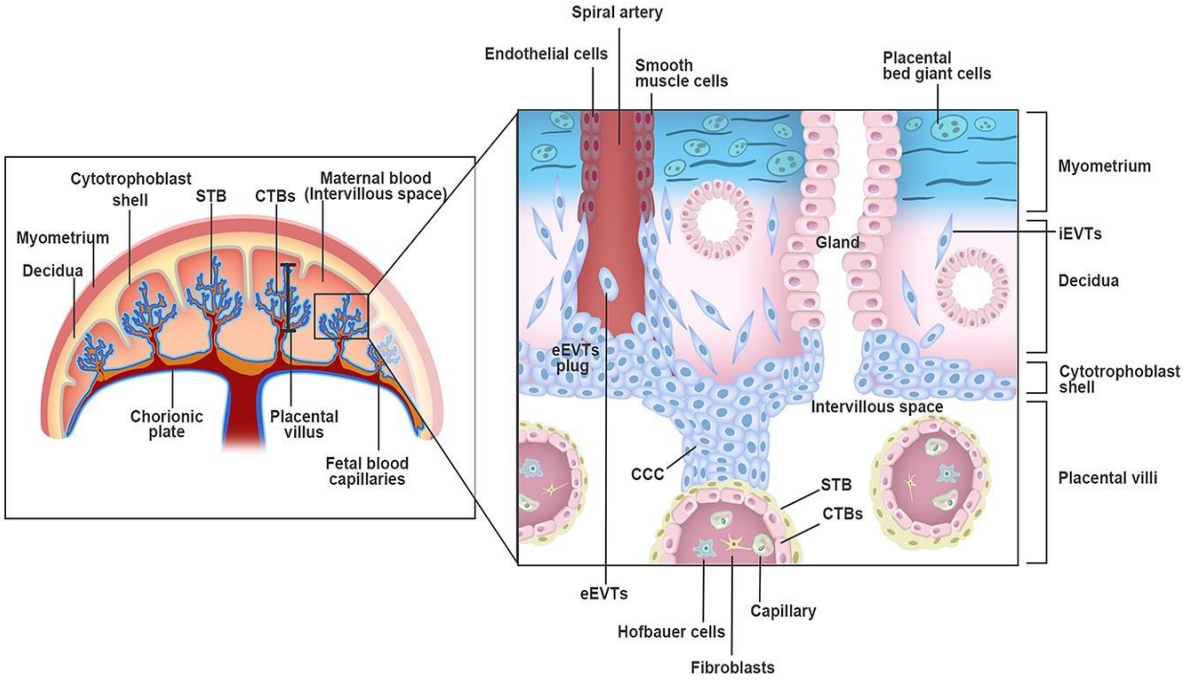

Figure 1. The anatomical structure of the human placenta. The left panel depicts the cross-sectional morphology of human placenta, with the right panel depicting an expansion of it's structure at the cellular level. CTBs (cytotrophoblast cells), STB (syncytiotrophoblast), CCC (cytotrophoblast cell column), iEVTs (interstitial extra-villous trophoblasts), eEVTs (endovascular extra-villous trophoblasts). Adapted from Li *et. al,* Biology of Reproduction, 2022 [8].

Table 1: Placental cell types and their functions

	Villous Compartment		**Extravillous Compartment**	
Structures	Villous trees surround by intervillous space		Decidua, anchoring villi/cell columns, spiral arteries	
Circulation	Feto-placental circulation		Utero-placental circulation	
Cell types	Syncytio-trophoblast (STB)	• Differentiated outer epithelial cell layer on surface of villi • Direct contact with maternal blood • Facilitates exchange of nutrient and other substances between mother and fetus	Columnar cytotrophoblast (CCC)	• Forms cytotrophoblast column • Differentiate into extravillous cytotrophoblasts (EVTs)
			Interstitial EVT (iEVT)	• Invade maternal decidua • Regulate maternal immune adaptation • Give rise to endovascular EVT • Remodels spiral artery
	Villous Cyto-trophoblast (CTB)	• Inner layer of trophoblast that differentiates into syncytiotrophoblasts • Plays a key role in the formation of villi and embryo implantation		
	Villous core stromal cells	• Mesenchymal cells- Differentiate into hematopoietic stem cells and vascular cells in the villi	Endovascular extravillous cytotrophoblast (eEVT),	• Forms plug in the maternal vessel • Remodels spiral artery • Replaces vascular endothelial cells
		• Hofbauer cells- Mesenchyme-derived macrophages • Confers immunity by antigen presentation • Release cytokines and growth factors that stimulate angiogenesis and trophoblast differentiation	Maternal cells	• Endothelial cells- Innermost lining of uterine vasculature • Adapt during various stages of pregnancy • Undergoes angiogenesis during placental expansion • Replaced by EVTs during spiral artery remodeling

		• Fibroblasts- Produce extracellular matrix and support villous architecture		• Vascular smooth muscle cells- Line spiral arteries during early implantation • Replaced by EVTs during spiral artery remodeling

1.1.2 Placental Function

The placenta is critical for fetal survival and appropriate maternal adaptation to pregnancy, supporting many vital functions across pregnancy, including: the regulation of materno-fetal exchange (gas, nutrients, waste products); hormone and growth factor production (required for uterine quiescence, maternal adaptation to pregnancy, fetal growth and development); serving as a selective barrier (fetal protection from xenobiotics, enzymatic inactivation of maternal hormones - i.e. cortisol) [9] [1, 2]. A brief description of these key function is provided below:

1.1.2.1 Maternal-fetal exchange

In the first trimester, the placenta trophoblast cells acquire nutrition via phagocytosis of endometrial secretions [10]. Following the establishment of the utero-placental circulation at ~ 12 weeks, maternal blood enters the intervillous space, where it bathes the chorionic villi structures. The exchange membrane, composing of the syncytiotrophoblast, underlying cytotrophoblast and the fetal vascular endothelium, progressively thins across gestations as fetal demands rise, and allows for the simple diffusion of oxygen, carbon dioxide, free fatty acids, glycerol, urea, uric acid and creatinine. Higher affinity of fetal hemoglobin for oxygen to carbon dioxide, compared to that of the mother's, aids in the exchange of respiratory gases with maternal blood. Glucose and lactic acid transport are facilitated by carrier-mediated diffusion. Active

transport is required for the exchange of amino acids [11], potassium, magnesium, calcium and phosphate. Cholesterol can be synthesized in the placenta but majority of it is obtained through receptor-mediated endocytosis of low-density lipoproteins. Some proteins and immunonoglobulin G antibodies are transported into placenta through pinocytosis [12]. Like the placentas function in nutrient transfer, the placenta also collects fetal waste products such as uric acid, urea, bile etc. and transfers to maternal circulation for their removal [13].

1.1.2.2 Partial and selective barrier functions

The placenta serves to protect the fetus from *in utero* exposure to (some) harmful substances and/or (most) pathogens via its selective barrier function. The transfer of xenobiotics – unnatural chemical substances found within an organism – from the maternal to fetal compartments is limited by a series of membrane transporters found on the syncytitotrophoblast cellular layer, which expel them back into the maternal circulation. Key xenobiotic membrane export transporters include the multidrug resistance ATP-binding cassette, the breast cancer resistance and the mitoxantrone resistance-associated proteins [14]. Xenobiotics and drugs that make it into the placenta can also be metabolized through phase-I, phase-II and cytochrome P450 enzymes [15]. The placenta further serves as a physical and immunological barrier, with the syncytiotrophoblast – the cellular layer in direct contact with the maternal circulation - capable of resisting placental and fetal infection from a number of diverse pathogens [16]. However, the placenta is only a partial pathogen barrier, with some well-known pathogens capable of vertical transmission through the placenta to the fetus (i.e. HIV, Zika, malaria etc) [16, 17]. Importantly, this barrier function is also selective in nature, for

example permitting the passage of maternal antibodies, essential for the establishment of passive immunity in the developing fetus [18].

1.1.2.3 Endocrine functions

Hormones play a key role in the communication between mother, fetus and placenta, as there is no nerve supply to the placenta. Specifically, the placenta is a primary site for the gestational production of: Progesterone - which inhibits uterine contraction and oestrus, and stimulates the release of luteinizing hormone from pituitary gland [19]; Estrogens – which enlarge breasts and the uterus to support milk production and growth of fetus respectively [20]; Human chorionic gonadotropin – which prevents shedding of the uterine lining and menses, and stimulates the release of estrogen and progesterone [21]; Lactogen – which supports growth, intermediary metabolism and development in fetus, stimulates the production of adrenocortical hormones, insulin, insulin-like growth factors (IGF), and pulmonary surfactant [22]; Placental growth hormone- which supports maternal adaptation to pregnancy, stimulates maternal IGF production to increase supply of nutrients to the fetus; Thyrotropin and corticotropin- which influences maternal metabolism [2]. In addition, the placenta also produces cytokines, chemokines, eicosanoids, proteins and acetyl choline that supports pregnancy adaptation and fetal growth [2].

1.1.3 Placental Metabolism

The metabolic activity of the placenta is similar to that of other highly energy demanding organs such as brain, liver, kidneys. Its specific nutrient and energy demands change across pregnancy and can be altered through nutritional availability, endocrine

signalling, and adverse environmental conditions. Alterations in the feto-maternal microenvironment signals the placenta to adapt and facilitate oxygen and nutrient transport for optimal fetal development [23-25].

Glucose is considered the main energy source for both the placenta and the developing fetus, with increasing metabolic requirements across gestation. Glucose is transported across the placenta by facilitated diffusion, with rates of transfer dependent on maternal-fetal concentration gradients and fetal-placental consumption. In humans it is estimated that ~ 30% of uteroplacental glucose uptake is consumed by the placenta in the third trimester of pregnancy, with the remainder left for the fetus [26]. Beyond glucose, the placenta also relies on fatty acids and amino acids as additional fuel sources for the placenta. Specifically, trophoblasts oxidize or esterify the free fatty acids taken up from maternal circulation. Moreover, microsomal enzymes in the placenta synthesize free fatty acids, glycolipids and some precursors to maintain energy stores for the placenta [20]. In a human placental *ex vivo* perfusion study, merely 6% of the labelled fatty acids were transported to the fetus, suggesting a crucial role for beta-oxidation of fatty acids in placental energy production. Glutamine, serine and other branched chain amino acids have been found in high levels in placenta. Metabolism of these amino acids generates α-ketoglutarate or succinyl CoA that can feed into the tri-carboxylic acid (TCA) cycle [27].

Given that the placenta is a highly heterogeneous organ, each of its functional structures are composed of numerous cell types, each demonstrating a distinct metabolic activity profile. The syncytiotrophoblast cellular layer of the chorionic villi - serving as the barrier between maternal and fetal circulations - has long been

considered the metabolic workhorse of the placenta, due to its direct roles in maternal-fetal exchange and hormone production. However, recent findings suggest that extravillous cytotrophoblast cells - responsible for invading into the uterine decidua and remodeling the uterine spiral arteries - have greater glycolytic and mitochondrial respiration rates, and higher ATP content than syncytiotrophoblast [28]. Further, when isolated in culture, cytotrophoblasts demonstrated gene expression profiles indicative of active lipid uptake and metabolism [28, 29], produced more lactate than syncytiotrophoblasts and showed high expression of lactate transporter, collectively suggesting a yet unknown role in nutrient metabolism and possibly transport of this overlooked trophoblast cell population [28-30].

1.2 Placenta Dysfunction and Placenta Mediated Diseases

Developmental and functional impairment of placenta can occur at any stage of gestation and can lead to the development of placenta-mediated diseases (PMD) of pregnancy. This term refers to an umbrella of common and debilitating gestational conditions that arise entirely, or in large part, from compromised placental development and function. Often, they arise as a result of defects in deep placentation – the process of spiral artery remolding at decidual and myometrial zones of placenta [31]. Depending on the type of PMD that emerges, the fetus, the mother or both can be at risk for adverse health outcomes. Two of the most common PMDs are preeclampsia (PE) and fetal growth restriction (FGR), collectively impacting upwards of 10-12% of all pregnancies [32, 33]. Other PMDs include gestational diabetes, placenta previa, abruption, and accreta, along with some cases of stillbirth and preterm birth.

1.3 Preeclampsia – Clinical Impact

Hypertensive disorders of pregnancy are leading contributors to the perinatal fatalities of mothers and newborns globally, with PE being the most common of these disorders, complicating 2-8% of pregnancies worldwide [34]. Preeclampsia is characterized by new onset hypertension after 20 weeks of pregnancy in addition to evidence of maternal end organ damage (i.e. proteinuria or altered liver function) [35].

Maternal mortality rates due to PE vary by geographical location and socioeconomic status, ranging from 9% to 26% [34, 36]. The western world is seeing increased rates of PE, where the frequency jumped by 25% between 1987 and 2004 [37], and the severe complication risk associated with PE increasing almost 7-fold [36, 38]. In 2010, for every 1000 Canadian deliveries, 11.5 were diagnosed with PE, while specifically in Ontario this number jumped to 42.1 [39]. In 2005 in Ontario, the estimated additional health expenditure of treating individuals with PE and their neonates, comprising of many premature births, was $3.5 million annually [40]. It should be noted that this calculation was performed almost 18 years ago, and it is predicted to be considerably higher today, making this condition of pregnancy a huge economic burden to our health care system.

1.4 Preeclampsia – Heterogeneity and Mechanisms of Disease

The exact etiology of PE is not entirely clear, however a central role for the placenta has been identified. The evidence to support this is the resolution of clinical symptoms upon

delivery of the placenta (in most cases), and clinical evidence of PE in patients with hydatidiform moles – in which no fetal tissue exists. In these latter studies, removal of the placental remnants resolved the symptoms [41]. Despite a clear set of diagnostic criteria for PE, this highly heterogenous disorder demonstrates a wide range of clinical presentations and pregnancy outcomes. As such, PE is often clinically described as being either “early-onset/severe” or “late onset”. However, even within these dichotomous groups there remains a high degree of heterogeneity, including in the frequency of adverse outcomes such as placenta abruption, fetal growth restriction, premature delivery and stillbirth [42]. This heterogeneity has led to speculation that subclasses of PE with distinct etiologies may be present.

In this vein, we and others have been able to identify and characterize subgroups of PE patients with distinct underlying molecular mechanisms leading to the development of common clinical features [43-49]. As described in great detail in Chapter 2, we have previously characterized the presence of at least three clinically distinct PE patient groups, including: 1) *A gestational parent-driven subclass of PE* (Subclass 1), likely arising from the presence of pre-existing parental risk factors that prevent appropriate adaptation to pregnancy; 2) *An hypoxia-driven subclass of PE* (Subclass 2), demonstrating hallmarks of insufficient trophoblast invasion and remodelling of uterine spiral arteries, leading to hypoxic placental injury; and 3) *An inflammation-driven subclass of PE* (Subclass 3), demonstrating heighten pro-inflammatory signalling at the maternal-fetal interface [48, 50].

1.5 Role of Mitochondria in Placental Health and Dysfunction

Being a highly metabolic organ, the placenta relies heavily on its mitochondrial pool to maintain its health and function [27, 51, 52]. Unsurprisingly, mitochondrial dysfunction has been widely described in cases of PMDs, particularly in cases of PE. Despite the strong evidence for distinct subclasses of PE etiology, as discussed in Chapter 2, there is a strong case to be made that mitochondrial dysfunction is a common feature across all PE subclasses, and as such may prove to be an important therapeutic target for the prevention or treatment of PE.

1.6 NAD^+ and Mitochondrial Health/Function

Nicotinamide adenine dinucleotide (NAD^+) is a metabolic derivative that plays direct and indirect roles in cellular functions [53, 54]. NAD^+/NADH, in addition to FADH/$FADH_2$, redox coupling is essential for many reactions including driving glycolysis, the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC). These redox events are therefore essential for the oxidation of protein, glucose or fats to generate reduced NADH and $FADH_2$. NADH is then used by mitochondrial Complex I (CI; NADH-dehydrogenase) to drive protons from the matrix into the intermembrane space (IMS) of the mitochondria and to transfer electrons via the electron carrier ubiquinone/ubiquinol (CoQ/CoQH2) to Complex III (CIII – cytochrome c oxidoreductase), regenerating NAD^+ in the process. CIII then transfers electrons received from CI (or from Complex II) to Complex IV (CIV; cytochrome *c* oxidase) through cytochrome C, while also driving additional protons into the IMS. CIV then donates electrons to oxygen to produce water

while the electrochemical gradient produced by protons being pumped into the IMS drives ATP generation via ATP-synthase (Complex V; CV). Overall, NAD^+ is an abundant metabolite in cellular and mitochondrial spaces, functions as a coenzyme for many redox reactions, and can be consumed as a substrate in a limited number of reactions - all of which are central to energy metabolism and cellular homeostasis [53, 54].

NAD^+ can be generated from dietary tryptophan, in an elaborate multistep enzymatic reaction called the *de novo* synthesis pathway [53, 54], or more efficiently through the intake of vitamin B3 derivatives such as Niacin, nicotinamide (NAM), nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR) [53, 54]. Enzymes that consume NAD^+ include those within the sirtuin family of proteins (SIRTs), the poly-ADP-ribose polymerases (PARPs) family of proteins, the monoADP-ribosyltransferases (ARTDs), the ADP ribose synthetases - CD38 and CD157; and sterile alpha and toll/interleukin-1 receptor motif-containing protein 1 (SARM1) [54]. Many of these NAD^+ dependent enzymes are critical for the maintenance of cellular health, particularly under conditions of heightened cellular stress, regulating processes such as oxidative damage repair, transcription, chromatin remodeling, post-translational modification, cell cycle regulation, cell survival/death pathways, inflammation, ROS regulation, oxidative phosphorylation (OXPHOS), mitochondrial biogenesis, cellular stress response and inflammation [55-61].

Under a state of cellular homeostasis, the activity of NAD^+-consuming enzymes and the NAD^+ biosynthesis and salvage pathways are tightly regulated, maintaining a steady state of NAD^+ levels. However, under pathological conditions, there can be serious

consequences of NAD^+ depletion, including the loss of sufficient mitochondrial function and cellular energy metabolism [53]. For example, mice that have a homozygous knockout of the NAD^+ salvage enzyme *Nampt* demonstrate embryonic lethality [62]. Further, tissue-specific loss of NAMPT, and the eventual loss of NAD^+ conversion through the salvage pathway, is detrimental to cellular health, with tissues showing phenotypic signs of advanced aging such renal fibrosis, neurodegeneration and loss of muscle mass [63-66], suggesting maintenance of NAD^+ level is highly critical for normal cellular function.

1.7 PARPs and Inflammation

Poly(ADP-ribose) polymerases (PARPs) are ADP-ribosyltransferase (ARTs) enzymes that utilizes NAD^+ to catalyze MonoADPribosylation (MARylation) or PolyADPribosylation (PARylation) of target proteins [67]. MARylation or PARylation is a reversible post-translational modification which plays crucial roles in various cellular processes [67]. The PARP family of enzymes consists of at least 17 distinct enzymatic members. PARP1, 2, 5a and 5b are commonly known as PARylating enzymes *in vivo* (PARP3 *in vitro*) [67-69]. Other PARPs are known for their MARylating activity, while PARP9 and PARP13 have no known catalytic function [67].

PARP functionality encompasses a wide range of biological process, for instance, DNA damage and repair, inflammatory response, cell death pathways, maintenance of chromatin structure, histone modifications and DNA methylation, mitosis, glycolysis, NAD^+ metabolism, aging and transcription [55]. PARP1 and PARP2 are the primary PARylating enzymes of the cell and are considered as the predominant NAD^+ consuming enzymes, playing an important role in the fluctuation of cellular NAD^+ stores

[70, 71]. PARP1/2/3/5a/5b/9/10/14 have all been implicated in DNA damage repair during oxidative stress [68]. Subsequently, inhibition of PARPs hinders DNA repair and activates the cAMP-STING-IRF3/NF-κB inflammatory pathway [72]. On the other hand, under pro-inflammatory disease conditions, inhibition of PARPs has been shown to reduce inflammation, suggesting that PARPs directly participate in the inflammatory pathways [73, 74]. For instance, lipopolysaccharide (LPS) or tumour necrosis factor (TNF-α) treatment has been shown to activate PARP1 which causes PARylation of p65 and activates the NF-kB pathway [75]. Beyond enzymatic activity, PARP1 has been shown to be a transcriptional co-activator of NF-kB mediated gene expression, where its direct binding to NF-kB subunits is required [76]. Moreover, PARylation of human antigen R (HuR) by PARP1 has been shown to enhance mRNA stability of pro-inflammatory genes [77]. The release of PAR by PARP1 has also been shown to activate other PARPs [78]. For example, under inflammatory or oxidative stress, PAR moieties along with several other PARPs are required for the formation of stress granules (SG). SGs are clusters of ribonucleoproteins containing untranslated mRNA, RNA-binding protein and translation factors [78, 79]. These stress granules are believed to act as a hub where they triage untranslated mRNA either for storage, degradation, or translation, thus, play a key role in stress adaptation [80]. In HeLa cells, following an oxidative stress induced by NaAsO2, PARP1 was shown to generate and release PAR moieties that enters the cytoplasm. In the cytoplasm, PAR binds to PARP12 and initiates its translocation to SG where they bind with PARP13 and PARP15 to complete SG formation [72, 78]. This suggests that PARPs are also needed downstream of stress response pathway. Such stress granules were also shown to form under chronic

inflammatory conditions such as atherosclerosis, with their presence decreased with the use of anti-inflammatory agents [79]. Transcript levels of other PARPs, such as PARP3, PARP4, PARP7, PARP8, PARP10, PARP11, PARP12, and PARP13, have been shown to be transcriptionally upregulated upon LPS-induction of macrophages, suggesting their role in inflammation [81, 82]. Several PARPs are shown to be involved in anti-viral immune response. For instance, PARP9 can act as a sensor of RNA virus and enhance interferon I (IFN-I) mediated immune response while PARP7 inhibits IFN-I production upon viral infection [72]. PARP14 appears to have anti-inflammatory properties as it was shown to inhibit the pro-inflammatory IFN-ɣ-STAT1 pathway by MARylating STAT1 in IFN-ɣ stimulated macrophages [83]. On the other hand, the non-catalytic PARP9 was shown to promote IFN-ɣ-STAT1 pathway by inhibiting PARP14 [83].

1.8 Therapeutically Targeting NAD^+ Signaling for Inflammatory Disorders

NAD^+ depletion and the associated mitochondrial dysfunction has been widely described in various inflammatory conditions in the non-pregnant population, including ageing, neurodegenerative disorders [i.e. Alzheimer's, Parkinson's, multiple sclerosis and amyotrophic lateral sclerosis (ALS)], obesity, diabetes, non-alcoholic fatty liver disease, inflammatory bowel disease, systemic lupus erythematosus and rheumatoid arthritis [60, 84-94]. These observations have been proposed to be the result of inflammation-mediated PARP activation, resulting in rapid NAD^+ depletion, compromised function of multiple NAD^+-dependent enzymes and subsequent alterations in cellular metabolism and homeostasis [Figure 2]. The PARylating PARP

enzymes (1, 2, 4, 5), in particular, are highly sensitive to chronic pro-inflammatory environments, with heightened activation that consumes a large amount of NAD^+ while attempting to repair inflammation-mediated oxidative DNA damage [68]. These same PARP enzymes also activate inflammatory pathways, again in an NAD^+-consuming fashion, that in some cases further exacerbate the inflammatory pathophysiology in these conditions [85, 93, 95]. In the face of depleted intracellular NAD^+ levels [91], it is thought that the enzymatic activity of sirtuins becomes compromised, resulting in decreased mitochondrial health and function, and loss of cellular antioxidant capacity (i.e. SOD2), but may also limit the beneficial actions of PARPs. As such, therapeutic NAD^+ replenishment to improve energy metabolism and cellular health is an active area of research, particularly in the context of chronic inflammatory conditions [60, 84-94]. While clinical trials examining the safety and therapeutic potential of NAD^+-boosting therapies have been carried out in non-pregnant populations, showing tremendous promise, to-date the use of these therapies in human pregnant populations have not been explored. In the current thesis, the potential of targeting NAD^+ signalling within the placenta, in the context of inflammation-driven PE, will be specifically explored.

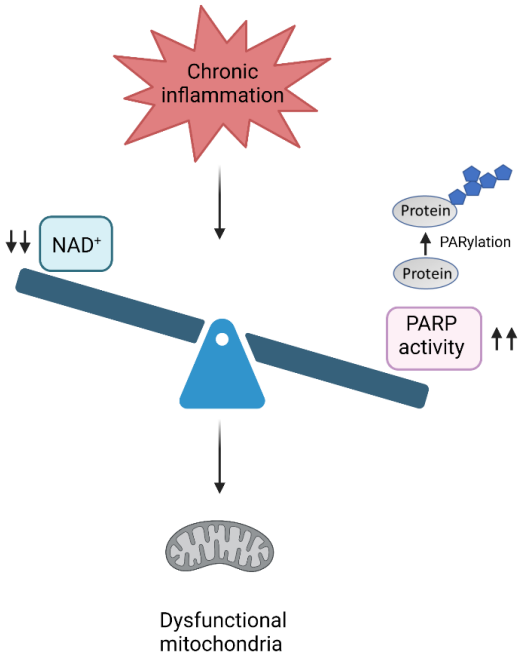

Figure 2. Effect of chronic inflammation on PARP activation and NAD^+ depletion. Chronic inflammatory conditions activate PARP enzymes causing a decline in total cellular NAD^+. Subsequently energy metabolism is impaired and the function of other NAD^+ dependent enzymes is compromised, leading to dysfunctional mitochondria.

Chapter 2 - Placental Mitochondrial Function and Dysfunction in Preeclampsia

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Abstract

The placenta is a vital organ of pregnancy, regulating adaptation to pregnancy, gestational parent/fetal exchange, and ultimately, fetal development and growth. Not surprisingly, in cases of placental dysfunction-where aspects of placental development or function become compromised-adverse pregnancy outcomes can result. One common placenta-mediated disorder of pregnancy is preeclampsia (PE), a hypertensive disorder of pregnancy with a highly heterogeneous clinical presentation. The wide array of clinical characteristics observed in pregnant individuals and neonates of a PE pregnancy are likely the result of distinct forms of placental pathology underlying the PE diagnosis, explaining why no one common intervention has proven effective in the prevention or treatment of PE. The historical paradigm of placental pathology in PE highlights an important role for utero-placental malperfusion, placental hypoxia and oxidative stress, and a critical role for placental mitochondrial dysfunction in the pathogenesis and progression of the disease. In the current review, the evidence of placental mitochondrial dysfunction in the context of PE will be summarized, highlighting how altered mitochondrial function may be a common feature across distinct PE subtypes. Further, advances in this field of study and therapeutic targeting of mitochondria as a promising intervention for PE will be discussed.

Keywords: disease subclasses; hypertension; mitochondria; placenta; preeclampsia; pregnancy; reactive oxygen species; therapies.

2.1 Introduction

The human placenta serves as a metabolic and endocrine exchange barrier between the gestational parent and fetus, regulating adaptation to pregnancy and fetal growth and development [1, 2]. Abnormalities in the development and function of this organ underlie the majority of obstetrical disorders faced today, many of which can be classified under the umbrella term of “placenta-mediated diseases”. One of the most common of these is preeclampsia (PE), a hypertensive disorder of pregnancy that affects ~3–5% of all pregnancies and to date has no effective therapeutic interventions [96]. A large body of research has focused on understanding the placental pathology(/ies) that lead to gestational parent hypertension and end-organ damage. Much of this research has focused on a paradigm of utero–placental malperfusion, placental hypoxia and oxidative stress resulting in shedding of placental debris, and proinflammatory and anti-angiogenic mediators into the circulation of the gestational parent. This initiates a heightened inflammatory response and endothelial dysfunction that precipitate the clinical symptoms observed in the gestational parent [48, 96].

The primary functions of the placenta are to regulate gestational parent–fetal exchange (gas exchange, nutrients/micronutrient and ion exchange, waste removal); hormone and growth factor production (required for uterine quiescence, gestational parent adaptation to pregnancy, fetal growth and development); and to act as a selective barrier to the fetus (fetal protection from xenobiotics, and enzymatic inactivation of gestational parent hormones, i.e., cortisol) [9]. Each of these functions is highly energy dependent and consequently highly reliant on mitochondria function.

Mitochondrial dysfunction has been widely implicated as a central component to the establishment of placental and gestational parent disease in cases of PE [97]. Mitochondria not only harvest energy to produce adenosine triphosphate (ATP) but also play a vital role in signaling pathways that are essential for growth, development, and homeostasis of any organ. Mitochondrial dysfunction is a central mediator of disease pathophysiology in many organ systems, owing to the numerous processes that can be directly influenced by mitochondrial function, including biosynthetic and bioenergetic pathways, reduction–oxidation (redox) signaling, antioxidant defense, hypoxia adaptations, inflammatory signaling, the mitochondrial unfolded protein response, calcium signaling, and apoptotic signaling [98]. Similarly, when placental mitochondrial functions become dysregulated, placental development and function may be compromised, resulting in detrimental health outcomes for both the developing fetus and the gestational parent [27, 52, 97, 99]. In the current review, literature on the central role of mitochondria in the establishment of placental disease will be summarized, highlighting the possibility that placental mitochondrial dysfunction may be a central and common aspect of pathophysiology across distinct subclasses of PE. Further, recent advancements in mitochondria-targeting therapeutics to treat PE will be discussed.

2.2 Mitochondria in the Healthy Placenta

Nutrient and energy demand of the placenta change across pregnancy to match and optimize fetal development and growth patterns [23-25]. Further, the placenta requires a considerable amount of ATP for its own growth and expansion, for example, during developmental processes that include vascularization, cell differentiation and the formation of function-specific structures within the placenta [100]. Mitochondrial content,

structure, and function play an important role in supporting the placenta's energetic demands during these highly dynamic transitions. A brief overview is provided below and summarized in Table 1, but a more comprehensive discussion on its role in healthy placental development and function can be found in the following reviews [97, 101-103].

Table 1. Overarching trends of placental mitochondria content, structure/dynamics, and function in healthy pregnancies (early and late) and PE pregnancies (early onset vs. late onset).

		Healthy Pregnancy		PE	
		Early	***Late***	***Early-Onset/Severe***	***Late-Onset/Mild***
Content		⇓ ◢	⇑	⇑⇓	⇑=
Structure and dynamics		• Large • Oval or circular	• Smaller • Irregular cristae	• Swelling • Impaired cristae • Fragmentation	• Similar to healthy • More fusion?
Function	***Respiration and ATP generation***	⇓ ◢	⇑	⇑⇓	⇑=
	ROS	⇓ ◢	⇑	⇑	⇑=
	Antioxidant capacity	⇓ ◢	⇑	⇑⇓	⇑

In healthy pregnancy, comparison was between early and late gestation. In PE-complicated pregnancy, comparison was with healthy term or preterm pregnancy. The symbol "=" was used when the finding was similar to healthy control. The symbol ↑ indicates increase and ↓ indicates decrease.

2.2.1 ***Placental Mitochondrial Content, Structure, and Function across Pregnancy***

During most of the first trimester of human pregnancy, feto-placental development takes place in a low-oxygen environment [104-106], accompanied by low placental mitochondrial content. At this gestational phase, mitochondria found within the chorionic villi structures are relatively large in size, with an oval or circular shape. At the onset of the second trimester, when the gestational parent–fetal circulation is established, placental mitochondrial biogenesis is upregulated, leading to increased mitochondrial content that continues to rise until the end of pregnancy [107]. Mitochondrial structure during this trimester likewise demonstrates dynamic remodeling, with the presence of smaller mitochondria with irregular cristae, likely a function of rapid placental mitochondrial turnover in response to increased oxygen supply [108]. During this gestational phase, placental respiration and ATP production increases; however, the ratio of respiration rates to mitochondrial content demonstrates a slowing within the third trimester of pregnancy, perhaps to maintain respiration capacity for acute energetic insults [107].

In addition to meeting the specific temporal energy demands, the placental mitochondria must be capable of maintaining cellular redox homeostasis across pregnancy in the face of a changing oxygen landscape. Mitochondria are a major source of reactive oxygen and nitrogen species (ROS, RNS) generation. They also house several key antioxidant enzymes, required to maintain cellular redox balance [109]. When compared with pre-pregnancy state, an increase in the maternal blood levels of superoxide is required for healthy pregnancy; and this level remains stable throughout the gestation

[110]. With the rapid increase in oxygen availability at the transition between first and second trimester, a burst of ROS production is also noted, with elevated ROS production maintained across the remainder of gestation. Importantly, placental ROS generation is considered an important second messenger system to promote development of the placental vasculature [111]. On the other hand, the expression and activity of mitochondrial superoxide dismutase (MnSOD) and glutathione peroxidase (GPX) enzymes could be upregulated in response to increased oxidative stress as an adaptive mechanism under both physiological and pathological conditions [108, 112]. In line with this, results from a mouse study showed that placental mitochondria are highly responsive to environmental changes, such as oxygen level, and can modulate its oxidative capacity and substrate preference accordingly [113].

Placental mitochondria are also involved in regulating cellular apoptosis. Upon cellular stimuli such as nutrient stress, mitochondrial membrane permeability may increase, leading to swelling and release of proapoptotic proteins to initiate the mitochondria mediated caspase 9 apoptotic cascade [114]. The mitochondrial-driven apoptotic cascade is a central biological process required for normal placental developmental processes, such as gestational parent–fetal immune tolerance, trophoblast differentiation, syncytium formation, trophoblast invasion and remodeling of uterine spiral arteries [115, 116]. Thus, aberrant regulation of apoptosis within the placenta at any point in pregnancy can disrupt placental development and function and contribute to the establishment of placental dysfunction and obstetrical disease.

2.2.2 *Mitochondrial Content, Structure, and Function in Distinct Placental Cell Types*

The human placenta demonstrates considerable anatomical complexity, with heterogenous structures and cellular composition—characteristics required to support the diverse functions of this critical organ of pregnancy. Unsurprisingly, the distinct placental cell populations demonstrate unique oxygen availability and energetic needs, dramatically influencing the mitochondrial content, structure, and function in the distinct populations. As such, in addition to temporal plasticity in the placental mitochondrial landscape, there is also considerable heterogeneity in mitochondrial content, structure and function across the distinct cell populations and structures within the placenta.

In humans, the syncytiotrophoblast (STB) cellular layer (also referred to as the syncytium) of the chorionic villi, serves as the barrier between the circulations of the gestational parent and the fetus. The syncytium has long been considered the metabolic workhorse of the placenta, due to its direct roles in gestational parent–fetal nutrient/gas exchange and hormone production. However, recent findings suggest that primary human placental villous cytotrophoblasts (CTBs)—cells that terminally differentiate and fuse into the overlying syncytium—have higher mitochondrial content and respiration rates than syncytiotrophoblast (STB) in culture. Mitochondria in CTBs are large and oval shaped with defined cristae, while mitochondria in differentiated STB are smaller and round with undefined cristae, which may reflect cell-specific roles and metabolic properties for these respective mitochondrial pools [28]. Functionally, mitochondria are important for steroid biosynthesis via their role in isoprenoid precursor synthesis and, in particular, mitochondria found in the STBs constitutively produce progesterone to

sustain pregnancy. It is thought that mitochondrial size and cristae structural changes occur during CTB to STB differentiation to support improved cholesterol import into the STB mitochondria, supporting efficient production of progesterone [117-120].

A third trophoblast cell population—the extravillous cytotrophoblasts (EVTs)—demonstrate high energy demands associated with their functionality, namely, invading the uterine decidua and remodeling the uterine vasculature. Using the immortalized EVT cell line (HTR-8/SVneo) *in vitro*, paracrine signaling from placenta-derived mesenchymal stem cells has been shown to increase expression of mitochondrial proteins important for ATP production, such as ATP-binding cassette sub-family B member 10 (ABCB10) and mitochondrial Ca^{+} uniporter (MCU), the latter of which also acts as a cellular glucose sensor [121, 122]. Furthermore, compared with the villous counterparts, the EVTs exhibit increased mitochondrial respiration and produce more ATP [123]. Moreover, activation of mitochondrial quality control process, mitophagy and balancing mitochondrial dynamics is known to be essential in successful EVT invasion and required for proper placentation [121, 122].

In summary, mitochondria play a central role in ensuring proper development and functioning of the human placenta, supporting the development and growth of the fetus and successful pregnancy outcomes. Conversely, dysregulation of mitochondrial content, structure and/or function can have negative consequences for placentation and pregnancy health, and has been identified as a significant contributor to the pathophysiology of all placenta-mediated obstetric complications, including preeclampsia (PE)—one of the most prevalent and devastating of the placenta-mediated diseases [122].

2.3 Preeclampsia

PE is one of the most common and serious placenta-mediated diseases in humans, contributing to approximately 76,000 gestational parent and 500,000 fetal/neonatal deaths per year worldwide [96, 124]. PE is characterized by de novo hypertension after 20 weeks of gestation, accompanied by proteinuria and/or evidence of end-organ damage in the gestational parent [125]. Fetal growth restriction is a common comorbidity, particularly in cases of early onset PE, estimated to occur in ~39% of PE cases [126]. Adverse neonatal health outcomes associated with PE include periventricular or intraventricular hemorrhage or subdural and cerebral hemorrhage, hypoxic–ischemic encephalopathy or periventricular leukomalacia, stillbirth, and perinatal death [125], in many cases the iatrogenic results accompanying medically-indicated preterm delivery. Aside from the immediate health risks associated with a PE diagnosis (i.e., HELLP, eclampsia), individuals who have had PE are seven times more likely to have a recurrence of PE in subsequent pregnancies, particularly with early onset disease [127]. Further, these individuals are at increased risk for cardiovascular disease in later life, with some estimates indicating the average age of first cardiac events as early as 38 years of age [127].

The underlying cause of PE is not entirely clear, however placental dysfunction is recognized as a critical component of pathophysiology. In a healthy pregnancy, uterine spiral arteries are remodeled by the EVT population, modifying them into large-diameter vessels that are no longer responsive to vasopressors in the circulation of the gestational parent; modifications aimed at increasing blood flow into the intervillous space of the placenta to promote fetal growth and development [128]. In the dogmatic

two-stage model of PE pathophysiology [129], it is proposed that this vital aspect of placentation becomes compromised, with shallow invasion of the EVTs and insufficient remodeling of the uterine spiral arteries [130-133]. This poor vascular remodeling results in placental ischemic–reperfusion injury; the high speed, pulsatile blood flow into the placenta causing damage to the delicate villous structures, promoting thrombus formation and compromising adequate gestational parent–fetal exchange of oxygen and nutrients. This injury is supported by findings of STB necrosis, damage and destruction of syncytial microvilli structures, hyperplastic and apoptotic CTBs, and bulged endoplasmic reticulum cisternae and mitochondria [52, 134, 135]. Damaged trophoblasts are proposed to respond to the structural damage and oxidative stress through the upregulation and release of pro-inflammatory cytokines and anti-angiogenic factors, along with extracellular vesicles, apoptotic debris and cell-free fetal DNA into the systemic circulation of the gestational parent [136-138]. In the second stage of the PE pathophysiology model, these placenta-derived factors illicit a heightened systemic immune response and endothelial dysfunction in the gestational parent, precipitating in the clinical manifestation of the disease [139, 140]. While there is certainly robust evidence to support this model of PE pathophysiology, it is important to note that not all cases of PE demonstrate evidence of insufficient invasion and uterine spiral artery remodeling, nor do all PE placentas demonstrate structural or molecular evidence of ischemia–reperfusion type injury [48].

4.3.1 Subclasses of Disease in Preeclampsia

PE is a heterogeneous disorder that demonstrates considerable variability in the timing of disease onset, presentation and severity of symptoms, gestational parent and fetal

health outcomes, and pathological evidence of placental disease—indicative of multiple disease subtypes with divergent underlying etiology and pathophysiology. In this vein, the scientific community has embarked on several lines of inquiry attempting to identify and characterize distinct subtypes of PE [43-49]. Discovery-driven approaches using robust omics datasets are particularly useful when trying to understand complex diseases with heterogeneous presentation and are proving useful in gaining insight into the presence of distinct PE subclasses. Using clustering approaches, groups of PE patients who share commonality in (epi-)genome [141], transcriptome [48] or proteome [46] profiles in relevant tissues (i.e., blood, placenta) have been identified. Our group has contributed to this body of work, completing multi-scale profiling of a robust sample of PE patients that spanned the clinical spectrum of disease [48, 50, 142]. Through integration of clinical, placental transcriptome and histopathology profiles, our group uncovered at least three clinically relevant PE subclasses (Table 2) with divergent forms of placental disease, or lack thereof, which are highly supported by the growing body of research in this domain (see [143] for a detailed review on this topic by the Global Pregnancy Collaboration).

Table 2. Overview of clinical features and mechanistic pathways involved in the pathophysiology of distinct PE subclasses.

	Canonical PE	**Immunological PE**	**Gestational Parent Driven PE**
Onset and severity of disease	• Early onset (many <34 weeks) and ↑ preterm delivery • More severe symptoms (↑ blood pressure, ↑ proteinuria, HELLP)	• Early–late onset (34–37 weeks) and near-term delivery • ↑↑ growth restricted infants (FGR)	• Late onset (>37 weeks) and term delivery • Less severe symptoms • Appropriate birth weight for gestational age newborns

	• Low birth weight newborns (SGA and FGR)		
Pathogenesis	• Impaired spiral artery remodeling • Placental ischemia–reperfusion injury	• Gestational parent/fetal immune incompatibility • Pro-inflammatory response	• No evidence of placental disease • Likely driven by gestational parent factors
Genes expressed in placenta	• Hypoxia and oxidative stress responses such as egl-9 family hypoxia–inducible factor 1 (EGLN1), BCL family of proteins, etc. • Anti-angiogenic molecules (FLT, ENG) • Hormone signaling (INHA)	• Immune and inflammatory responses (TNF-α, IFN-Ɣ, CXCL-10)	• Unchanged from healthy controls
Placenta pathology	• Smaller placenta • Maternal vascular malperfusion lesions (infarcts, distal villous hypoplasia, excessive syncytial knots)	• Chronic inflammation lesions (i.e., villitis of unknown etiology, massive perivillous fibrin deposition) • Infiltration of immune cells	• Normal weight placenta • No evidence of placental disease
Potential risk factors	• Advanced age of gestational parent • Pre-existing chronic hypertension and diabetes • Genetic heredity	• Type A blood • Short duration paternal antigen exposure • Non-Caucasian ethnicity	• Nulliparity • Advanced age of gestational parent • High pre-pregnancy BMI • Pre-existing chronic hypertension

The “↑” arrow indicates an increase.

2.3.1.1 Canonical PE Subclass

The largest PE subclass characterized by our previous work demonstrates a phenotype which tightly aligns with the traditional framework of PE pathophysiology. These patients have small placentas with lesions of maternal vascular malperfusion (MVM) and gene expression profiles consistent with hypoxia–reperfusion injury—including upregulation of hypoxia-inducible factors and anti-angiogenic molecules commonly associated with PE. Clinically, these patients have an earlier onset and more severe form of gestational parent disease and are more likely to experience preterm delivery (<34 weeks) [48, 50].

2.3.1.2 Immunological PE Subclass

Heightened systemic inflammation has long been identified as a key component of PE pathophysiology [12, 144, 145], and, more recently, it is becoming clear that an immune-driven subclass of PE exists [146-148]. This subclass of PE patients demonstrates evidence of chronic inflammatory insults at the utero–placenta interface, with heightened immune cell infiltration, a propensity for gestational parent–fetal HLA-discordance, heightened placental expression of pro-inflammatory cytokines and chemokines, and placental lesions consistent with chronic inflammation and gestational parent–fetal interface disturbances [48].

2.3.1.3 Gestational Parent-Driven PE Subclass

Despite being widely identified as a placenta-mediated disease, historical reports have long described a handful of PE cases with little evidence of placental disease [48, 50, 149]—a PE subclass driven in large part by underlying and predisposing factors in the

gestational parent, which compromise appropriate physiological adaptation to pregnancy. This subclass of PE patients demonstrates normally grown placentas with gene expression profiles that mimic healthy tissues and little histological evidence of pathology. Fetuses in this subclass demonstrate normal growth trajectories and gestational parent symptoms are relatively mild with a later onset (>37 weeks gestation) [48, 50]. Collectively, this phenotype suggests minimal placental involvement, but rather predisposing gestational parental factors as the primary drivers of PE pathophysiology.

While there is convincing evidence supporting distinct mechanistic pathways driving placental and/or gestational parent disease across PE subclasses, there is likewise convincing evidence to suggest that mitochondrial dysfunction may be a central point of convergence within the pathophysiology of PE across all subclasses of patients.

2.4 Placental Mitochondrial Dysfunction in Preeclampsia

Compromised placental mitochondrial biogenesis, structure, and functionality contribute to placental dysfunction in PE. Below, the existing evidence for placental mitochondrial dysfunction as a key component of PE pathophysiology—as a singular clinical diagnosis—is reviewed (Table 1).

2.4.1 Mitochondrial Content

The evidence related to mitochondrial content in cases of PE is not clear cut. Decreased placental mitochondrial DNA content and citrate synthase protein activity have been described in cases of PE, compared with healthy term controls, coupled to a downregulation of genes that regulate mitochondrial biogenesis, such as PGC1- αa, NRF1 and Tfam [150]. It should be noted, however, that when PE placentas are

compared against gestational age-matched controls, the reported difference in citrate synthase levels are no longer apparent, and an increase in mitochondrial complex II and III protein levels are observed [151]. In a subsequent prospective case control study, placental mtDNA copy number was in fact found to be elevated in cases of preterm/severe PE, compared with both term PE and healthy term controls, possibly indicative of a compensatory upregulation of mitochondrial biogenesis in the face of severe placental disease [152]. Systemically, lower cell-free (membrane bound and non-membrane bound) plasma mtDNA content has been measured in the circulation of individuals with PE in the third trimester, compared with gestational age-matched healthy controls [153]. However, others have reported elevated whole blood, plasma and serum mtDNA content in cases of PE compared with health controls, with the mtDNA content elevations significantly associated with female fetal sex according to one of these studies [154-156]. On its own, the evidence surrounding placental mitochondrial content in the context of PE does not appear to be highly informative—however the evidence suggests the timing of disease onset, severity of disease, gestational sampling time and fetal sex may all influence the necessary interpretations of mitochondrial content measurements.

2.4.2 Mitochondrial Structure and Dynamics

To maintain mitochondrial homeostasis in the face of environmental stress, mitochondria go through dynamic morphological changes via two processes, fusion and fission. The fusion of separate mitochondria is driven by outer mitochondrial membrane proteins mitofusins 1 and 2 (Mfn1 and 2), and the inner membrane protein optic atrophy 1 (OPA1) [157]. The benefits of fusion have been linked to the diffusion of metabolites,

protein and mtDNA across the network of mitochondria, enabling mitochondria to produce energy more efficiently. Fission, on the other hand, permits the separation of damaged portions of mitochondria for degradation via mitochondrial autophagy (i.e., mitophagy), and is mediated by in large part by mitochondrial fission 1 (FIS1) and dynamin-1-like (DNM1L) proteins [158].

Evidence for dysregulated mitochondrial dynamics (fission and fusion) has been collected from placenta tissues of pregnancies impacted by PE. Term PE placenta tissues demonstrate decreased mRNA expression of the fusion protein Mfn2, coupled to decreased ATP content, when compared with gestational age-matched controls [159]. While not explicitly examined in that study, similar decreases in Mfn2 expression have been associated with mitochondrial fragmentation in both human and rodent models of pulmonary arterial hypertension [160]. Structurally, placental mitochondria from cases of severe/early onset PE demonstrate swelling, impaired cristae and fragmentation when visualized with electron microscopy [161]. Fission processes are also likely impacted in early onset PE cases, with reported measurements of increased DNM1L expression relative to term controls [150]. Interestingly, cultured first trimester immortalized human placenta cells (TEV-1) likewise exhibit decreased expression of Mfn2, decreased mitochondrial membrane potential, decreased ATP production, mitochondrial fragmentation, and mitophagy, when exposed to hypoxic insults in vitro [159]—similar to what is proposed to occur within the context of PE pathophysiology. Damaged mitochondrial clearance via mitophagy is described in cases of PE, particularly those with severe/early onset disease [162]. This finding is paralleled by an increase in placental expression of mitophagy-related proteins, such as PTEN-induced putative

kinase 1 (PINK1), Parkin, B-cell lymphoma-2 (BCL-2) interacting protein 3 (BNIP3) and BNIP3 like (BNIP3L), when compared with preterm controls [150, 162].

2.4.3 Mitochondrial Function- Respiration and ATP Generation

The mitochondria primarily act as an ATP generation hub through their ability to perform oxidative respiration. Altered placental mitochondrial respiration is widely reported in cases of PE, however the direction of this functional change varies across studies. It should be noted that performing mitochondrial functionality assays using fresh samples (immediately after collection) is ideal; however fresh tissue collection can pose a difficulty in multi-site clinical studies or when performing retrospective studies, especially in pregnancy research. Unfortunately, freezing of samples can lead to damage of mitochondrial membranes, uncoupling ETC activity and loss of cytochrome c. Thus, historical results must be interpreted with caution when frozen tissue was used. To mitigate such issues, newly reported techniques that restore electron transfer components lost during freeze/thaw and correct for membrane permeabilization, have demonstrated considerable success, preserving 90–95% of the maximal respiratory capacity in frozen samples, and may serve as tremendously useful for future research in this area (see reference [163] for method).

In cryopreserved placental samples collected from cases of early onset PE (onset at <34 weeks), a considerable decrease in mitochondrial respiration is described, when compared with healthy term controls [164]. Supporting these findings, enzymes critical to the glycolytic pathway have been found to be upregulated in placentas from PE pregnancies, indicative of suppressed mitochondrial respiration [150]. Muralimanoharan et al. more specifically described decreased mitochondrial complex-I and complex-III

activity in isolated mitochondria from frozen PE term placentas, when compared with gestational age-matched controls, functional findings that were coupled to decreased expression of respiratory complex proteins [165].

In contrast, others have described an increase in placental mitochondrial respiration activity in cases of PE. For example, Vishnyakova et al. [166] demonstrated a modest increase in complex-I mediated respiration, with no change in complex-II mediated respiration, when studying freshly isolated mitochondria from placentas of early onset PE patients compared with healthy term controls. It should be noted, however, this same study found no measurable differences in mitochondrial activity in late onset PE samples, when compared with gestational age-matched controls—highlighting the importance of considering the temporal changes of mitochondrial content and activity across pregnancy when interpreting this data. However, others have reported an increase in complex-I and complex-I + II mediated respiration in term PE permeabilized placenta tissue that occurred in conjunction with increases in the expression of respiratory protein complexes, when gestational age was appropriately matched in the healthy control group [151]. Importantly though, the reserve respiratory capacity—the amount of extra ATP that can be produced by OXPHOS with an energetic demand—was reduced in these same PE samples [151], suggesting these mitochondria were likely functioning close to their maximal capacity. These findings may also imply that PE cases that do reach term may do so because of a unique capacity to induce compensatory increases in mitochondrial function.

There are several possible reasons for the high degree of study finding discrepancies on this topic. One possible interpretation may be that those placentas capable of

initiating a compensatory increase in mitochondrial respiratory function at times of cellular stress, can limit the severity of placental disease and allow the pregnancy to progress to term. Conversely, placentas unable to initiate this response succumb to a more severe form of placental disease and dysfunction, initiating an earlier onset and more severe form of PE. Alternatively, these discrepancies may in fact be further evidence of distinct subclasses of PE, with divergent underlying pathophysiology and placental disease. It should be noted that in all studies described, no efforts to identify distinct PE subclasses were attempted, aside from the clinical distinction of early vs. late onset disease.

2.4.4 Mitochondrial Function—Redox Homeostasis and Apoptosis

Mitochondrial respiratory complexes I and III; and matrix enzymes such as 2-oxoglutarate dehydrogenase (OGDH) and pyruvate dehydrogenase (PDH) are among the major sites of cellular ROS generation [167, 168], particularly under hypoxic conditions [169, 170]. Oxidative stress, due to excessive ROS generation, is a well described hallmark of PE pathophysiology [52, 171]. For instance, elevated levels of oxidative stress markers such as 8-oxoDG, an indicator of DNA damage, and lipid peroxides in the placenta and maternal plasma in PE cases have been reported [172, 173]. Elevated levels of ROS, such as H_2O_2, have been measured in both the placentas and circulating serum of PE patients compared with healthy controls, however it is not entirely clear where the systemic ROS is originally generated [174]. Evidence of increased mitochondrial lipid peroxidation in PE placentas corresponds to high levels of lipid peroxide in the circulation of the gestational parent [173, 175-177]. Moreover, higher levels of dichlorofluorescin (DCF) oxidants, reactive nitric oxide metabolites such

as NO_2^- and NO_3^-, and protein carbonyl, have been observed in the placental tissue mitochondria from preterm/severe PE patients compared with term healthy controls, implying a major contribution of mitochondrial ROS in the oxidative stress observed in PE [178].

Coupled to increased ROS generation, there is also evidence of adaptive changes in antioxidant capacity in the context of PE. For example, mitochondrial superoxide dismutase (MnSOD) enzymatic activity is decreased in preterm/severe PE placentas compared with gestational age-matched controls, while its mRNA levels have been shown to be upregulated [151, 179]. Some studies suggest that there is an overall increase in antioxidant capacity of PE placenta through activation of other antioxidant enzymes present in cytoplasm and mitochondria such as glutathione peroxidase (GPx) [150, 151]. This suggests that an adaptive response is required in order to maintain placental function in the oxidative stress condition.

Intracellular stress such as oxidative damage can activate the mitochondria mediated caspase-9 apoptotic pathway [180]. Intracellular stress induces activation of proapoptotic proteins Bax and Bak that inactivate anti-apoptotic Bcl-2 and Bcl-xL proteins, leading to opening of outer mitochondrial membrane [181]. PE placentas show increased presence of apoptotic syncytial knots with evidence of oxidative DNA damage [182-184]. Holland et al. showed that the ratio of BAX/BCL2 protein expression was increased in preterm/severe PE placentas compared with gestational age-matched controls [151]. On the other hand, in term PE placentas, the ratio of BAX/BCL2 expression was relative to term controls while the levels of antioxidant enzymes increased [151]. These findings suggest that increased antioxidant activity in PE

placentas may keep oxidative stress at bay and, in turn, reduce cellular apoptosis and facilitate reaching term pregnancy.

2.5 Mitochondrial Dysfunction—A Point of Convergence across Preeclampsia Subclasses

To date, most research focused on understanding placental mitochondrial function and dysfunction in PE combines all cases together or uses the clinical classification scheme of “early-onset” vs. “late-onset”. While this foundational work has been tremendously helpful in gaining an appreciation for the central role of mitochondrial dysfunction in PE, it is important to note that even within these clinically distinct groups of patients, there remains a high degree of heterogeneity in: (1) the types of placental disease and damage noted at the time of pathological review; (2) circulating concentrations of placenta-derived biomarkers of disease (i.e., sFLT, sENG); (3) placental gene expression profiles; and (4) impact on fetal growth profiles. Collectively, this data would indicate that even within these clinically distinct populations, their very likely exists different underlying etiology and pathophysiology, and detailed profiling work conducted by our group [4,61,63,64] and others [65], certainly supports this conclusion. Hence, to gain a true appreciation for the role of mitochondrial dysfunction in the establishment and progression of PE, it is important to consider the underlying disease processes at play.

2.5.1 Canonical PE Subclass—Ischemia-Reperfusion Induced Mitochondrial Dysfunction

The historical dogma of PE pathophysiology includes defective EVT invasion of the decidua and impaired spiral artery remodeling, leading to hypoxia–reoxygenation events

and oxidative damage in the placenta [185, 186]. The canonical PE subclass previously described by our group demonstrated gene expression, histopathology and clinical findings aligned to this disease model [48]. Data collected in rodent models of PE established by surgical reductions in uterine blood flow—the RUPP model—demonstrated a 30% increase in placental H_2O_2 production, compared with sham treated controls [187], highly indicative of placental mitochondrial redox dysfunction. Likewise, in primary human trophoblast cells, hypoxic culture conditions (2–5% O_2) increased the production of mitochondrial ROS, triggering HIF1-α-mediated expression of the anti-angiogenic PE marker sFLT-1—a circulating vascular endothelial growth factor signaling inhibitor [188, 189]. Increase in sFLT-1 not only causes systemic endothelial dysfunction in the gestational parent but also induces further mitochondrial dysfunction and oxidative stress in placenta [190]. A recent study of placentas from early onset ‘canonical PE’ with evidence of ischemia–reperfusion injury, demonstrated that activation of mitochondrial unfolded protein response (UPRmt) led to a decrease in the expression of mitochondrial quality control protease Caseinolytic peptidase P (CLPP) [164]. In vitro, knockdown of CLPP in the BeWo trophoblast cell line led to increased mitochondrial fission and reduced respiration, mimicking findings of canonical human PE placentas. Recently, another study demonstrated that chronic hypoxia in ovine pregnancy induced ER and cytoplasmic UPR, however, activation of mitochondrial UPR was not investigated [191].

2.5.2 *Immunological PE Subclass—Inflammation Induced Mitochondrial Dysfunction*

Preeclampsia has long been associated with a pro-inflammatory state, however a clearer identification of PE cases that demonstrate profound immune dysregulation at the utero–placenta interface, in the absence of traditional hallmarks of ischemia–reperfusion injury, has helped to clarify the existence of this unique subclass of PE patients [48, 151, 192, 193]. Due to the understudied nature of this PE subclass in general, our current state of knowledge on the mechanisms through which chronic inflammation at this interface establishes placental dysfunction, and more specifically, what role placental mitochondria may play in the placental pathophysiology within this subclass, are not currently well understood. However, a wealth of literature has identified mitochondrial dysfunction as a common feature of many chronic inflammatory diseases in non-pregnant populations, as well as within the aging process [194-196]. Inflammatory mediators have been shown to alter the activities of respiratory complexes, ATP production, mitochondrial membrane potential and its morphology [196]. Proinflammatory cytokines such as TNF-α have been shown to be an inducer of both cytoplasmic and mitochondrial ROS generation [197-200]. A recent study suggests that inflammation induced by TNF-α causes reverse electron flow through mitochondrial complex I and, thus, stimulates mitochondrial ROS production [200].

Increased levels of TNF-α have been measured in placentas of PE cases [192, 201, 202], and profiling work carried out by our group demonstrated a very high placental gene expression of both TNF-α and TNF-α receptors uniquely within the ‘immune-driven PE subclass’ samples [48]. Interestingly, daily infusions of TNF-α (50 ng/day) in a

pregnant rat model was capable of eliciting PE clinical features, including hypertension and decreases in both glomerular filtration rate and renal plasma flow [203]. In vitro treatment of first trimester placental explants with TNF-α (10 ng/mL) compromised key aspects of placental function, including migratory and invasive properties [204]. In the JEG3 trophoblast cell line, TNF-α treatment (0.2–5.0 ng/mL) prevented cell integration into endothelial networks and tube formation [205]. While there is certainly strong evidence to suggest that TNF-α, in addition to other pro-inflammatory signaling molecules, can initiate placental dysfunction and contribute to adverse pregnancy outcomes, the specific role of placental mitochondria, or mitochondrial dysfunction, in the context of chronic inflammation in pregnancy is not yet as clear. In vitro, TNF-α treatment in primary human trophoblasts, isolated from female offspring originating from overweight or obese pregnant individuals, caused decreased expression of mitochondrial complex-I subunit NDUFA4 and iron–sulfur cluster assembly enzyme (ISCU) and inhibited mitochondrial respiration [206]. In an LPS-induced mouse model of intrauterine inflammation, placental transcriptomic analysis demonstrated dysregulated expression of upstream regulators of mitochondrial function. Further, metabolomics analysis showed alterations in TCA cycle and increase in acylcarnitine levels, known to induce mitochondrial dysfunction and oxidative stress [207, 208]. Collectively, this body of work that is still in its infancy, certainly suggests that inflammation alone can initiate mitochondrial dysfunction in placental tissues, and as such would lend support to the hypothesis that mitochondrial dysfunction may in fact be a common point of convergence in the establishment of placental disease across multiple PE subclasses.

2.5.3 *Gestational Parent Driven PE—Predisposing Factors and Mitochondrial Dysfunction*

Contrary to the traditional dogma of PE pathophysiology, in which placental dysfunction is central to disease establishment, there have long been reports of PE cases with minimal evidence of placental disease [48, 209, 210]. Our group has confirmed the existence of this patient population through detailed clinical and placental profiling, concluding this group of patients likely arrive at a PE diagnosis (hypertension and end organ damage) as a result of poor gestational parent adaptation to pregnancy, rather than placental dysfunction [48]. In healthy pregnancies, the process of placentation and normal placental function results in the release of some level of placenta-mediated factors into the gestational parent's circulation, including: antiangiogenic factors (i.e., sFLT-1), cytokines and chemokines, microparticles/microvesicles, exosomes, apoptotic bodies and cell-free fetal DNA (to name a few) [211-214]. In a healthy pregnancy, the gestational parent responds to these signaling factors in a way that promotes appropriate adaptation to pregnancy. However, predisposing risk factors (i.e., diabetes, obesity, advanced age, kidney disease, systemic lupus, antiphospholipid syndrome, chronic hypertension) may prevent the appropriate adaptive response in the gestational parent and instead may trigger systemic pathophysiology, including a heightened immune response, endothelial damage and dysfunction and the development of hypertension. As discussed in the previous section, a pro-inflammatory environment may be a potent trigger for mitochondrial dysfunction. Further, the dysregulated role of mitochondria within the context of endothelial dysfunction and hypertension has already been widely described, even in the context of pregnancy (see reviews [103, 215, 216]

for more details). As such, while there may be a subclass of PE patients in which placental mitochondrial dysfunction may not be a major contributing factor to disease, systemic mitochondrial dysfunction in the gestational parent is still likely to be of major concern—and this is likely true of all subclasses of PE described. In fact, elevated mutational loads of circulating cell-free (ccf)-mtDNA have been identified in blood (buffy coat) collected from third trimester patients with PE and gestational hypertension alike. As ccf-mtDNA is often regarded as a marker of systemic inflammation, this result suggests that dysregulated mitochondria from gestational parent origin is a likely contributor to PE pathophysiology [217]. In vitro studies have shown that treatment of human umbilical vascular endothelial cells (HUVECs) with serum of PE patients or exogenous placenta-mediated factors (sFLT-1 or angiotensin II type 1 autoantibodies), leads to decreased mitochondrial respiratory capacity, increased superoxide formation and induces a glycolytic phenotype [156, 218, 219]. Thus, with or without the direct contribution of placental disease, systemic mitochondrial dysfunction within the gestational parent is likely to be another common point of convergence of pathophysiology across all PE subclasses.

Collectively, the current literature supports a role for mitochondrial dysfunction, both within the placenta and within gestational parent tissues, in the pathophysiology of PE. The underlying etiology of disease may in fact be different across PE subclasses, but we propose that dysregulation of mitochondrial biogenesis and function may be a common feature to all cases of PE (Figure 1), and as such, serve as an important therapeutic target in the identification of effective treatments for this common and debilitating disorder of pregnancy.

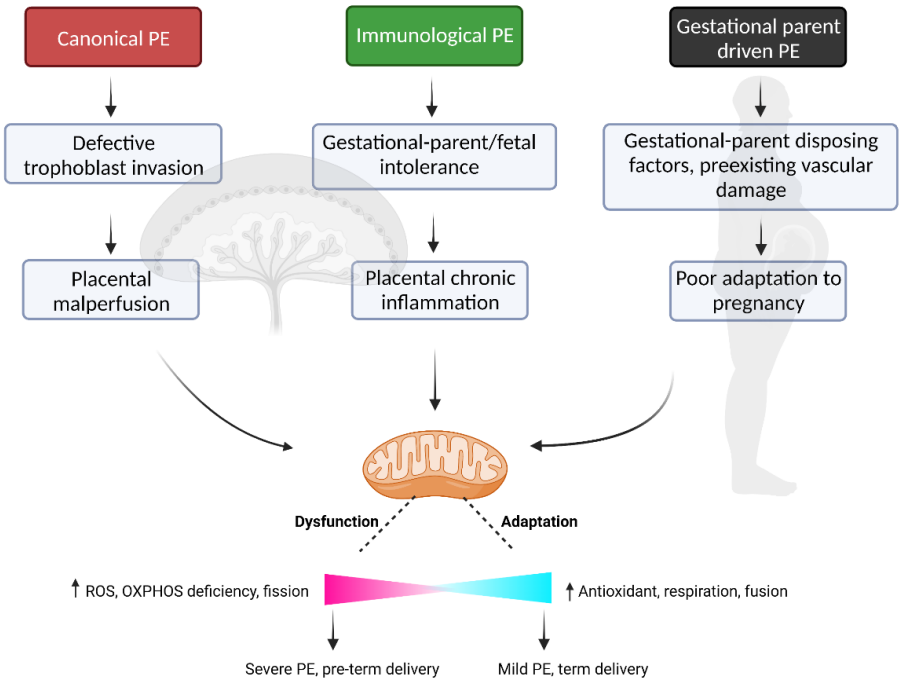

Figure 1. Mitochondrial dysfunction as a common feature across all PE subclasses. PE disease driven by either placental malperfusion, chronic inflammation at the utero–placental interface and/or poor gestational parent adaptation, likely all demonstrate altered mitochondrial respiration, increased ROS production and mitochondrial fragmentation within the placenta and across numerous gestational parent tissues. The inherent ability of the mitochondria to adapt during pregnancy to various pathological insults may in part dictate the clinical outcome of the pregnancy, with high degrees of mitochondrial adaptation associated with a milder clinical presentation of the disease, prolonged gestation, and improved fetal growth profiles. The "↑" arrow indicates an increase. Figure was created with Biorender.com, accessed on 24 January 2023.

2.6 Potential for Mitochondrial Targeting Therapies in Preeclampsia

Currently, there are no highly effective treatments for PE. Antihypertensive drugs are used to manage clinical symptoms in the gestational parent, but these treatments are not aimed at targeting the source of disease and as such these treatments neither serve to prolong the pregnancy nor ameliorate adverse impacts of PE on fetal growth profiles [220]. As there is sufficient evidence to implicate mitochondrial dysfunction in the establishment and/or progression of PE, consideration should be given to how

therapeutic strategies aimed at improving mitochondrial function may be applied in cases of PE [27, 97]. In this section, some of the most recent advancements on this topic will be discussed (Figure 2).

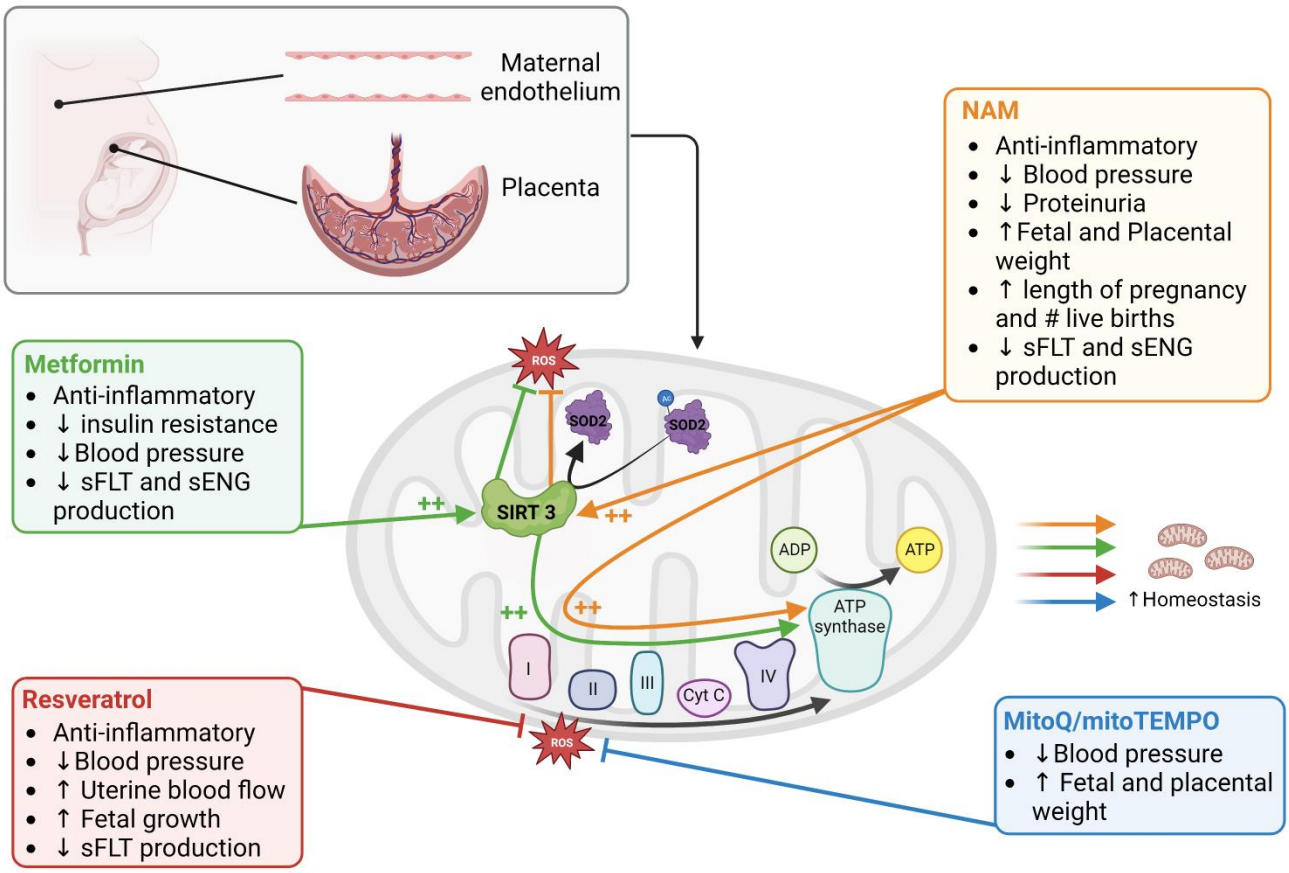

Figure 2. Mitochondria targeting treatments in preeclampsia. Among the drugs and nutraceuticals tested in PE studies, the most promising ones that target mitochondria are metformin, resveratrol, nicotinamide (NAM), and mitoQ/mitoTEMPO. Metformin, resveratrol, and NAM function on multiple pathways that improve mitochondrial biogenesis, function, quality control (by mitophagy), and reduce ROS; thereby establishing mitochondrial homeostasis under pathological conditions. MitoQ/mitoTEMPO act by reducing mitochondrial ROS which eventually improve mitochondrial homeostasis. The symbol ++ indicates activation, # indicates numbers, ↑ indicates increase and ↓ indicates decrease. Figure was created with Biorender.com, accessed on 13 February 2023.

2.6.1 Resveratrol

Several studies have shown that mitochondrial complex-I inhibitors may provide benefits in disease conditions by reducing superoxide generation through complex-I. Resveratrol (3,4,5-trihydroxy-trans-stilbene) has been described as one of them. It is a plant-based polyphenol found in some fruits such as grapes, berries, and peanuts. It has antioxidant, anti-inflammatory and anti-microbial properties. Studies also suggest that it enhances mitochondrial homeostasis by activating the master regulator of mitochondrial biogenesis, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) (see the following review for more information [221]). Notably, it reduced blood pressure in hypertensive rodent models [222]. Resveratrol administration also improved uterine artery blood flow velocity and increased fetal weight in a pregnant mouse model of uterine blood flow defect [223]. Similarly, resveratrol has shown some beneficial effects in various in vitro PE models. In one study, first trimester HTR-8/SVneo trophoblast cell lines were treated with angiotensin II, TNF-α, protein kinase C (PKC) activator, phorbol-12-myristate-13-acetate (PMA) for 24 h. These treatments resulted in increased expression of sFLT1—a major contributor to gestational parent endothelial dysfunction in PE. However, co-treatment with resveratrol significantly reduced the expression of this potent antiangiogenic factor and did not demonstrate any cytotoxic effect on the cells [224]. Interestingly, in a randomized control trial carried out with 400 patients diagnosed with PE, the use of resveratrol as an adjuvant treatment alongside oral nifedipine (an anti-hypertensive drug commonly prescribed in severe PE) was shown to further improve both systolic and diastolic blood pressure compared with nifedipine treatment alone. With the resveratrol-combination therapy, time required for

controlling blood pressure was reduced and time before developing new hypertension was delayed; thus, indicating a beneficial effect of resveratrol [225]. Interpretation of these results must be taken with caution, however, as there was no resveratrol alone in the study arm, rather the patients all received nifedipine as well. Thus, it is not clear based on these study results alone that resveratrol may serve as an effective solo therapy.

2.6.2 Metformin

Metformin is an effective treatment for type 2 diabetes that lowers blood glucose levels and reduces insulin resistance. It has anti-inflammatory and antioxidant effects [226, 227]. Metformin has been shown to increase the expression of mitochondrial sirtuin 3, an NAD^+ dependent deacetylase, that regulates oxidative phosphorylation and activates mitochondrial antioxidant MnSOD [56, 227]. It also activates adenosine 3′,5′-monophosphate (AMP) protein kinase (AMPK), a major regulator of mitochondrial fission, mitophagy and biogenesis [228, 229]. Metformin has been used during pregnancy to improve pregnancy outcomes in individuals with obesity, gestational diabetes, type 2 diabetes and polycystic ovary syndrome. Interestingly, metformin has been shown to reduce the production of the anti-angiogenic factors sFLT1 and sENG (endoglin) in both villous cytotrophoblasts and endothelial cells in culture [230]. Moreover, third trimester placental villous explants collected from PE patients treated with metformin in culture demonstrated a decrease in the secretion of sFLT1 and sENG, and co-treatment with succinate blocked this effect, indicating metformin-mediated regulation of sFLT1 and sENG expression is likely mediated via mitochondrial function [230]. In a PE mouse model induced via a high fat diet, metformin administration was

also shown to reduce blood pressure and increase circulating levels of vascular endothelial growth factor (VEGF) [231]. The data in humans collected to date, however, is not as clear. A number of clinical trials, observational cohort studies and subsequent meta-analyses have been carried out to assess the ability of metformin treatment to reduce the establishment of PE in individuals at high risk, with mixed results [232-236]. Collectively, the data suggest that metformin may prove to be beneficial for a specific subset of patients, specifically those that have polycystic ovary syndrome (PCOS) [233-235]. Moreover, metformin treatment in the context of gestational diabetes has been associated with low birth weights, indicating a potential fetal risk with this form of treatment [97, 237].

2.6.3 MitoQ and MitoTEMPO

MitoQ is a synthetic ubiquinone that is intracellularly converted to ubiquinol by the enzymatic activity of mitochondrial complex II. Ubiquinol serves to neutralize free radicals and can be continuously recycled to maintain a potent antioxidant presence in the mitochondria. Similarly, MitoTEMPO, which can specifically be targeted to the mitochondria via a bound lipophilic cation, contains piperidine nitroxide (Tempo) that mimics superoxide dismutase and catalyzes the dismutation of the superoxide radical into molecular oxygen and hydrogen peroxide. These mitochondria-targeted antioxidants have shown therapeutic promise in the RUPP rodent model of PE, where they have been shown to significantly reduce the maternal blood pressure and increase both placental and fetal weights. [187]. In another study, MitoTEMPO improved placental mtDNA stability and mitochondrial dynamics in a PE-like rat model [238]. In cell culture, it reduced mitochondrial ROS production in HUVECs treated with plasma

from PE patients. Both MitoQ and MitoTEMPO have been tested in humans and found to be tolerable with no adverse effect [239-242]. MitoQ has been reported to reduce liver inflammation in patients with chronic hepatitis C virus (HCV) infection [240]. On the other hand, MitoTEMPO has been shown to improve cutaneous vascular conductance (CVC) in patients with chronic kidney disease [241]. However, caution should be taken when considering therapeutic application of such antioxidants in pregnancy as one of the rodent studies indicated that MitoQ administration early in pregnancy could hinder normal placenta development by reducing physiological ROS levels [243].

2.6.4 Nicotinamide (NAM)

Nicotinamide or NAM is a derivative of vitamin B3 and has antioxidant and anti-inflammatory properties [244, 245]. NAM is a known cellular nicotinamide adenine dinucleotide (NAD^+) donor and NAD^+ level is critical for maintenance of OXPHOS function, mitochondrial biogenesis, and homeostasis [246]. NAD^+ is also important for DNA damage response, inflammation, and oxidative stress pathways as it functions as a cofactor for certain enzymes such as PARPs and sirtuins [54]. The therapeutic potential of NAM treatment has been investigated using three different rodent models of PE: RUPP, sFLT1 overexpression and ASB4 (ankiryn-repeat-and-SOCS-box-containing-protein) knockout mice. In all three models, NAM administration (500 mg/kg per day) was shown to decrease blood pressure, proteinuria and prevent glomerular endotheliosis in the mothers. Further, NAM treatment increased fetal and placental weight, prolonged pregnancy duration and increased the number of live births. NAM also decreased HIF1-alpha expression; and increased the levels of NAM, NAD^+ and ATP in fetal brains [247]. As NAM is a NAD^+ donor, it is likely that the observed

beneficial effects were through improving mitochondrial function at various maternal or fetal tissue levels, including placenta. Thus, more research needs to be performed to understand the mechanism. Moreover, there is a safety concern regarding the use of NAM at high dose during pregnancy. Studies performed in rats showed that NAM administration at 500 mg–4 g/kg/day dose was associated with liver toxicity [248, 249]. Maternal NAM intake led to DNA hypomethylation in rat placenta and fetal liver [250]. Indeed, in humans, NAM intake at 300 mg/day was shown to elevate plasma levels of homocysteine, an indicator for methyl donor consumption due to NAM methylation [251]. However, there are additional NAD^+ donors that can be examined for their efficacy in improving mitochondrial and placental function, and ultimately pregnancy outcomes, such as nicotinamide riboside (NR), nicotinamide mononucleotide (NMN), dihydronicotinamide riboside (NRH)—each of which may demonstrate a more appropriate safety profile for use in pregnancy.

2.7 Future Perspective

Findings from animal and human studies indicate that mitochondrial dysfunction is a central component to the establishment of both placental disease and gestational parent disease in PE. The degree of mitochondrial dysfunction and inherent ability to adapt to cellular stress signals may dictate the severity of placental or gestational parent tissue damage and, ultimately, pregnancy outcome. Novel therapeutic interventions aimed at targeting such common disease features will likely prove to be highly effective at the population level regardless of their subclass in a clinical setting. However, there are still many hurdles to overcome in this domain. Using animal models and human studies, future research should aim to: (1) identify specific aspects of mitochondrial dysfunction

that are common across PE and should be the focus of therapies moving forward; (2) determine the critical window when mitochondrial dysfunction occurs during PE pathogenesis in order to maximize therapeutic benefits, and (3) vigorously test the safety profiles of these treatments, as any novel treatments must not interfere with placental or fetal development.

Chapter 3 – Hypothesis and Research Objectives

Preeclampsia is a life-threatening hypertensive disorder of pregnancy, to which there is no cure. Our group has identified three subclasses of PE disease, one of which demonstrates evidence of heightened and chronic inflammation within the placenta. In non-pregnant populations, numerous chronic inflammatory disorders demonstrate PARP-mediated NAD^+ depletion and subsequent mitochondrial dysfunction. Preliminary data collected by our group has likewise demonstrated increased PARP expression in placentas from this inflammatory-driven PE subclass, suggesting that NAD^+-signalling processes linked to cellular homeostasis and function, such as mitochondrial health, may be compromised in this PE subclass.

The overarching hypothesis of the current thesis is that PARP-mediated NAD^+ depletion and subsequent mitochondrial dysfunction is causal to establishment of placental dysfunction in the inflammatory-driven subclass of PE. Further, it is postulated that placental NAD^+ replenishment may be an effective subclass-specific therapeutic approach to prevent or treat inflammation-mediated PE.

These hypotheses were specifically examined through the completion of the following research objectives:

Objective 1 - Profile PARP activity, NAD^+ availability, and mitochondrial health in human cases of all three PE subclasses.

Objective 2 – Examine the causal relationship between chronic inflammatory insults and dysregulated NAD^+ signalling in both an *in vitro* human trophoblast culture model and in a rodent model of inflammation-driven PE.

Objective 3 - Evaluate the therapeutic potential of NAD^+ boosters to improve placental health and function in a rodent model of inflammation-driven PE.

In the completion of this thesis a comprehensive review of the literature specific to the role of placental mitochondrial dysfunction in PE pathogenesis, including the potential for therapeutic targeting, was carried out and published (Chapter 2; Int J Mol Sci. 2023 Feb 20;24(4):4177). The main body of this thesis comprises an additional two manuscripts that present the results obtained in the completion of the stated research objectives 1-3, with one manuscript in the final stages of peer-review (Chapter 4) and the other in the final stages of manuscript preparation for submission (Chapter 5).

- In Chapter 4, three rat models of inflammation-driven PE were characterized and compared (e.g. Tumor necrosis factor-α infusion and intraperitoneal injections of polyinosinic:polycytidylic acid or lipopolysaccharide during gestation), to identify and validate a rodent model that most closely mimics human cases of inflammation-mediated PE. This manuscript is relevant to the completion of objectives 2 and 3 and is currently under peer-review for publication in Frontiers in Endocrinology (Jahan et al., 2023).

- In Chapter 5, the characterization of NAD^+-signalling and mitochondrial health in human cases of PE is presented, along with the establishment of a causal relationship between inflammatory signalling and dysregulated placental NAD^+ signalling in both an in vitro cell culture model and a rodent model of inflammation-mediated PE. Further, the therapeutic potential of NAD^+ boosting to improve placental mitochondrial function and prevent the establishment of inflammatory PE is presented. This manuscript is relevant

to the completion of all three research objectives and is currently being prepared for submission to Science Translational Medicine (Jahan et al., 2023).

Chapter 4 - A comparison of rat models that best mimic immune-driven preeclampsia in humans

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Statement of contribution:

F.J. contributed to conceptualization, data collection and analysis, writing, reviewing, and editing. All authors have read and agreed to the published version of the manuscript.

Abstract

Preeclampsia (PE), a hypertensive pregnancy disorder, can originate from varied etiology. Placenta malperfusion has long been considered the primary cause of PE. However, we and others have showed that this disorder can also result from heightened inflammation at the maternal-fetal interface. To advance our understanding of this understudied PE subtype, it is important to establish validated rodent models to study the pathophysiology and test therapies. We evaluated three previously described approaches to induce inflammation-mediated PE-like features in pregnant rats: 1) Tumor necrosis factor-α (TNF-α) infusion via osmotic pump from gestational day (GD) 14-19 at 50ng/day/animal; 2) Polyinosinic:polycytidylic acid (Poly I:C) intraperitoneal (IP) injections from GD 10-18 (alternate days) at 10mg/kg/day/animal; and, 3) Lipopolysaccharide (LPS) IP injections from GD 13-18 at 20ug-70ug/kg/day per animal. Maternal blood pressure was measured by tail-cuff. Upon sacrifice, fetal and placenta weights were recorded. Placenta histomorphology was assessed using H&E sections. Placenta inflammation was determined by quantifying TNF-α levels and inflammatory gene expression. Placenta metabolic and mitochondrial health were determined by measuring mitochondrial respiration rates and placenta NAD^+/NADH content. Of the three rodent models tested, we found that Poly I:C and LPS decreased both fetal weight and survival; and correlated with a reduction in region specific placenta growth. As the least effective model characterized, TNF-α treatment resulted in a subtle decrease in fetal/placenta weight and placenta mitochondrial respiration. Only the LPS model was able to induce maternal hypertension and exhibited pronounced placenta metabolic and mitochondrial dysfunction, common features of PE. Thus, the rat LPS model was most effective for recapitulating features observed in cases of human inflammatory PE.

Future mechanistic and/or therapeutic intervention studies focuses on this distinct PE patient population may benefit from the employment of this rodent model of PE.

Keywords: preeclampsia, disease subclasses, pregnancy, hypertension, immune, pro-inflammatory, TNF-α, Poly I:C, LPS, rat model

4.1 Introduction

Preeclampsia (PE) is a pregnancy associated disorder characterized by a sudden onset of hypertension at ≥20 weeks of gestation and evidence of maternal end organ dysfunction. It is a highly heterogenous condition in humans, with variable pathogenesis, timing of onset, maternal symptoms, and fetal outcomes – indicative of distinct subclasses of disease [1-8]. Detailed clinical and placenta profiling by our group, and others, has identified the presence of a unique subclass of PE patients that demonstrate profound pro-inflammatory signalling at the maternal-fetal interface [1, 5, 6, 8, 9]. Unlike the traditional dogma of PE pathogenesis, these patients do not demonstrate evidence of poor utero-placenta perfusion and hypoxia, or placenta up-regulation of anti-angiogenic factors (i.e. sFLT, sENG). Rather, placentas from these PE patients highly express pro- inflammatory cytokines and their receptors, such as tumor necrosis factor-α (TNF-α), interferon gamma (IFN-γ), TNF-α induced Proteins 2, 3, 6 (TNFAIP2, TNFAIP3, TNFAIP6), and interleukin 6 cytokine family signal transducer (IL-6ST). Further, these placentas demonstrate histopathology lesions consistent with chronic inflammatory insults, such as villitis of unknown etiology and/or massive perivillous fibrin deposition [1, 6, 7, 10]. Clinically, this PE subclass demonstrates late pre-term deliveries (34-37 weeks) with milder maternal symptoms, however, fetal growth is most significantly impacted in the subclass [1, 7].

Placenta mitochondrial dysfunction is a recognized contributor to placenta dysfunction across all PE subclasses and is thus an intriguing therapeutic target for this disorder [11- 13]. Interestingly, nicotinamide adenine dinucleotide (NAD^+) consuming enzymes are highly and uniquely expressed in placentas from pregnancies with inflammatory PE

–with NAD^+ being a critical co-enzyme required for cellular reduction-oxidation (redox) status and homeostatic maintenance of energy metabolism and mitochondrial function [1, 14-17]. These placentas likewise demonstrate a profound depletion of cellular NAD^+ stores, suggesting a disruption of NAD^+ signalling as a key mechanism through which mitochondrial function becomes disrupted in this PE subclass [14]. These results are not entirely surprising, given that several non-pregnant pro-inflammatory diseases (e.g., non-alcoholic fatty liver disease, colitis, optic neuritis, central nervous system autoimmunity) and ageing tissues demonstrate NAD^+ depletion with paralleled mitochondrial impairment and organ dysfunction [15, 16, 18-23]. Collectively, this set of data are important biological observations which hint at the molecular mechanisms through which placenta dysfunction may be established and/or propagated in this inflammatory PE subclass. However, further work focused on causal mechanisms of placenta disease in this understudied PE subclass is required, and as such animal model systems which can closely mimic the pathophysiology of this unique patient population are needed. Once established and validated, these models can help push forward the discovery of subclass (/etiology)-specific screening and treatment modalities for PE [1].

Spontaneous onset of PE is unique to humans and some non-human primates, however the establishment of animal models which mimic various aspects of PE pathophysiology have been established in dogs, sheep, rhesus monkeys and most frequently in rodents – mice and rats. Several comprehensive reviews [24, 25] on this topic nicely describe the most widely used of these models, including those established via induction of placenta ischemia, angiogenic imbalance (by increasing levels of anti-

angiogenic factors such as s-FLT1, sENG) and genetic manipulations of the renin-angiotensin system [24, 25]. In large part, these animal models lean heavily on a conceptual framework of PE pathogenesis that included poor utero-placenta perfusion and placenta hypoxia as central features. These well-established models certainly demonstrate utility in expanding our understanding of human PE with these same features (i.e., the canonical PE subclass), however their utility is likely limited for explorations of the smaller and less characterized subclasses of PE pathophysiology, including the inflammatory PE subclass described.

There are, however, a handful of immune-mediated, non-genetic animal models of PE that have been established, which may effectively serve this purpose. These models have been established in rodents, generated via continuous TNF-α infusion or daily injections of Toll-like receptor (TLR) agonists across pregnancy (e.g. lipopolysaccharide-LPS, Polyinosinic:polycytidylic acid- Poly I:C). TNF-α is an important mediator of inflammation in human PE [1, 26, 27], with well described elevations in serum and placenta [1, 27, 28]. TNF-α infusion in pregnant rats from gestational day (GD) 14 to 19 has been reported to successfully induce maternal hypertension, with no described changes to litter size or fetal weight [29]. Alternatively, mouse and rat models of PE have been established via TLR agonist activation, as TLR signalling cascades have been implicated in placenta inflammation described in human cases of PE [30]. In this vein, some groups have carried out daily injections of LPS to illicit an immune response. LPS, a bacterial cell-wall component, is a potent inducer of TLR-4 mediated inflammatory cascade [31] and is proposed to induce PE-like symptoms in this model through TNF-α mediated trophoblast dysfunction and maternal systemic inflammation

[24, 32-34]. As with TNF-α infusion, LPS administration to rats during pregnancy increased maternal blood pressure, however decreased fetal growth and survival was also described in this model [32, 35-38]. Several other TLR agonists have been tested, including Poly I:C – a TLR-3 agonist; Imiquimod (R837) - a TLR-7 agonist; CLO97 (Imidazoquinoline compound) – a TLR7/8 agonist; and synthetic CpG oligonucleotide (ODN 2395)- a TLR-9 agonist. In all cases, treatment with these agonists resulted in increased maternal blood pressure with variable reported effects on fetal survival and growth [30, 39, 40].

In the current study, three previously published inflammation-mediated rodent models of PE were evaluated for their potential utility in future investigations of the inflammatory subclass of PE. Specifically, rat PE models established in the following ways were directly compared: 1) TNF-α infusion via osmotic pump, 2) Poly I:C treatment by intraperitoneal (IP) injection, and 3) LPS treatment by IP injection. PE-like features (i.e., maternal hypertension), fetal health outcomes, evidence of chronic placenta inflammation and metabolic dysfunction were compared across all models. This study will serve as a solid resource for other groups aiming to establish an appropriate in vivo animal model of PE to investigate underlying mechanisms of disease, biomarker discovery or therapeutic interventions for the inflammatory subclass of PE.

4.2 Methods and Materials

Model establishment and pregnancy outcomes

All animal studies were performed in accordance with University of Ottawa animal care ethics and guidelines (protocol# HS2923). Sprague Dawley rats were purchased from

Charles River Laboratories International, Inc. Animals were housed in a room with stable temperature of 23°C and a 12:12-hr light-dark cycle was maintained. Animals were timed- mated and vaginal smears were performed to confirm pregnancy [41]. Body weights were recorded each day to monitor pregnancy progression. Animals were allocated to one of the model treatment groups described below.

I. TNF-α model (n=10): Recombinant rat TNF-α protein (510-RT-010, R&D systems) was infused at 50ng/day/animal concentration by implanting osmotic mini pump (Alzet Model 2001) on GD 13 (infusion days GD 14-19), as previously described [29, 42].

II. Poly I:C model (n=10): Polyinosinic–polycytidylic acid sodium salt (P1530-100MG, Sigma-Aldrich) was solubilized in saline and injected intraperitoneally (IP) every alternate day between GD 10-18 at 10mg/kg/day, as previously described [39].

III. LPS model (n=10): Lipopolysaccharides from Escherichia coli O55:B5 (L2880-10MG, Sigma-Aldrich) was solubilized in saline and injected by IP from GD 13-18 at 20ug-70ug/kg/day incremental dose, as previously described [35].

IV. Saline control (n=10): Controls were randomly allocated for saline osmotic pump infusion or saline IP injections. (n=3 osmotic pump, n=3 IP from GD10-18 alternate days and n=4 IP from GD 13-18)

Necropsy of pregnant rats was performed on GD 19, with collection of litter size, number of resorption sites and fetal and placenta weights.

Maternal blood pressure measurement

For all models, blood pressure (BP) measurements were performed using tail-cuff plethysmography using the CODA® high-throughput non-invasive system (Kent Scientific Corporation). Measurements were recorded from GD 15-19. Animals were acclimatized to the tail-cuff system for 4 days prior. On the day of recording, 5-10min was allowed for body temperature stabilization and a continuous 20min blood pressure recording was taken. The collected BP measurements for the TNF-α model using this method did not align with previous reports, and as such BP measurements were confirmed using the radiotelemetry method (HD-S10 Implant, Data Sciences International, USA) in a subsequent set of TNF-α treated rats (n=4; 50ng/day, GD14-19) and saline treated controls (n=4). In this subset, 10 days prior to mating, animals were subjected to surgery and a blood pressure transducing catheter was place into the femoral artery and the transmitter body was placed subcutaneously. A detailed method of the surgery is described by Huo et. al, 2014 [43].

Placenta histomorphometry

Rat placentas were bisected at the point of umbilical cord insertion, fixed in 4% PFA [in phosphate buffer saline (PBS)], paraffin-embedded, sectioned at 4um thickness and stained with hematoxylin and eosin (H&E). The slides were scanned using a Zeiss Axio Scan Z1 slide scanner, and the images were analyzed using the ZEISS ZEN lite software. The placenta diameter was determined by drawing a straight line perpendicular to the umbilical cord insertion and extending to both placenta extremities. Similarly, to estimate the depth of the decidua, junctional zone, and labyrinth placenta layers, three lines located in the middle, right lateral, and left lateral regions of the

placenta were drawn perpendicular to the placenta diameter line in each layer. The results were averaged and expressed in micrometers (um).

Placenta inflammation

Quantikine® Rat TNF-α Immunoassay immunosorbent assay (R&D Systems, Minneapolis, MN) was used for measuring placenta TNF-α protein levels. 40 mg of placenta tissue was homogenised in PBS-EDTA 5mM extraction buffer containing protease inhibitor cocktail (complete™ Roche 11836170001) and the immunoassay was performed according to manufacturer's instruction.

RNA was extracted to assess gene transcript expression of key pro-inflammatory signalling mediators and growth factors previously linked to placenta inflammation [44-50] [e.g., TNF-α, IFN-γ, macrophage migration inhibitory factor (Mif), intercellular ddhesion molecule 1 (ICAM-1), placental growth factor (PIGF), chemokine ligand 2 (CCL2), and inducible nitric oxide synthase (iNOS or NOS2)]. TRIzol (invitrogen) was used to extract total RNA from rat placenta, with RNA concentrations measured using NanoDrop technology (Thermo Fisher Scientific). Subsequently, gDNA was eliminated and 1µg of total RNA was transcribed to cDNA with iScript™ gDNA Clear cDNA Synthesis Kit (Bio- Rad; #1725035). In a 20µL reaction, SsoAdvanced Universal SYBR Green Supermix was used to amplify cDNA following the manufacture's instructions (Biorad). Selected gene expression was analyzed in triplicate with SYBR green chemistry in a CFX384™ Real- Time PCR Detection System. Then, using comparative cycle threshold method (ΔΔCq), quantification cycle (Cq) values were normalized based on beta-actin expression. Primer sequences used for qPCR can be found in the supplementary 2 (table 1).

Placenta Mitochondrial Function

High-resolution oxygraphy was used to assess oxygen respiration rates of mitochondria isolated from fresh rat placenta samples. Isolation of mitochondria was performed according to Frezza et al. with slight modifications as noted below [51]. Animals were decapitated [52] and 6-8 placentas/litter were quickly removed and pooled, washed in cold PBS and placed in ice cold buffer A [300mM sucrose mM, 10mM Tris-HCl, 1mM EGTA-Tris Base and 0.1% bovine serum albumin (BSA, fatty acid-free); pH 7,2]. Placentas were first cut into large pieces and washed twice with buffer A to remove blood. These samples were then minced in a small volume of buffer A. The sample was transferred to a 30ml Potter-Elvehjem homogeniser, topped up with buffer A, and homogenised at 500rpm for 6 strokes. The suspension was centrifuged at 1000Xg and 4 °C for 10 min. The supernatant was collected in a new tube and the centrifugation step was repeated. To omit floating fat layers, a syringe was used to collect the supernatant. The combined supernatant was transferred to a new tube and centrifuged at 8000Xg and 4 °C for 10 min. Following aspiration of the supernatant, buffer B (300mM sucrose, 10mM Tris-HCl and 0.05mM EGTA-Tris Base; pH 7,) was added to the pellet and gently mixed by pipetting, followed by centrifugation at 8000Xg and 4 °C for 10 min. This step was repeated. The resulting mitochondrial pellet was re-suspended in 200µl of MiR05 respiration buffer (110 mM D-sucrose, 60 mM lactobionic acid, 20 mM taurine, 20 mM HEPES, 10 mM KH2PO4, 3 mM MgCl2, 0.5 mM EGTA, 1 g/L fatty-acid free BSA; pH 7.1 at 23 °C). An aliquot was taken from the mitochondrial suspension to perform detergent compatible colorimetric (DC) protein assay (Biorad) to quantify protein concentration.

Oxygen consumption rate was recorded using Clark-type electrodes according to a previously published protocol [53]. 2 mg/ml of mitochondrial protein content was added to the MiR05 respiration buffer. Baseline traces were recorded, and mitochondrial substrates and inhibitors were injected in sequence: i) 5mM glutamate and 2,5mM malate were added as a substrate for Complex-I mediated oxygen consumption, ii) ADP (1 mM) was added to record Complex-I driven state-III respiration, iii) 1 mM amytal was added to inhibit Complex-I mediated oxygen consumption, iv) 5mM succinate was added to record Complex-II driven state-III respiration v) 5 µM antimycin A was added to inhibit Complex-III mediated electron transfer in order to measure complex-IV mediated oxygen consumption vi) Tetramethyl-p-phenylenediamine (TMPD)/Ascorbate (5/0.3 mM), was added to record Complex-IV driven state-III respiration, and vii) Potassium cyanide was added to stop oxygen consumption by mitochondria.

Placenta Metabolic Signalling

Placenta NAD^+ and NADH was quantified using a Biovision NAD^+/NADH kit (K337). 30-40mg of pooled (by litter) pulverized placenta tissue was added to 300ul of extraction buffer. Tissues were further homogenized using 21-gauge syringe needles. Quantification was performed according to the manufacturer's instructions.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software (Version 9.5). All values are reported as mean ± standard deviation (SD). Comparison was performed between saline control and treatment groups using a one-way ANOVA with a Fisher

Least Significant Difference (LSD) test and a p value of < 0.05 was considered statistically significant.

4.3 Results

Inflammatory PE-like features and pregnancy outcomes were most consistently demonstrated in the LPS rat model

As development of hypertension during pregnancy is a key clinical feature of PE [54], we measured BP in the pregnant rats using a tail-cuff blood pressure system. Results show that mean BP was significantly increased only in LPS treated pregnant rats compared to saline treated controls (figure 1A). In our hands, neither Poly I:C or TNF-α treatments showed an effect on BP. Given that TNF-α infusion was previously shown to increase BP by GD 19 [29, 42], we set out to confirm our TNF-α tail-cuff data using radio-telemetry, considered the gold standard of BP measurement techniques. Similar to tail-cuff results, radio-telemetry of TNF-α treated pregnant rats exhibited no change in BP compared to their corresponding controls (supplementary figure 1). Fetal and placenta growth was decreased in all treatment models, compared to saline controls, with Poly I:C and LPS treatments displaying the most prominent fetal and placenta growth restriction phenotype (figure 1C-D). Unsurprisingly, these same two treatment models also demonstrated decreased fetal survival, with increased number of resorption sites at GD19, whereas no fetal losses were observed in the TNF-α group (figure 1B).

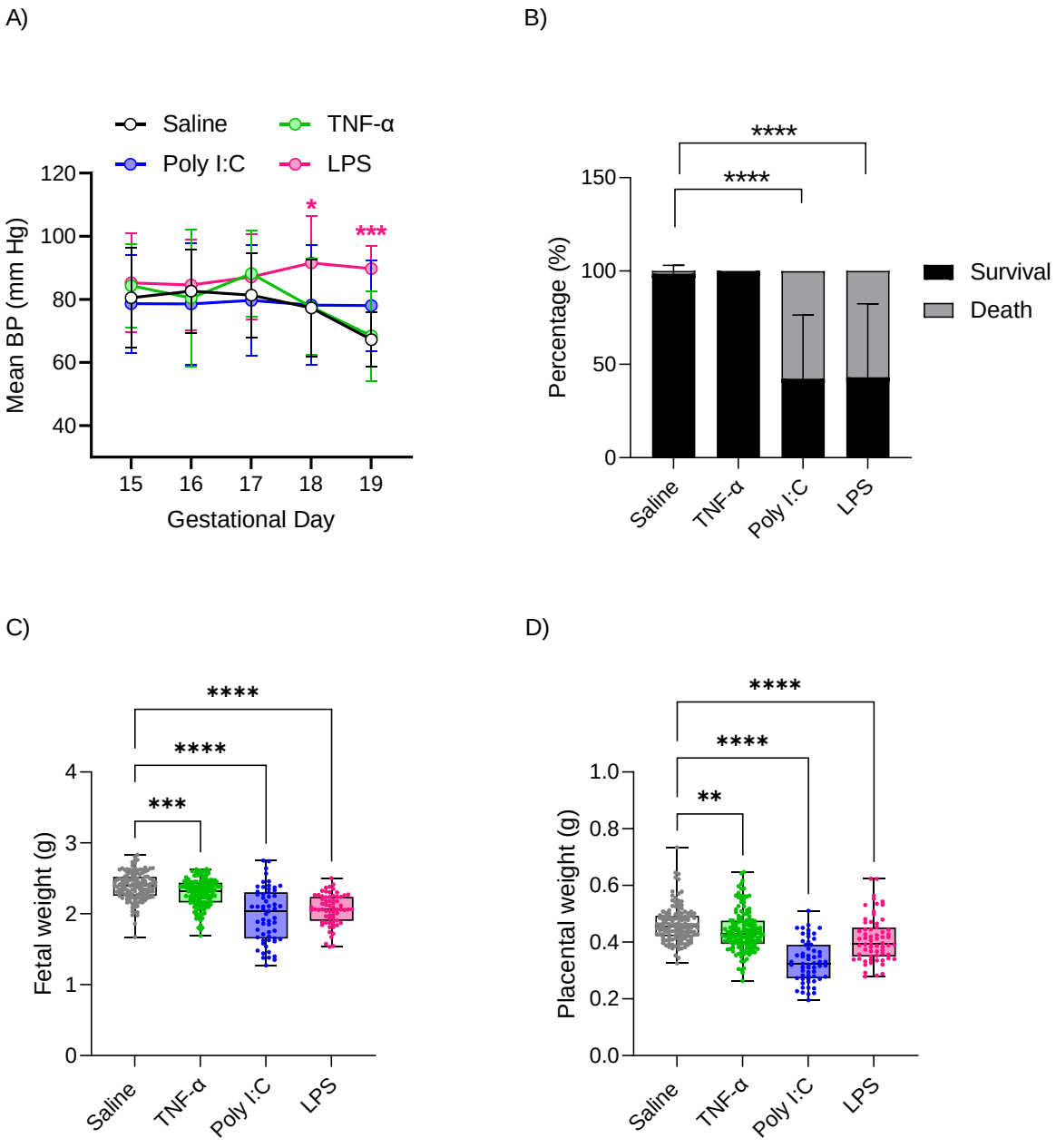

Figure 1: Evaluation of preeclampsia (PE)-like features in TNF-α, Poly I:C and LPS treated rat models of inflammatory PE. A) Mean blood pressure by tail-cuff system measured from gestational day 15-19 in treated TNF-α (green), Poly I:C (blue) and LPS (pink) pregnant rats. n=8 pregnant rats/treatment group B) Average fetal survival (percentage) in each treatment group at GD 19. C-D) Fetal and placenta weight. n=10 litters/treatment group; n=1-16 pups or placentas/litter. *P＜0.05,**P＜0.01,***P＜0.001. Error bar indicates standard deviation (SD). Tumor necrosis factor (TNF-α), Polyinosinic:polycytidylic acid (Poly I:C) and Lipopolysaccharide (LPS).

Placenta morphometry was altered in LPS and Poly I:C treated pregnant rats

H&E-stained placenta cross-sections were used for histomorphology assessments. Placentas from Poly I:C and LPS treatment groups demonstrated a reduced placenta width and depth (figure 2A-C). Interestingly, placentas from the LPS treatment group demonstrated a reduced depth of the labyrinth exchange region of the placenta (figure 2F), whereas placentas from Poly I:C treated rats showed junctional zone depth deficits (figure 2E). TNF-α treatment had no effect on the above measures, while none of the treatments had an effect on the decidual depth (figure 2D).

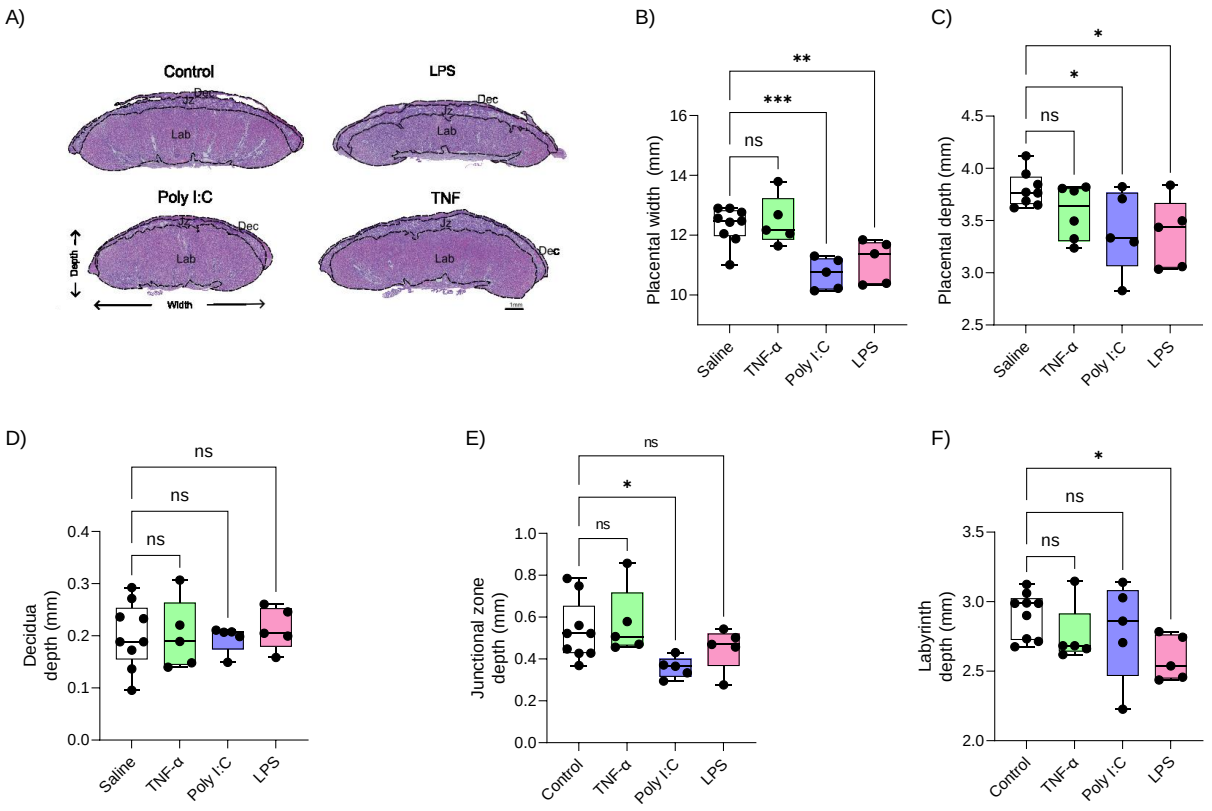

Figure 2: Placenta morphometry in TNF-α, Poly I:C and LPS treated pregnant rats. Representative images of placenta cross-section from saline control (white), TNF-α (green), Poly I:C (blue) and LPS (pink) treatment groups (A). Measurement of placenta width (B), depth (C), decidua (D), junctional zone (E) and labyrinth (F) on Hematoxylin

and eosin (H&E) stained sections. n=5-9 litters/treatment group; n=1 placenta/litter. *P＜0.05,**P＜0.01,***P＜0.001 Error bar indicates standard deviation (SD). Tumor necrosis factor (TNF-α), Polyinosinic:polycytidylic acid (Poly I:C) and Lipopolysaccharide (LPS).

LPS treatment induces TNF-α protein expression and inflammatory gene expression in the placenta

Gene expression patterns associated with TNF-α mediated pro-inflammatory signalling have been described in placentas from patients with immune-driven PE [1, 7]. Thus, we measured placenta TNF-α protein levels by performing an ELISA. We found that only LPS treated rats had significantly higher placenta TNF-α protein levels when compared to our saline control (figure 3A). We further measured placenta gene expression for a set of pro- inflammatory and growth factor mediators (3B-H). Only the placentas from the LPS treatment model demonstrated a pattern of gene expression consistent with pro- inflammatory signalling, including elevated expression of IFN-γ, PIGF, CCL2 and iNOS (figure 3B-H). Whereas Poly I:C treated placentas had significantly higher gene expression of ICAM-1 (figure 3E).

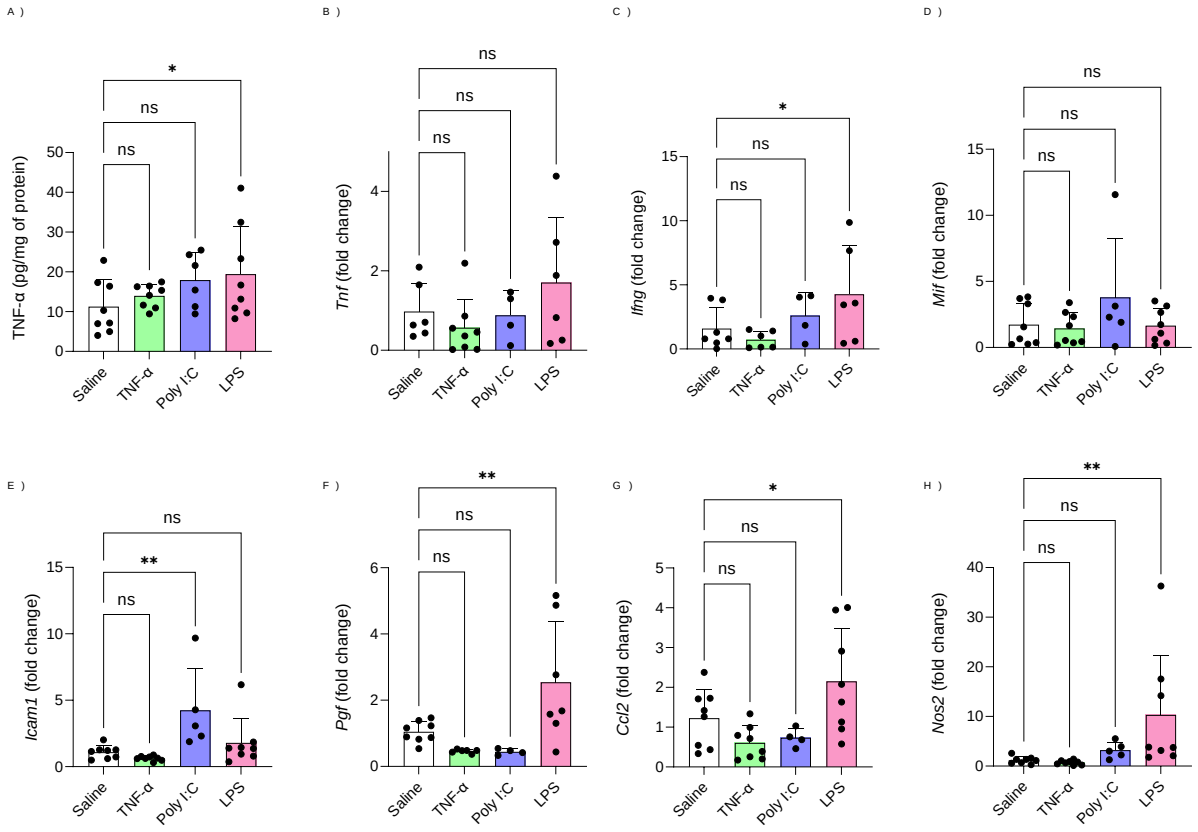

Figure 3: Effect of TNF-α, Poly I:C and LPS treatment in mediating placenta inflammation. A) Measurement of placenta TNF-α protein by ELISA. B-H) Gene expression of *TNF-α, IFN-γ, Mif, ICAM-1, iNOS, PGF, CCL2,* and *NOS2* (iNOS) by qPCR. n=4-8 litters/treatment group; n=1-16 pooled placenta/litter. *P＜0.05 and **P＜0.01. Error bar indicates standard deviation (SD). Tumor necrosis factor (TNF-α), Polyinosinic:polycytidylic acid (Poly I:C) and Lipopolysaccharide (LPS).

Placenta mitochondrial function and NAD(H) redox status were most adversely impacted by LPS treatment in pregnant rats

Oxygen respiration rates of isolated placenta mitochondria were measured and compared across all treatment models, given that metabolic dysfunction is a signature of placenta dysfunction in human PE [11-13]. Consistent with the ability of LPS treatment to replicate multiple phenotypes of inflammation-mediated PE, this model also demonstrated decreased placenta state-III respiration or oxygen consumption rates

through mitochondrial complex-I and complex-II. TNF-α treatment showed a moderate decrease in complex-I driven state-III respiration or oxygen consumption rates and Poly I:C treatment did not affect placenta mitochondrial respiration at all (figure 4A-B). Mitochondrial complex-IV driven oxygen consumption rates did not change for any of the treatment groups (figure 4C). We also determined the ratio and content of placenta NAD^+ and NADH, as altered levels NAD(H) redox status is strong a marker of mitochondrial dysfunction and altered cellular redox status [14, 15, 18]. Placentas from LPS treated rats showed decreased NAD^+ content and NAD^+/NADH ratio compared to saline control placentas (figure 4D-F). Poly I:C treatment only exhibited a decrease in placenta NAD^+ levels, while TNF-α treatment had no effect.

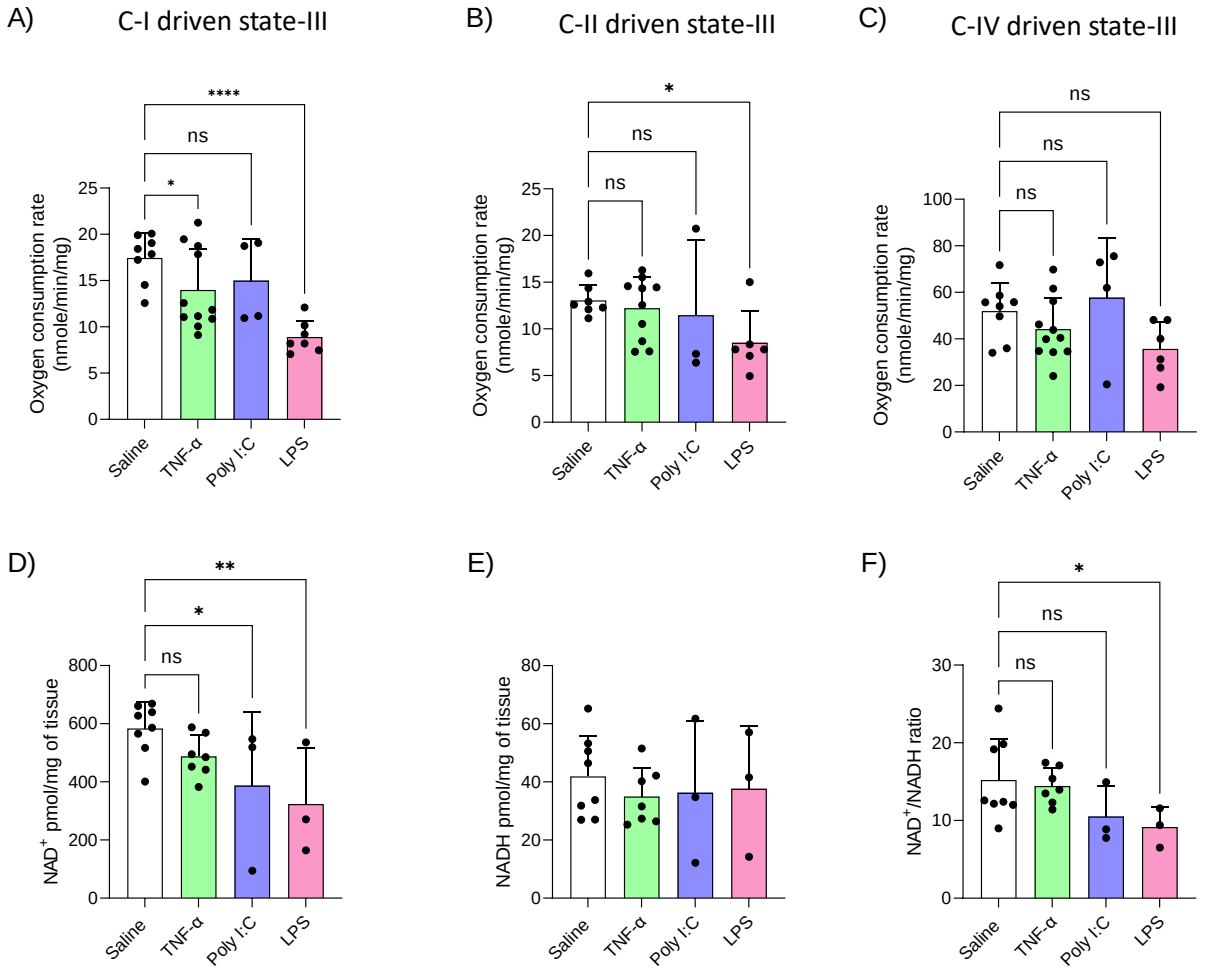

Figure 4: Placenta mitochondrial function in TNF-α, Poly I:C and LPS treated pregnant rats. A-C) Mitochondrial oxygen consumption rates were measured by Oxygraph system. Mitochondrial complex-I, II and IV driven state-III respiration or oxygen consumption rates were determined**.** n=4-9 litters/treatment group, n=6-8 placentas pooled/litter. *P＜0.05,**P＜0.01,***P＜0.001. D-F) Placenta NAD^+/NADH levels were measured and their ratio was determined. n=3-8 litters/treatment group, n=1 placenta/litter. *P＜0.05 and **P＜0.01. Error bar indicates standard deviation (SD). Tumor necrosis factor (TNF-α), Polyinosinic:polycytidylic acid (Poly I:C) and Lipopolysaccharide (LPS).

4.4 Discussion

The current study was undertaken to evaluate the utility of three previously described immune-mediated rodent models of PE [29, 35, 39, 42], determining which of these models may be best suited for future investigations of the inflammatory PE subclass described recently in human populations [1, 7]. Collectively, the PE-like model established by daily IP injections of LPS from GD 13-18 most closely mimicked clinical and pathological findings described in human cases of inflammatory PE, including the establishment of maternal hypertension, evidence of fetal growth restriction and morphometric and functional evidence of inflammation-mediated placenta dysfunction. Conversely, the other two models examined (Poly I:C injection and TNF-α infusion) demonstrated some, but not all, key features attributed to this unique PE subclass and as such may not be as well-suited for future investigations of inflammatory-driven PE.

Maternal hypertension is the keystone finding of PE and is a necessary feature for any PE-like rodent model. In our study, only LPS treated pregnant rats showed elevations in maternal BP across pregnancy (figure 1A). Our data suggests that LPS treatment led to induction of several pro-inflammatory mediators, such as TNF-α, IFN-ɣ, PGF, CCL2 and iNOS – all critical for sustaining inflammatory signalling (figure 3A, 3C, 3F-H) [46, 48-50, 55-57]. We think that together, such a robust inflammatory stimulus may significantly contribute to maternal endothelial dysfunction, resulting in hypertension [32, 50, 55-58]. On the other hand, Poly I:C treatment showed a non-significant trend towards increased BP on GD 19. This is aligned with our finding that Poly I:C treatment could only increase the transcript levels of ICAM-1 but not the other pro-inflammatory genes tested (figure 3).

The TNF-α treatment had no effect on maternal BP when measured via tail cuff (figure 1A). Previous reports describe an induction of hypertension in this rat model – using the same dosage (50ng/day/animal) and exposure time of the current study. To confirm the discrepancy in results, we performed radio-telemetry on an additional subset of TNF-α-treated pregnant rats, and controls. In line with our tail-cuff data, BP measurements via radio-telemetry likewise demonstrated no significant increase in BP with TNF-α infusion (supplementary figure 1). When looking at placenta genes expression for markers of pro- inflammatory signalling in the TNF-α treated rats, surprisingly no evidence of placental inflammation was observed, indicating that this treatment was insufficient to illicit inflammation-mediated placental dysfunction or systemic hypertension (figure 3). Treatment with higher concentrations of TNF-α may be needed in order to prompt/initiate this type of pathology.

The placental dysfunction established in human cases of inflammatory PE profoundly impacts fetal and placenta growth trajectories, more so than observed in the other two described PE subclasses [1]. Both the Poly I:C and LPS rodent models tested here likewise demonstrated evidence of fetal and placental growth restriction, while TNF-α treatment had minimal effect on these outcomes (figure 1C-D). Due to the degree of growth restriction observed, it is unsurprising that fetal survival was also decreased in the Poly I:C and LPS models (figure 1B) [29, 30, 32, 35, 36, 38-40]. When examining the types of placental defects established in these two models, Poly I:C treatment appeared to have the largest impact on the junctional zone (JZ). The JZ produces hormones, growth factors and cytokines, thus, regarded as a critical endocrine region of the rodent placenta. Further, this region of the placenta serves as an energy reserve

through glycogen storage [59]. In several different rodent models, JZ defects have been linked to fetal growth restriction via dysregulated hormone production – particularly those hormones required to support utero-placenta and feto-placenta angiogenesis [60, 61]. In the current study no significant differences in size of the vascularized labyrinth region were observed in the Poly I:C model, however, in-depth morphometric analysis of utero-placenta and feto- placenta vascular structures and/or angiogenic signalling were not specifically evaluated. As such, we cannot rule out this mechanism in the development of fetal growth restriction in this model. It should be noted, however, that deficits in placenta angiogenic and/or vasculogenic pathways have not been reported to date in human cases of inflammatory PE, and therefore this rodent model may not best replicate the placenta deficits observed in human populations. In the tested LPS model, the most prominent placenta defects observed were in the labyrinth exchange region, coupled to increased expression of numerous pro-inflammatory mediators (i.e. TNF-α, CCL2, IFN-ɣ; figure 3). Previously, descriptions of immune cell infiltration into the placental exchange region, and up- regulated placenta expression of similar pro-inflammatory mediators have been demonstrated in rodent models of LPS treatment in pregnancy [62, 63] - findings that closely mimic observations made in human placentas from cases of inflammatory PE [1, 6, 64]. However, to date, very little work has been carried out to clarify region-, or function-specific deficits in placental developmental, and how they may contribute significantly to the observed fetal growth restriction in this rodent model.

Placenta mitochondrial impairments, specifically deficits in mitochondrial respiration, are well described pathological features of PE [11, 12, 65], with our group proposing that

mitochondrial dysfunction may be a common feature across all PE subclasses [13, 65]. As such, any rodent PE model should likewise demonstrate dysregulated mitochondrial function. Through ex vivo measurements of mitochondrial oxygen consumption rates we determined that placentas from the LPS-induced PE model had reduced complex I and II driven state-III respiration, indicative of significant mitochondrial dysfunction considering complex I and II are the two main electron transporters in the mitochondrial ATP synthesis process (figure 4A-B). Interestingly, despite having only minimal observable impacts on placenta and fetal growth, the TNF-α induced PE model demonstrated a moderate decrease in the mitochondrial complex-I driven state-III respiration (figure 4A). Metabolic mitochondrial flexibility may allow for dose- and tissue-dependent activation of compensatory pathways which ensure cellular energy production is maintained in the face of mild-moderate decreases in complex I activity [66-68]. This may be particularly true in tissues like the placenta, which requires widespread functional redundancy to protecting fetal growth and development in the face of different in utero stresses [69, 70]. Alternatively, our findings may point to a TNF-α dose-dependent impact on the respiration rates of different mitochondrial complexes within the placenta [71]. Specifically, the LPS- treatment model demonstrated the highest placenta protein content of TNF-α of all models tested, and likewise demonstrated the most profound deficits in mitochondrial respiration. As such, it is possible that establishment of a rodent TNF-α model using higher doses than those tested here may in fact, be capable of further compromising metabolic activity of the placenta, and therefore may have more profound impacts on fetal and placenta growth profiles.

Ongoing work in our group has also identified dysregulated NAD^+-mediated metabolic signalling and NAD^+ depletion in placentas of human cases of inflammatory PE [14]. Depletion of cellular NAD^+ pools is associated with metabolic impairment and mitochondrial dysfunction [15, 18]. NAD^+ is essential for many redox reactions and is consumed by key metabolic signalling pathways, such as that driven by NAD^+ dependent sirtuin deacetylases that regulate mitochondrial biogenesis and homeostasis [15, 16, 18]. Inflammation-driven activation of NAD^+ consuming enzymes and a subsequent drop in NAD^+ levels have been described in many studies. Such dysregulated NAD^+ metabolism not only affects mitochondrial function but also fosters inflammatory conditions, ultimately causing organ dysfunction [15, 19-22]. As such, methods that help maintain NAD(H) homeostasis with NAD^+ boosting strategies, such as treating with vitamin B3 derivatives [72], presents an attractive therapeutic strategy for chronic inflammatory diseases [15-17, 22]. Our results showed that placentas from LPS treated pregnant rats exhibited a significant decrease in NAD^+ levels, and in the NAD^+/NADH ratio, which correlated to a reduction in mitochondrial respiration at multiple complexes, as a marker of mitochondrial function (figure 4D-F). Poly I:C treatment on the other hand, did not show placenta respiratory deficiency but did demonstrate decreases in placenta NAD^+ levels, suggesting an underlying metabolic imbalance (figure 4D-F) in this model. In the TNF-α model no changes in placenta NAD(H) status were observed, further indicating this may not be the most appropriate model for the study of this distinct human PE subclass.

In conclusion, this study evaluated three inflammatory PE models, aiming to identify a model that may closely recapitulate findings described in human cases of inflammatory

PE. The clinical features and impacts on fetal and placenta development differ significantly across the tested models, with the rodent model of inflammatory PE initiated by daily LPS injections from GD 13-18 deemed most suitable for this purpose. In this model, mothers demonstrated increased BP across pregnancy, evidence of inflammation- mediated placental dysfunction and fetal growth restriction. It is important that future mechanistic or therapeutic intervention studies for PE carried out using rodent models must thoughtfully consider the underlying etiology and pathophysiology they are aiming to study, and ensure they choose a rodent model that most closely mirrors the findings in the patient population of interest.

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References

1. Leavey, K., et al., Unsupervised Placental Gene Expression Profiling Identifies Clinically Relevant Subclasses of Human Preeclampsia. Hypertension, 2016. 68(1): p. 137-47.

2. von Dadelszen, P., L.A. Magee, and J.M. Roberts, Subclassification of preeclampsia. Hypertens Pregnancy, 2003. 22(2): p. 143-8.

3. Redman, C.W., I.L. Sargent, and A.C. Staff, IFPA Senior Award Lecture: making sense of pre-eclampsia - two placental causes of preeclampsia? Placenta, 2014. 35 Suppl: p. S20-5.

4. Myatt, L. and J.M. Roberts, Preeclampsia: Syndrome or Disease? Curr Hypertens Rep, 2015. 17(11): p. 83.

5. Powers, R.W., et al., Low placental growth factor across pregnancy identifies a subset of women with preterm preeclampsia: type 1 versus type 2 preeclampsia? Hypertension, 2012. 60(1): p. 239-46.

6. Benton, S.J., et al., The clinical heterogeneity of preeclampsia is related to both placental gene expression and placental histopathology. Am J Obstet Gynecol, 2018. 219(6): p. 604 e1-604 e25.

7. Leavey, K., S.A. Bainbridge, and B.J. Cox, Large scale aggregate microarray analysis reveals three distinct molecular subclasses of human preeclampsia. PLoS One, 2015. 10(2): p. e0116508.

8. Roberts, J.M., et al., Subtypes of Preeclampsia: Recognition and Determining Clinical Usefulness. Hypertension, 2021. 77(5): p. 1430-1441.

9. McElrath, T.F., et al., Circulating microparticle proteins obtained in the late first trimester predict spontaneous preterm birth at less than 35 weeks' gestation: a panel validation with specific characterization by parity. Am J Obstet Gynecol, 2019. 220(5): p. 488 e1-488 e11.

10. Leavey, K., D. Grynspan, and B.J. Cox, Both "canonical" and "immunological" preeclampsia subtypes demonstrate changes in placental immune cell composition. Placenta, 2019. 83: p. 53-56.

11. Aye, I., et al., Placental energy metabolism in health and disease-significance of development and implications for preeclampsia. Am J Obstet Gynecol, 2022. 226(2S): p. S928-S944.

12. Hebert, J.F. and L. Myatt, Placental mitochondrial dysfunction with metabolic diseases: Therapeutic approaches. Biochim Biophys Acta Mol Basis Dis, 2021. 1867(1): p. 165967.

13. Jahan, F., et al., Placental Mitochondrial Function and Dysfunction in Preeclampsia. Int J Mol Sci, 2023. 24(4).

14. Jahan, F., et al., PARP mediated NAD+ depletion and Placental Dysfunction in Preeclampsia. Placenta, 2021. 112: p. e12.

15. Amjad, S., et al., Role of NAD(+) in regulating cellular and metabolic signaling pathways. Mol Metab, 2021. 49: p. 101195.

16. Srivastava, S., Emerging therapeutic roles for NAD(+) metabolism in mitochondrial and age-related disorders. Clin Transl Med, 2016. 5(1): p. 25.

17. Rajman, L., K. Chwalek, and D.A. Sinclair, Therapeutic Potential of NAD-Boosting Molecules: The In Vivo Evidence. Cell Metab, 2018. 27(3): p. 529-547.

18. Lauritzen, K.H., et al., Instability in NAD(+) metabolism leads to impaired cardiac mitochondrial function and communication. Elife, 2021. 10.

19. Shim, D.W., et al., Intracellular NAD(+) Depletion Confers a Priming Signal for NLRP3 Inflammasome Activation. Front Immunol, 2021. 12: p. 765477.

20. Meyer, T., et al., NAD(+) metabolism drives astrocyte proinflammatory reprogramming in central nervous system autoimmunity. Proc Natl Acad Sci U S A, 2022. 119(35): p. e2211310119.

21. Novak, E., et al., NAD+ depletion in the intestinal epithelium results in mitochondrial dysfunction and influences the pathogenesis of experimental colitis. Inflammatory Bowel Diseases, 2023. 29(Supplement_1): p. S5-S6.

22. Guo, J., et al., Protective effect and mechanism of nicotinamide adenine dinucleotide against optic neuritis in mice with experimental autoimmune encephalomyelitis. Int Immunopharmacol, 2021. 98: p. 107846.

23. Gariani, K., et al., Inhibiting poly ADP-ribosylation increases fatty acid oxidation and protects against fatty liver disease. J Hepatol, 2017. 66(1): p. 132-141.

24. Taylor, E.B. and E.M. George, Animal Models of Preeclampsia: Mechanistic Insights and Promising Therapeutics. Endocrinology, 2022. 163(8).

25. LaMarca, B., et al., Placental Ischemia and Resultant Phenotype in Animal Models of Preeclampsia. Curr Hypertens Rep, 2016. 18(5): p. 38.

26. Roberts, J.M., et al., Sera from preeclamptic women specifically activate human umbilical vein endothelial cells in vitro: morphological and biochemical evidence. Am J Reprod Immunol, 1992. 27(3-4): p. 101-8.

27. Weel, I.C., et al., Association between Placental Lesions, Cytokines and Angiogenic Factors in Pregnant Women with Preeclampsia. PLoS One, 2016. 11(6): p. e0157584.

28. Conrad, K.P. and D.F. Benyo, Placental cytokines and the pathogenesis of preeclampsia. Am J Reprod Immunol, 1997. 37(3): p. 240-9.

29. Alexander, B.T., et al., Tumor necrosis factor-alpha-induced hypertension in pregnant rats results in decreased renal neuronal nitric oxide synthase expression. Am J Hypertens, 2002. 15(2 Pt 1): p. 170-5.

30. Chatterjee, P., et al., Placental Toll-like receptor 3 and Toll-like receptor 7/8 activation contributes to preeclampsia in humans and mice. PLoS One, 2012. 7(7): p. e41884.

31. Song, D.H. and J.O. Lee, Sensing of microbial molecular patterns by Toll-like receptors. Immunol Rev, 2012. 250(1): p. 216-29.

32. Cotechini, T., et al., Inflammation in rat pregnancy inhibits spiral artery remodeling leading to fetal growth restriction and features of preeclampsia. J Exp Med, 2014. 211(1): p. 165-79.

33. Todt, J.C., et al., Effects of tumor necrosis factor-alpha on human trophoblast cell adhesion and motility. Am J Reprod Immunol, 1996. 36(2): p. 65-71.

34. Renaud, S.J., et al., Activated macrophages inhibit human cytotrophoblast invasiveness in vitro. Biol Reprod, 2005. 73(2): p. 237-43.

35. Hu, J., J. Zhang, and B. Zhu, Protective effect of metformin on a rat model of lipopolysaccharide-induced preeclampsia. Fundam Clin Pharmacol, 2019. 33(6): p. 649-658.

36. Xue, P., et al., Single administration of ultra-low-dose lipopolysaccharide in rat early pregnancy induces TLR4 activation in the placenta contributing to preeclampsia. PLoS One, 2015. 10(4): p. e0124001.

37. Faas, M.M., et al., Glomerular inflammation in pregnant rats after infusion of low dose endotoxin. An immunohistological study in experimental pre-eclampsia. Am J Pathol, 1995. 147(5): p. 1510-8.

38. Faas, M.M., et al., A new animal model for human preeclampsia: ultra-low-dose endotoxin infusion in pregnant rats. Am J Obstet Gynecol, 1994. 171(1): p. 158- 64.

39. Tinsley, J.H., et al., Toll-like receptor 3 activation during pregnancy elicits preeclampsia-like symptoms in rats. Am J Hypertens, 2009. 22(12): p. 1314-9.

40. Chatterjee, P., et al., Cotreatment with interleukin 4 and interleukin 10 modulates immune cells and prevents hypertension in pregnant mice. Am J Hypertens, 2015. 28(1): p. 135-42.

41. Cora, M.C., L. Kooistra, and G. Travlos, Vaginal Cytology of the Laboratory Rat and Mouse: Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears. Toxicol Pathol, 2015. 43(6): p. 776-93.

42. LaMarca, B.B., et al., Role of endothelin in mediating tumor necrosis factor-induced hypertension in pregnant rats. Hypertension, 2005. 46(1): p. 82-6.

43. Hou, S., A. Blesch, and P. Lu, A radio-telemetric system to monitor cardiovascular function in rats with spinal cord transection and embryonic neural stem cell grafts. J Vis Exp, 2014(92): p. e51914.

44. Juliano, P.B., M.H. Blotta, and A.M. Altemani, ICAM-1 is overexpressed by villous trophoblasts in placentitis. Placenta, 2006. 27(6-7): p. 750-7.

45. Shi, C. and E.G. Pamer, Monocyte recruitment during infection and inflammation.

Nat Rev Immunol, 2011. 11(11): p. 762-74.

46. Rao, R.M., et al., Endothelial-dependent mechanisms of leukocyte recruitment to the vascular wall. Circ Res, 2007. 101(3): p. 234-47.

47. Bui, T.M., H.L. Wiesolek, and R. Sumagin, ICAM-1: A master regulator of cellular responses in inflammation, injury resolution, and tumorigenesis. J Leukoc Biol, 2020. 108(3): p. 787-799.

48. Kang, M., et al., Placental growth factor (PlGF) is linked to inflammation and metabolic disorders in mice with diet-induced obesity. Endocr J, 2018. 65(4): p. 437-447.

49. Albonici, L., et al., PlGF Immunological Impact during Pregnancy. Int J Mol Sci, 2020. 21(22).

50. Vishnyakova, P., et al., Role of the Monocyte-Macrophage System in Normal Pregnancy and Preeclampsia. Int J Mol Sci, 2019. 20(15).

51. Frezza, C., S. Cipolat, and L. Scorrano, Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. Nat Protoc, 2007. 2(2): p. 287-95.

52. Kishikawa, J.I., et al., General anesthetics cause mitochondrial dysfunction and reduction of intracellular ATP levels. PLoS One, 2018. 13(1): p. e0190213.

53. Kuznetsov, A.V., et al., Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. Nat Protoc, 2008. 3(6): p. 965-76.

54. Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. Obstet Gynecol, 2013. 122(5): p. 1122-1131.

55. Carvalho-Galvao, A., et al., Central Inhibition of Tumor Necrosis Factor Alpha Reduces Hypertension by Attenuating Oxidative Stress in the Rostral Ventrolateral Medulla in Renovascular Hypertensive Rats. Front Physiol, 2019. 10: p. 491.

56. Benson, L.N., et al., IFN-gamma Contributes to the Immune Mechanisms of Hypertension. Kidney360, 2022. 3(12): p. 2164-2173.

57. da Silva, G.M., et al., Nitric Oxide as a Central Molecule in Hypertension: Focus on the Vasorelaxant Activity of New Nitric Oxide Donors. Biology (Basel), 2021. 10(10).

58. Peracoli, J.C., M.V. Rudge, and M.T. Peracoli, Tumor necrosis factor-alpha in gestation and puerperium of women with gestational hypertension and pre- eclampsia. Am J Reprod Immunol, 2007. 57(3): p. 177-85.

59. Woods, L., V. Perez-Garcia, and M. Hemberger, Regulation of Placental Development and Its Impact on Fetal Growth-New Insights From Mouse Models. Front Endocrinol (Lausanne), 2018. 9: p. 570.

60. Tunster, S.J., M. Van de Pette, and R.M. John, Impact of genetic background on placental glycogen storage in mice. Placenta, 2012. 33(2): p. 124-7.

61. Salas, M., et al., Placental growth retardation due to loss of imprinting of Phlda2.

Mech Dev, 2004. 121(10): p. 1199-210.

62. Chen, Y.H., et al., Vitamin D3 inhibits lipopolysaccharide-induced placental inflammation through reinforcing interaction between vitamin D receptor and nuclear factor kappa B p65 subunit. Sci Rep, 2015. 5: p. 10871.

63. Zhao, M., et al., Folic acid protects against lipopolysaccharide-induced preterm delivery and intrauterine growth restriction through its anti-inflammatory effect in mice. PLoS One, 2013. 8(12): p. e82713.

64. Robineau-Charette, P., et al., Fibrinogen-Like Protein 2-Associated Transcriptional and Histopathological Features of Immunological Preeclampsia. Hypertension, 2020. 76(3): p. 910-921.

65. Jahan, F.V., G.; Green, A.E.; Bainbridge, S.A.; Menzies, K.J., Placental Mitochondrial Function and Dysfunction in Preeclampsia. Preprints 2023, (2023010538).

66. Zielinski, L.P., et al., Metabolic flexibility of mitochondrial respiratory chain disorders predicted by computer modelling. Mitochondrion, 2016. 31: p. 45-55.

67. Lan, J., et al., Discovery of Mitochondrial Complex I Inhibitors as Anticancer and Radiosensitizer Drugs Based on Compensatory Stimulation of Lactate Release. Cancers (Basel), 2022. 14(21).

68. Garmier, M., et al., Complex I dysfunction redirects cellular and mitochondrial metabolism in Arabidopsis. Plant Physiol, 2008. 148(3): p. 1324-41.

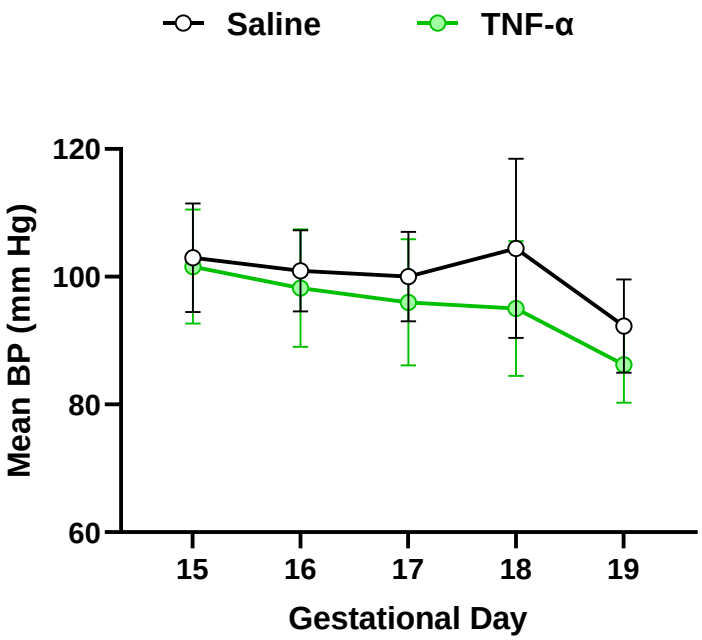
69. Sferruzzi-Perri, A.N., J. Lopez-Tello, and E. Salazar-Petres, Placental adaptations supporting fetal growth during normal and adverse gestational environments. Exp Physiol, 2023. 108(3): p. 371-397.

70. Foster, C.S.P., et al., Different Genes are Recruited During Convergent Evolution of Pregnancy and the Placenta. Mol Biol Evol, 2022. 39(4).

71. Mariappan, N., et al., TNF-induced mitochondrial damage: a link between mitochondrial complex I activity and left ventricular dysfunction. Free Radic Biol Med, 2009. 46(4): p. 462-70.

72. Montllor-Albalate, C., Z. Song, and D. Chen, The therapeutic promises of NAD(+) boosters. Cell Metab, 2021. 33(7): p. 1274-1275.

Supplementary 1



Supplementary Figure 1: Blood pressure measurement in TNF-α treated pregnant rats by telemetry. Mean blood pressure was measured from gestational day 15-19. n=4 pregnant rats/treatment group. Error bar indicates standard deviation (SD). Tumor necrosis factor (TNF-α)

Supplementary 2

Table 1: Primers of inflammation markers

TNF

NM_012675.3 https://doi.org/10.1186/s12974-015-0248-1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GCAGATGGGCTGTACCTTATC	Plus	21	535	555	58.23	52.38	4.00	2.00

Reverse primer	GGCTGACTTTCTCCTGGTATG	Minus	21	655	635	58.09	52.38	3.00	0.00
Product length	121bp								

IFN-g

NM_138880.3

	Sequence (5'->3')	**Template strand**	**Length**	**Tm**	**GC %**	**Self complementarity**	**Self 3' complementarity**
Forward primer	TGTTACTGCCAAGGCACACT	Plus	20	59.82	50.00	6.00	1.00
Reverse primer	ACCGTCCTTTTGCCAGTTCC	Minus	20	60.82	55.00	3.00	0.00
Product length	129bp						

MIF

NM_031051.2

	Sequence (5'->3')	**Template strand**	**Length**	**Start**	**Stop**	**Tm**	**GC%**	**Self complementarity**	**Self 3' complementarity**
Forward primer	TCACGTAGCTCAGGTCCCAG	Plus	20	4	23	60.97	60.00	4.00	2.00
Reverse primer	ACTGCGATGTACTGTGCCG	Minus	19	203	185	60.45	57.89	4.00	2.00
Product length	200bp								
Exon junction	192/193 (reverse primer) on template NM_031051.2								

PIGF

NM_053595.2

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GAGGAACCCCACCTGTGATG	Plus	20	764	783	60.04	60.00	4.00	2.00
Reverse primer	CTTGTCCTTCCATGCCCCTT	Minus	20	956	937	59.96	55.00	4.00	0.00
Product length	193								
Exon junction	777/778 (forward primer) on template NM_053595.2								

iNOS

NM_012611.3 https://www.jimmunol.org/content/jimmunol/192/12/6009.full.pdf?with-ds=yes

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TTCCTCAGGCTTGGGTCTTGT	Plus	21	104	124	61.33	52.38	5.00	0.00
Reverse primer	GGCAAGCCATGTCTGTGACTT	Minus	21	200	180	61.16	52.38	6.00	3.00
Product length	97								

MCP-1/CCL2

NM_031530.1 https://d-nb.info/1220602175/34

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TGTTCACAGTTGCTGCCTGT	Plus	20	107	126	60.39	50.00	4.00	2.00
Reverse primer	CGACTCATTGGGATCATCT	Minus	19	215	197	54.03	47.37	5.00	3.00

Product length	109bp

ICAM-1

NM_012967.1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CACCATCACTGTGTATTCGTTC	Plus	22	345	366	57.42	45.45	5.00	0.00
Reverse primer	CTACTGAGAGCTGTGTCCGC	Minus	20	475	456	60.18	60.00	4.00	2.00
Product length	131								
Exon junction	361/362 (forward primer) on template NM_012967.1								

Bactin

NM_031144.3

https://patentimages.storage.googleapis.com/9e/06/cf/6c6cde27b1c952/US20160256514A1.pdf pag.36

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CTGTGTGGATTGGTGGCTCT	Plus	20	1091	1110	59.96	55.00	2.00	0.00
Reverse primer	GCTCAGTAACAGTCCGCCTA	Minus	20	1223	1204	59.18	55.00	3.00	2.00
Product length	133bp								

Chapter 5 - NAD^+ depletion and altered mitochondrial function are key to the establishment of placental dysfunction in an inflammatory-driven subclass of preeclampsia.

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Statement of Contribution

F.J. contributed to conceptualization, data collection and analysis, writing, reviewing, and editing.

One sentence summary: Boosting NAD^+ prevents inflammatory-driven preeclampsia by improving placental mitochondrial function.

Abstract

Preeclampsia (PE) is a pregnancy associated hypertensive disease. It is one of the major causes of pregnancy-related maternal/perinatal adverse health outcomes, with a lack of highly effective preventative strategies and/or therapeutic interventions. Our group has previously identified distinct subclasses of pathophysiology underlying a PE diagnosis, one of which exhibits heightened immune activation at the maternal-fetal fetal interface, identified as Inflammatory-driven PE. In non-pregnant populations, chronic inflammation is associated with reduced cellular availability of NAD^+, a vitamin B3-derived metabolite involved in energy metabolism and mitochondrial function. Interestingly, specifically in placentas from women with inflammatory-driven PE, we observed increased activity of NAD^+-consuming PARP enzymes and reduced NAD^+ content. Moreover, these placentas had decreased expression of several mitochondrial oxidative phosphorylation (OXPHOS) proteins and evidence of oxidative damage. This human data was supported by cell culture findings, which likewise demonstrated increased PARP activity, coupled to decreased mitochondrial respiration rates and decreased invasive function of cultured HTR8 human trophoblast cells, following inflammatory induction by TNF-α. Importantly, these adverse inflammatory effects were attenuated by boosting cellular NAD^+ levels with nicotinamide riboside (NR). Finally, using an LPS-induced rodent model of inflammatory-driven PE, we demonstrated that NR administration (200mg/kg/day) from gestational day (GD) 1-19 could prevent the development of maternal hypertension and fetal/placental growth restriction, improve

placental mitochondrial function, reduce placental inflammation and oxidative stress. Thus, this study demonstrates the critical role of NAD^+ metabolism in maintaining healthy placental function and identifies NAD^+ boosting as a promising preventative strategy for the inflammatory-driven subclass of PE.

5.1 Introduction

Preeclampsia (PE) is a complex hypertensive disorder that occurs during pregnancy (at ≥20 weeks of gestation) [1]. The disease affects almost 10 million women each year, making it one of the major causes of mortality and morbidity in the mother and baby [1]. Unfortunately, there is no cure for PE. Removal of the affected organ, the placenta, is the only 'cure', often resulting in premature delivery and associated iatrogenic outcomes [2]. Historically, PE has been studied as a singular disease. However, there is a considerable heterogeneity in the PE patient population based on the clinical profile, onset of disease, pregnancy outcome, fetal growth impacts and placental histopathology. It is now acknowledged that PE likely develops because of several distinct underlying etiologies [3-7]. Our group has previously characterized three different subclasses of PE pathophysiology using a multiscale profiling approach that incorporated gestational- parent/fetal clinical features and detailed placental profiling (molecular and histological). Specifically identified subclasses of PE disease included: 1) Parental-driven PE (PE1) – likely the result of underlying gestational parent predisposing factors (involving minimal placental pathology); 2) Hypoxia-driven PE (PE2) – resulting from placental ischemia- reperfusion injury (~ 60% of the PE patient population); and 3) Inflammation-driven PE (PE3) – resulting from aberrant inflammation at the gestational parent-fetal interface [3, 4]. Existence of such disease subclasses

necessitates the requirement for studies focused on better understanding subclass-specific disease processes and/or subclass-specific interventions. To date, most research on PE has focused almost exclusively on understanding the hypoxia-driven pathophysiology of PE, while the etiological relevance of parental factors and/or inflammatory processes have been given less attention. The current study aims to address this gap in knowledge, specifically focusing on better understanding placental dysfunction associated with inflammation-driven PE, and subclass-specific therapeutic interventions for this unique patient population.

Placental mitochondrial dysfunction has long been recognized as a key component of PE pathophysiology [8-12]. Altered placental mitochondrial respiration and/or total content is observed in tissues collected from pregnancies that span the clinical spectrum of PE, associated with altered oxidative phosphorylation (OXPHOS) protein expression, mitochondrial structural damage, defects in fusion/fission dynamics and increased oxidative stress [8, 13-15]. There is strong evidence to suggest that placental mitochondrial dysfunction may be a common feature across all PE subclasses [8], however a lack of subclass-specific research endeavors has limited our current understanding of this pathology, specifically in the context of inflammation-driven PE. In non-pregnant populations, several inflammatory disease conditions have been tightly linked to mitochondrial dysfunction, in large part driven by profound overactivity of nicotinamide adenine dinucleotide (NAD^+) consuming enzymes, leading to depletion of intracellular NAD^+ stores [16-28]. In the current study, this body of work will be expanded to determine if NAD^+ depletion may likewise initiate placental mitochondrial dysfunction in inflammation-mediated PE.

NAD^+, a vitamin B3 redox metabolite, is a critical coenzyme central to metabolic function throughout the body [29-31]. NAD^+ maintains mitochondrial health by regulating energy metabolism and by inducing pathways, such as the mitochondrial unfolded protein response [29-31]. Enzymes including poly(ADPribose) polymerases (PARP), Sirtuins (SIRT), cluster of differentiation 38/157 (CD38/CD157), and sterile alpha and toll/interleukin-1 receptor motif-containing protein 1 (SARM1), require NAD^+ to carry out their enzymatic functions [19, 29-31]. Among these, two of the most well-described NAD^+- consuming enzyme families are the PARPs and the SIRTs. A primary function of PARPs is the post-translational modification of proteins, via the covalent addition of poly- adenosinediphosphate (ADP)-ribose polymers to various proteins – a process known as PARylation. This NAD^+-dependant process helps to regulate chromatin organization, DNA repair, cellular replication, glycolysis and inflammation processes [32-35]. SIRTs, on the other hand, are known to be vital for mitochondrial metabolism, biogenesis, and ROS detoxification [19, 29-31]. Under normal conditions, cellular NAD+ content is tightly regulated, achieving an optimal balance between NAD^+ biosynthesis/salvage and NAD^+ consumption pathways. However, under proinflammatory conditions some of the major NAD^+ consumers, such as PARPs, become highly activated, tilting the scales of NAD^+ homeostasis towards excessive consumption and quickly deplete intracellular NAD^+ stores, leading to profound mitochondrial damage and impaired energy metabolism [16- 28, 30, 36]. Interestingly, in a mouse model of hypoxia-driven PE, replenishment of whole- body NAD+ stores via nicotinamide (NAM) treatment has been shown to rescue several PE-like features (i.e., hypertension, fetal growth restriction) [37, 38]. However, the role of placenta-specific

NAD^+ depletion in the pathogenesis of PE and whether supplementation with an NAD^+ precursor will be an attractive preventative strategy for other PE subclasses is unknown.

In the current study, the potential role(s) of inflammation-mediated NAD^+ depletion and subsequent mitochondrial dysfunction in the establishment of placental disease in PE were explored using complementary studies on human placenta tissues, a human trophoblast cell culture model and in a rodent model of inflammatory-driven PE. Further, the therapeutic potential of NAD^+ boosting, via NR treatment, on placental health, fetal growth and clinical manifestations of inflammation-mediated PE were evaluated.

5.2 Results

Human placenta tissues from inflammation-driven PE demonstrate evidence of PARP-mediated NAD^+ depletion and mitochondrial dysfunction.

Previous gene set enrichment carried out by our group using a genome-wide microarray dataset from human placenta samples (Table 1) annotated according to PE subclass, identified an overexpression of several pro-inflammatory signaling pathways uniquely within one PE subclass – labelled inflammation-mediated PE (PE3) [3, 4]. Using this same dataset, here we show that this pro-inflammatory gene expression profile is coupled to elevated expression of several PARP transcripts uniquely in these same PE3 cases, findings not observed in the placentas from the other PE subclasses (PE1 and PE2; Fig 1A). PARP hyperactivation has been widely described in non-pregnant inflammatory conditions, leading to excess protein PARylation and NAD^+ depletion [16-28, 30, 36]. As such the degree of protein PARylation was assessed in human placentas collected from each of the three PE subclasses, along with term, preterm and chronic

hypertension controls. Global protein PARylation was also found to be uniquely higher in the placentas from PE3, compared to other PE subclasses and all control groups (Fig 1B-C). In parallel, LC/MS measurements demonstrated significant depletion of NAD^+ and total NAD(H) in these placentas, compared to control preterm and term placentas (Fig 1D-E). However, there was no significant reduction in the NAD^+ and total NAD(H) content in the other PE subclasses (PE1 and PE2) or in the chronic hypertension groups (Fig 1D-E). NADH levels alone, NAD^+/NADH ratio and NAM, an NAD^+ related metabolite, on the other hand, did not change in any of the groups (Fig 1F-G, Supplementary Fig 1A).

Placental mitochondrial dysfunction and oxidative stress have been described as key contributors to placental dysfunction in PE when considered as a single clinical disease [8-12]. Thus, we aimed to determine if a reduced NAD^+ pool in PE3 placentas may be associated with placenta mitochondrial dysfunction and/or oxidative stress. We found no indications of altered placental mitochondrial content between any of the three PE subclasses and all control groups when using citrate synthase expression and mitochondrial complex IV (COX-IV) activity assays as surrogates of mitochondrial content [39, 40] (Supplementary Fig 1B-C). However, using a proteomic approach on a subset placenta samples, numerous mitochondrial proteins were found to be down-regulated in inflammation-mediated PE (PE3) placentas compared to controls, including: subunits of OXPHOS protein complexes [NADH:Ubiquinone Oxidoreductase Subunit V3 (NDUFV3), Ubiquinol-Cytochrome C Reductase Core Protein 1 (UQCRC1), UQCR Rieske Iron-Sulfur Polypeptide 1(UQCRS1)], and proteins that effect mitochondrial dynamics [Optic atrophy type 1 (OPA1)], mitochondrial import (TOMM7),

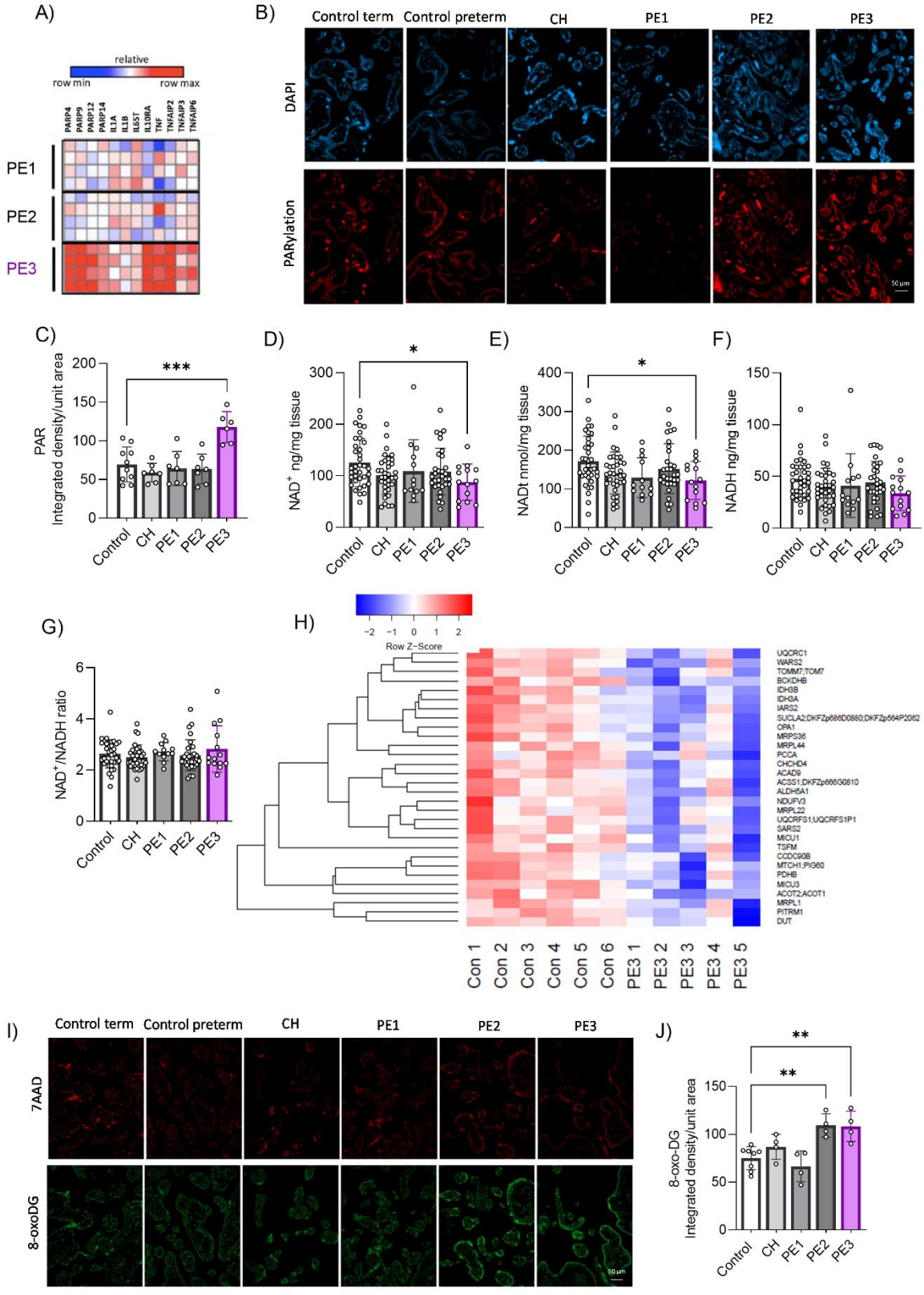
mitochondrial translation (mitochondrial ribosomal proteins, MRPS36, MRPL22, MRPL1), and others (Fig 1H). Placenta samples from cases of hypoxia-drive PE (PE2) likewise demonstrated a decrease in some mitochondrial proteins compared to controls, such as OXPHOS protein- ATP synthase membrane subunit f (ATP5J2), mitochondrial translation elongation factor (TSFM), MRPL22 etc. (Supplementary Fig 1D). However, placentas from parental-driven PE (PE1) did not show any changes in mitochondrial protein profiles compared to healthy controls.

Mitochondrial impairment is thought to be linked to oxidative stress and placental dysfunction in PE [8-12], however this relationship has not been explicitly examined in a subclass-specific manner. For this purpose, we performed 8-Oxo-2'-deoxyguanosine (8-oxo-DG) staining - detecting oxidized guanine residues in DNA [41] - of placenta sections collected from all three PE subclasses. Interestingly, both hypoxia-driven PE (PE2) and inflammation-driven PE (PE3) exhibited increased levels of oxidized nucleotides, when compared to parental-driven PE (PE1) and controls (Fig 1I-J).

Table 1: Study population demographics

	Control (Preterm and term) (n=43)	Chronic hyper-tension (n=23)	PE1 (n=16)	PE2 (n=42)	PE3 (n=17)
Maternal Age[a]	31.4±4.7	36.1±4.6[c]	32.2±5.4	33.1±6.3	31.4±4.2
Gestational Age at Delivery (weeks)[a]	34.1±5.3	33.2±4	35±3.1	31±3.1[c]	33.3±2.80
Female Fetal Sex (%)[b]	21/43 (48.8)	14/23 (60.9)	7/16 (43.8)	23/42 (54.8)	7/16 (43.8)
Birthweight (g)[a]	2481.3±1115.09	1649.8±867.7[d]	2292.2±813.1	1290.6±611.1[e]	1707.7±602.7[c]
Birthweight Centile <10th Percentile (%)[b]	2/43 (4.7)	15/23 (65.2)	5/16 (31.3)	30/42 (71.4)	10/17 (58.8)
Placental Weight (g)[a]	541.09±180.4	344.1±164.8[e]	454.7±133.5	312.3±128.2[e]	372.7±147.1[d]
Placental Weight Centile <10th Percentile (%)[b]	0/43 (0)	9/23 (39.1)	1/16 (6.3)	15/42 (35.7)	4/16 (25)
Maternal Systolic Blood Pressure (mmHg)[a]	118.4±15.4	162.8±19.6[e]	158.3±15[e]	162±16.9[e]	150.7± 15.6[e]
Maternal Diastolic Blood Pressure (mmHg)[a]	71.9±11.6	101±11.6 [e]	100.7±7.8 [e]	103.7±12.8[e]	95.8±11.3[e]
C-section Mode of Delivery (%)[b]	21/43 (48.8)	20/23 (87)	9/16 (56.3)	40/42 (95.2)	16/17 (94.1)

[a] For mean ± standard deviation; [b] for number of pregnancies out of total number per group; [c] for p value <0.01; [d] for p value <0.001 and [e] for p value <0.0001


A)
relative
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PARP4
PARP9
PARP12
PARP14
IL1A
IL1B
IL6ST
IL10RA
TNF
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TNFAIP3
TNFAIP6
PE1
PE2
PE3
B)
Control term
Control preterm
CH
PE1
PE2
PE3
DAPI
PARylation
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Integrated density/unit area
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Control
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PE1
PE2
PE3
D)
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NAD+ ng/mg tissue
E)
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NADt nmol/mg tissue
F)
NADH ng/mg tissue
G)
NAD+/NADH ratio
H)
Row Z-Score
UQCRC1
WARS2
TOMM7;TOM7
BCKDHB
IDH3B
IDH3A
IARS2
SUCLA2;DKFZp686D0880;DKFZp564P2062
OPA1
MRPS36
MRPL44
PCCA
CHCHD4
ACAD9
ACSS1;DKFZp666G0810
ALDH5A1
NDUFV3
MRPL22
UQCRFS1;UQCRFS1P1
SARS2
MICU1
TSFM
CCDC90B
MTCH1;PIG60
PDHB
MICU3
ACOT2;ACOT1
MRPL1
PITRM1
DUT
Con 1
Con 2
Con 3
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Con 5
Con 6
PE3 1
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PE3 4
PE3 5
I)
Control term
Control preterm
CH
PE1
PE2
PE3
7AAD
8-oxoDG
50 µm
J)
**
**
8-oxo-DG
Integrated density/unit area

Figure 1. Identification of altered NAD^+/PARP-signalling, mitochondrial proteomes and oxidative damage in human placentas with inflammation-driven PE (PE3). A) Human placenta gene expression of inflammatory markers and NAD^+ consuming enzymes in human placenta biopsies from all three PE subclasses (n=4 placentas/group), B-C) Representative immunofluorescence images and quantification of protein PARylation in human placenta tissue sections from all three PE subclasses and various control groups (n=7-8 placentas/group). D-G) LC/MS quantification of human placental NAD(H) levels (n=14-36 placentas/group). H) Heatmap of downregulated mitochondrial proteins in inflammatory PE3 (term) placentas compared to control term placentas (n=5-6 per group). Shown are only proteins that met the False Discovery Rare (FDR) threshold of 0.05. I-J) Placenta oxidative stress was determined by immunofluorescence staining of human placenta tissue sections with anti-8 oxoDG antibody and quantification by integrated intensity/area of trophoblasts (n=4 per group). One-way ANOVA with Holm-Šídák's multiple comparisons test *P＜0.05 and **P＜0.01. Error bar indicates standard deviation (SD). Control= control term and preterm, chronic hypertension control= (CH), Gestational parent-driven PE subclass = PE1, Hypoxia-driven PE subclass = PE2, Inflammation-driven PE subclass = PE3.

Boosting NAD^+ decreases protein PARylation and prevents human trophoblast cell dysfunction under inflammatory culture conditions.

To examine if inflammation-mediated NAD^+ depletion alters human trophoblast health and function, an TNF-α induced inflammatory insult was applied to the SVneo/HTR8 trophoblast cell culture model – representative of the invasive extravillous cytotrophoblast population, long considered a key trophoblast population contributing to the establishment of PE pathophysiology [42-45]. Following 24 hrs of treatment with 10ng/ml of TNF-α, we observed a significant increase in protein PARylation (Fig 2A-B), coupled to a decrease in total cellular NAD^+ levels and NAD^+/NADH ratio (Fig 2C). Interestingly, when intracellular NAD^+ content was boosted via NR treatment (150uM-1mM; Supplementary Fig 2A), despite the continued presence of an inflammatory insult (TNF-α treatment), excessive protein PARylation was inhibited (Fig 2A-B). This would indicate that higher intracellular NAD^+ concentrations are either inhibiting inflammation-

mediated protein PARylation or are promoting faster flux of protein (de)PARylation. To determine if inflammation-mediated NAD^+ depletion had any effect on mitochondrial function in this model, XFe96 seahorse assay was conducted to measure mitochondrial oxygen consumption rates [46]. TNF-α treatment caused a decrease in basal and ATP-linked respiration, while NAD^+ boosting with NR co-treatment was able to rescue these respiration rates (Fig 2D-E). No difference in maximal respiratory capacity was observed with either TNF-α or NR co-treatment (Fig 2F). Simultaneously, glycolytic capacity of these cells was assessed via measurements of extracellular acidification rates. However, there was no significant decrease in glycolytic capacity in any treatment groups (Fig 2G). An assessment of the expression of key OXPHOS subunit proteins [succinate dehydrogenase B (SDHB), Ubiquinol-Cytochrome C Reductase Core Protein 2 (UQCRC2), Mitochondrially Encoded Cytochrome C Oxidase I (MTCO1), ATP synthase subunit alpha of Complex V (vATP5A)], revealed no change in protein content with NR co-treatment, suggesting the improved mitochondrial respiration rates observed are likely related to mitochondrial functional capacity improvements rather than structural capacity improvements (Fig 2H-L) . Finally, an assessment of the impact of inflammation-mediated NAD^+ depletion on trophoblast function was carried out using a matrigel-coated boyden chamber invasion assay. The primary function of the extravillous cytotrophoblast cell lineage is invasion of the decidua and remodelling of the uterine spiral arterioles, ensuring adequate utero-placental blood supply to support fetal growth [47]. Previous studies have demonstrated an inhibitory effect of TNF-α treatment on the invasive capacity of these cells using both the HTR8 cell culture model and using first trimester ex vivo placenta explants [48-50]. We were able to replicate

these findings, demonstrating a 50% reduction in trophoblast invasion with TNF-α treatment. Importantly, intracellular NAD^+ boosting with NR co-treatment rescued this finding, returning the invasive capacity of these inflammatory insult-exposed cells back to those observed in untreated controls (Fig 2M- N). Collectively, these cell culture findings provide preliminary evidence which suggests that NAD^+ replenishment via NR treatment may improve human trophoblast health and function under pro-inflammatory conditions.

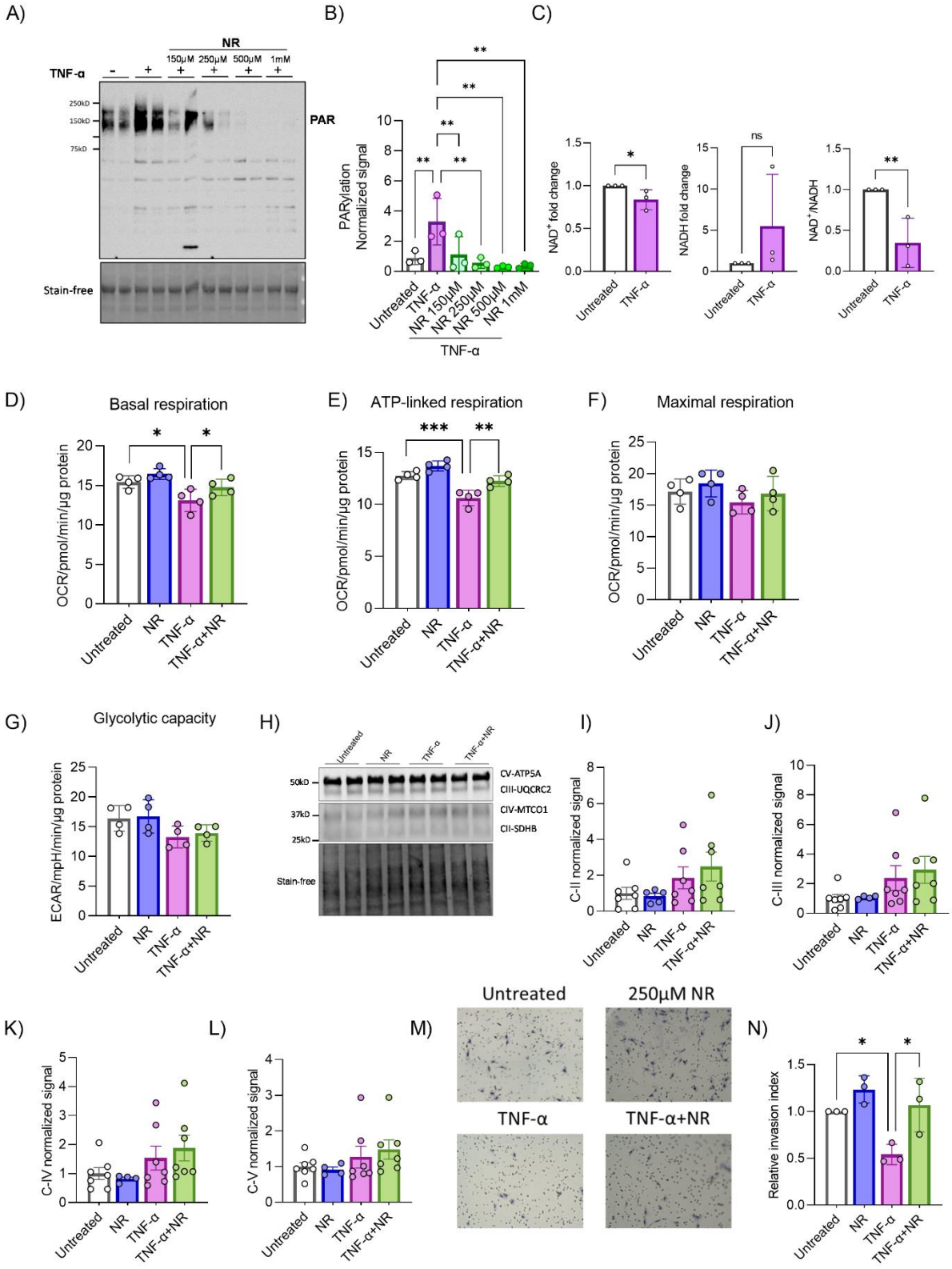
A)
B)
C)
NR
150µM 250µM 500µM 1mM
TNF-α
250kD
150kD
75kD
PAR
Stain-free
PARylation Normalized signal
Untreated
TNF-α
NR 150µM
NR 250µM
NR 500µM
NR 1mM
TNF-α
NAD+ fold change
NADH fold change
NAD+/NADH
ns
D)
Basal respiration
E)
ATP-linked respiration
F)
Maximal respiration
OCR/pmol/min/µg protein
Untreated
NR
TNF-α
TNF-α+NR
G)
Glycolytic capacity
ECAR/mpH/min/µg protein
H)
CV-ATP5A
CIII-UQCRC2
CIV-MTCO1
CII-SDHB
50kD
37kD
25kD
Stain-free
I)
C-II normalized signal
J)
C-III normalized signal
K)
C-IV normalized signal
L)
C-V normalized signal
M)
Untreated
250µM NR
TNF-α
TNF-α+NR
N)
Relative invasion index

Figure 2. Potential of NAD^+ boosting in improving HTR8/SVneo human trophoblasts health and function under an inflammatory *in vitro* condition. A-B) Trophoblast protein PARylation was determined by western blot with anti-PAR antibody on cells treated with 10ng/ml TNF-α and/or 150μM-1mM NR for 24 hrs (n=3). One-way ANOVA with Holm-Šídák's multiple comparisons test C) Total cellular NAD^+(H) and NAD^+/NADH ratio quantification was performed on cells treated with or without 10ng/ml TNF-α for 24 hrs (n=3). One-tailed unpaired t-test. D-G) XFe96 seahorse Mito stress test was performed on cells treated with 10ng/ml TNF-α and/or 250μM NR for 24 hrs. Basal, ATP-linked and maximal mitochondrial respiration rates and extracellular acidification rates were measured (n=4). H-L) Expression of oxidative phosphorylation (OXPHOS) proteins were determined by western blot with OXPHOS antibody cocktail on cells treated with 10ng/ml TNF-α and/or 250μM NR for 24 hrs (n=4-7). M-N) Trophoblast invasion capacity was determined by performing Matrigel invasion assay on cells treated with 10ng/ml TNF-α and/or 250μM NR for 72 hrs (n=3). Two-way ANOVA with Holm-Šídák's multiple comparisons test. *$P<0.05$,**$P<0.01$,***$P<0.001$. Error bar indicates standard deviation (SD). TNF-α= Tumor necrosis factor, NR= nicotinamide riboside.

Boosting NAD^+ levels during pregnancy using an NR therapeutic intervention prevents the development of PE-like features in rat model of inflammation-driven PE.

As NAD^+ depletion was observed in placentas from cases of inflammatory-driven PE, and NAD^+ supplementation *in vitro* improved human trophoblast health and function under pro-inflammatory conditions; we next sought to determine if NR could be used therapeutically to prevent the development of PE-like clinical features in a rat model of inflammatory-driven PE. Our group has previously characterized and compared several rodent models of inflammation-mediated PE, determining the lipopolysaccharide (LPS)-induced rat model was able to most closely recapitulate features observed in cases of human inflammation-driven PE [51]. In this model, LPS (20-70ug/kg/day) is injected daily from GD13 to GD18 intraperitoneally (IP). In our therapeutic intervention group, NR was administered using a preventative strategy by oral gavage from gestational day

(GD) 1-19 (200mg/kg/day). Control pregnant rats received oral gavage of drinking water and saline IP injections using the same schedule (Fig 3A). As previously described, maternal BP was heightened at the end of pregnancy (GD17-GD19) with LPS treatment compared to saline controls, however this phenotype was rescued with NR intervention (Fig 3B).

In the LPS-treated pregnancies, there was a significant decline in fetal survival to term (50.88%, Fig 3C), with the remaining viable fetuses demonstrating evidence of fetal growth restriction (Fig 3D). Importantly, these adverse fetal outcomes were attenuated with NR intervention (Fig 3C-D). Placental weights were decreased with LPS treatment (Fig 3E), with morphometric analysis demonstrating a decrease in total depth (Fig 3F), largely attributed to decreased depth of the labyrinth compartment of the placenta (Fig 3G-H) – the site of maternal-fetal exchange. NR intervention during pregnancy was able to rescue this adverse placenta phenotype (Fig 3E-H).

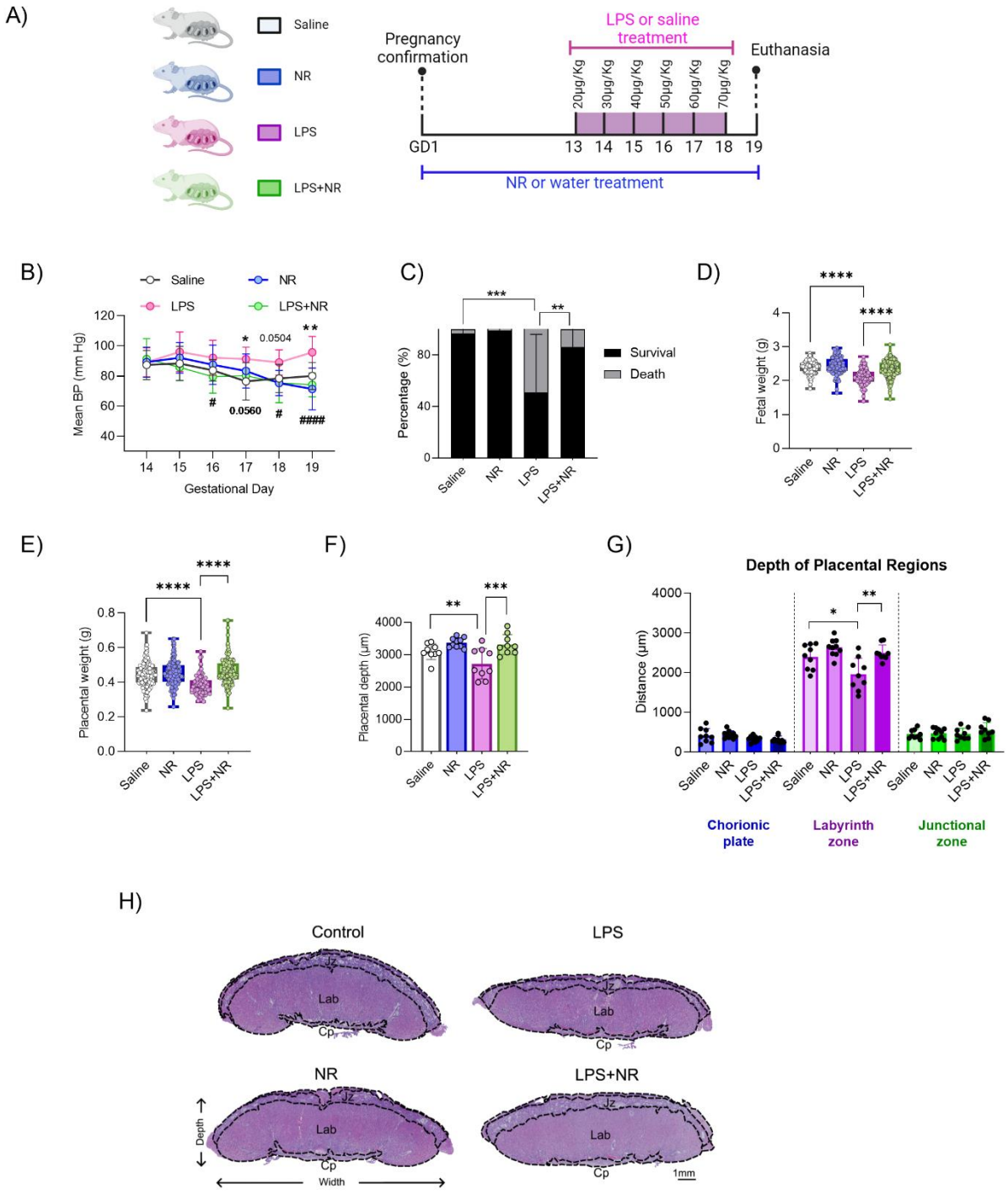

Figure 3. Effect of NAD⁺ boosting in preventing inflammatory PE in a rat model. A) Study design: Pregnant rats were given either NR or drinking water from GD1-19 and either LPS (20-70ug/kg/day) or saline were injected from GD13-18 and euthanasia was performed on GD 19 (Created with BioRender.com). B) Blood pressure was measured

from GD 14-19 by tail-cuff method (n=6-8 pregnant rats/treatment). * Indicates P values between saline and LPS and # indicates p value between LPS and LPS+NR. C) Fetal survival and death percentage. Two-way ANOVA with Holm-Šídák's multiple comparisons test. D-E) Fetal and placental weights were measured (n=10 litters/treatment). One-way ANOVA with Holm-Šídák's multiple comparisons test. F-H) Placental depth along with individual regions such as labyrinth, junctional zone and chorionic plate were measured (n=10 litter/treatment, 1 placenta/litter). Two-way ANOVA with Holm-Šídák's multiple comparisons test, *P＜0.05,**P＜0.01,***P＜0.001. Error bar indicates standard deviation (SD). LPS= Lipopolysaccharide, NR= nicotinamide riboside.

Boosting placenta NAD$^+$ levels using an NR therapeutic intervention prevents PARP-mediated NAD$^+$ depletion and mitochondrial dysfunction in a rat model of inflammation-driven PE.

To determine whether LPS treatment can induce placental inflammation, we first examined placental TNF-α levels by ELISA. TNF-α is considered to be a key contributor to the pathogenesis of hypertension in pregnancy [52-54]; and according to our microarray data, the TNF-α pathway is upregulated in the inflammatory PE3 placentas (Fig 1A). Our result demonstrates that LPS treatment similarly induces rat placental TNF-α expression, which can be normalized with NR intervention (Fig 4A). RNA-seq analysis performed on a subset of placentas further confirms that NR treatment in the presence of LPS modulates the placental transcriptome and reduces the pro-inflammatory response (Supplementary Fig 3 A-D).

To test if placental PARylation is hyperactivated in inflammatory PE, and if it causes a decline in NAD$^+$ content, we examined rat placentas from LPS-induced PE for PARylated proteins and NAD$^+$ content. We found that LPS led to increased placental protein PARylation while NR intervention normalized this change (Fig 4B-C). The levels

of ADP- ribose, a moiety produced from the hydrolysis of PAR chains by ADP-ribosyl-acceptor hydrolases [55, 56], were higher in placentas from LPS-induced rats and normalized with NR intervention, indicating that NR intervention likely did not increase PAR hydrolysis to result in lower PARylation levels (Fig 4D). Interestingly, during inflammation an increase in the expression of the NAD^+-dependent cADPR synthase CD38 produces ADPR and causes a reduction in NAD^+ levels [57, 58]. We, however, did not observe any significant increase in CD38 expression in LPS treated placentas, suggesting that PARPs are likely the primary driver of placental NAD^+ consumption with LPS treatment (Supplementary Fig 4A-B). Next, we measured placental NAD(H) levels. We further found a decrease in NAD^+, NADH and the NAD^+/NADH ratio in placentas from LPS treated pregnant rats, compared to saline controls, and an NR intervention prevented such declines in NAD^+ and NADH levels (Fig 4E-G). However, NAD^+/NADH ratio was not significantly rescued with NR supplementation (Fig 4G). Given that NR alone did not alter NAD(H) levels in rat placentas, we measured the levels of NAM, which is a by-product of NAD^+ utilizing enzymatic reactions, which can be converted back to NAD^+ by the salvage pathway [31]. As expected, we found high levels of NAM in both NR alone and NR with LPS treated placentas (Supplementary Fig 4C).

To determine if LPS treatment leads to placental mitochondrial dysfunction and whether NR exerts its beneficial effect by improving mitochondrial function we performed oxygraphy respirometry on freshly isolated mitochondria from these placentas. LPS treatment decreased complex I and II mediated oxygen consumption rates while NR intervention normalized respiration rates (Fig 4H-I). Complex IV mediated oxygen consumption was unaltered by LPS or NR treatments (Supplementary Fig 4D). Similar

to measurements in human HTR8 cells, these functional changes were not a result of altered expression of NADH:Ubiquinone Oxidoreductase Subunit B8 (NDUFB8), UQCRC2, vATP5A OXPHOS proteins (Supplementary Fig 4E-H). Given that placental mitochondrial fusion is impaired in PE [8] and that the expression of the fusion protein OPA1 was reduced in human inflammatory PE placentas (Fig 1H), we examined the expression of OPA1 in all conditions. Although OPA1 protein expression is significantly reduced in placentas from LPS treated rats, NR intervention did not attenuate this effect (Supplementary Fig 4I-J). Compromised mitochondrial quality control pathway has been shown to contribute to placental dysfunction in canonical/hypoxia driven PE2 [59]. Thus, we determined the expression levels of two mitochondrial quality control proteases- YME1 like 1 ATPase (YME1L1) and Caseinolytic Mitochondrial Matrix Peptidase Proteolytic Subunit (CLPP). Both YME1L1 and CLPP expression levels were unaltered in each of the different treatment conditions (Supplementary Fig 4K-M).

As chronic inflammation and mitochondrial dysfunction can result in oxidative stress [60-63] and our results show that human inflammatory PE placentas exhibit more oxidative DNA damage (Fig 1I-J), we next investigated if LPS treatment leads to rat placental oxidative DNA damage. We performed western blotting to determine the levels of the phosphorylated form of histone H2A.X (p-H2A.X), a marker of DNA double stranded break [64]. Placentas from LPS treated rats exhibit higher levels of phospho-H2A.X protein (Fig 4J-K), while NR intervention significantly attenuated this effect (Fig 4J-K). This is consistent with studies that have shown that NAD^+ replenishment can lead to the activation of the antioxidant enzyme mitochondrial superoxide dismutase (mnSOD2) [65]. We further confirmed this by immunohistochemistry showing that LPS treated

placentas have more p-H2A.X positive cells in the labyrinth and maternal decidua whereas NR intervention normalized this effect to control levels (Fig 4L-M).

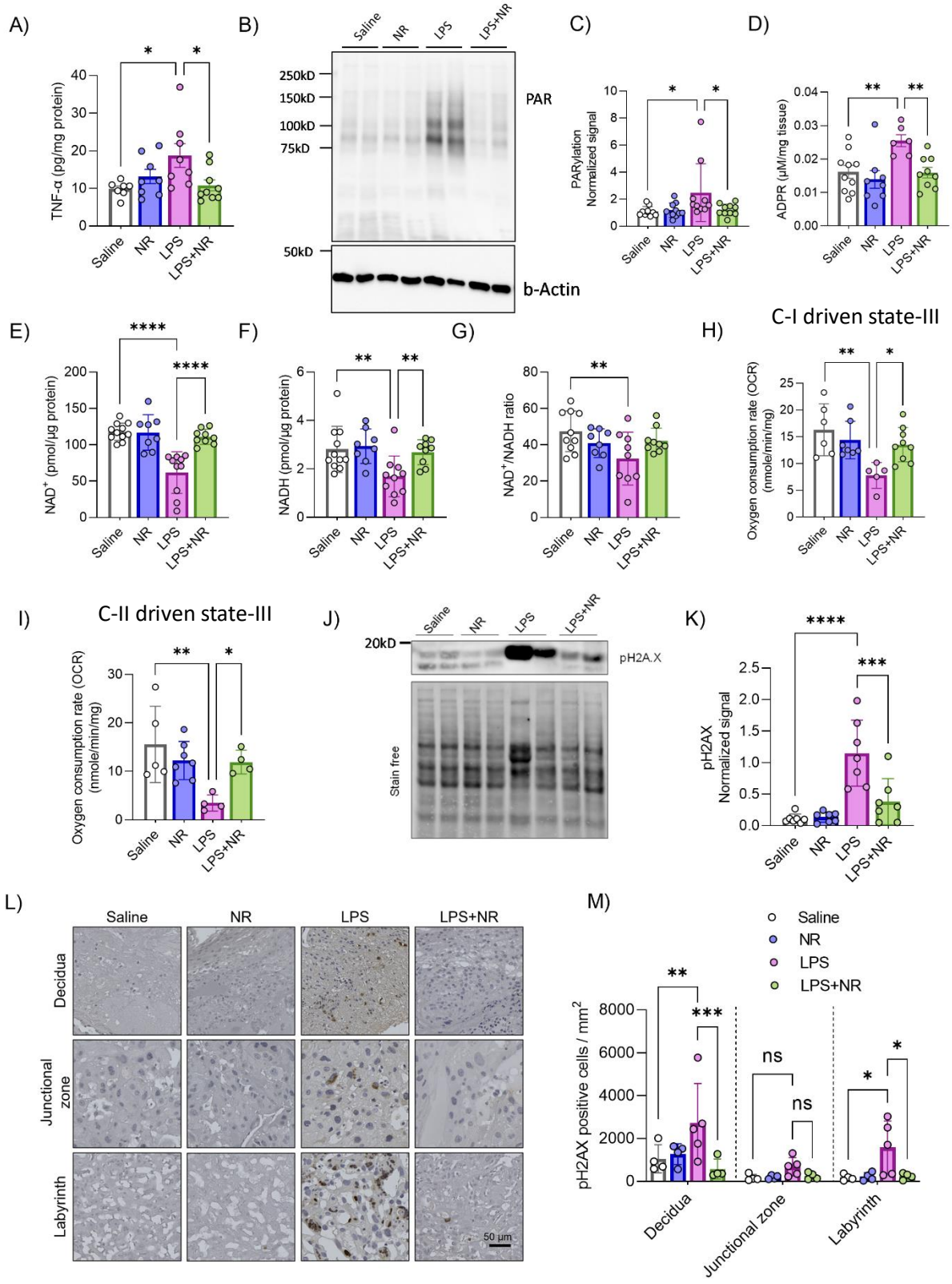
A)
TNF-α (pg/mg protein)
Saline
NR
LPS
LPS+NR
B)
250kD
150kD
100kD
75kD
50kD
PAR
b-Actin
C)
PARylation Normalized signal
D)
ADPR (µM/mg tissue)
E)
NAD+ (pmol/µg protein)
F)
NADH (pmol/µg protein)
G)
NAD+/NADH ratio
H)
C-I driven state-III
Oxygen consumption rate (OCR) (nmole/min/mg)
I)
C-II driven state-III
J)
20kD
pH2A.X
Stain free
K)
pH2AX Normalized signal
L)
Decidua
Junctional zone
Labyrinth
50 µm
M)
pH2AX positive cells / mm2
ns

Figure 4. Effect of NAD^+ boosting on placental dysfunction in a rat model of inflammatory PE. A) Placental TNF-α levels were determined by ELISA (n=7-9 litter/treatment, 1 placenta/litter). B-C) Placental protein PARylation was determined by western blot with anti-PAR antibody (n=10 litter/treatment, 1placenta/litter). D) Placental ADPR levels were determined by LC/MS (n=6-10 litter/treatment, 1placenta/litter). E-G) Placental NAD^+(H) levels were determined by a colorimetric assay (n=8-10 litter/treatment, 1placenta/litter). H-I) Placental mitochondrial function was determined by performing Oxygraph assay on isolated mitochondria. Complex-I and II driven state-III respiration or oxygen consumption rates were determined (n=4-8 litter/treatment, 6-8 placentas/litter). J-K) Placental oxidative stress was determined by western blot with anti-pH2A.X antibody, a marker of oxidative DNA damage (n=7 litter/treatment, 1placenta/litter). L-M) Placental sections were stained with anti-pH2A.X antibody to determine the number of pH2A.X positive cells in different regions of placenta (n=5 litter/treatment, 1placenta/litter). Two-way ANOVA with Holm-Šídák's multiple comparisons test, *P＜0.05,**P＜0.01,***P＜0.001. Error bar indicates standard deviation (SD). ADPR= ADP ribose, LPS= Lipopolysaccharide, NR= nicotinamide riboside.

5.3 Discussion

We show for the first time that NAD^+ levels are depleted in both human and rat placentas that are affected by inflammation. Our results indicate that this reduction in NAD^+ may be precipitated by the hyperactivity of NAD^+-consuming PARP enzymes. In vitro, we have shown that boosting NAD^+ levels with NR can improve trophoblast health and function under inflammatory conditions. Using the LPS-induced inflammatory PE rat model, we demonstrated that an oral pretreatment with NR prevents the development of PE, which was evident by lowered blood pressure, decreased inflammation, increased placental and fetal weights; and increased fetal survival.

One of the major NAD^+ consumers of the cell is PARP1 which accounts for about 90% of the total cellular PARP activity [66-68]. Among the other PARPs, PARP2/5a/5b are also known to have PARylating activity, while rest of the PARP family (PARP3/4/6/7/8/10/11/12/14) are known to cause mono-ADPribosylation (MARylation).

In contrast, PARP9/13 have no enzymatic activity [33]. PARP1 has been shown to play a critical role in inflammation demonstrating an attractive therapeutic target [69-72]. PARP1 is a transcriptional coactivator of NFkB. Upon LPS or TNF-α exposure, activated PARP1 PARylates p65, which is required for NFkB-mediated inflammatory gene transcription [73]. PARP1 PARylates human antigen R (HuR), a protein that increases mRNA stability of pro-inflammatory genes upon LPS exposure [74]. Moreover, upon LPS induction, PARP1 can also PARylate high-mobility group box protein 1 (HMGB1), a proinflammatory molecule, and facilitate its extracellular release to sustain inflammation [75]. Gene expression of other MARylating PARPs such as PARP3/4/7/8/10/11/12/13 were also found to increase upon LPS induction. MARylating and non-enzymatic PARPs such as PARP9/11/12/13/14 have broad spectrum antiviral properties [76]. PARP14 was shown to MARylate STAT1 and inhibit its phosphorylation and downstream inflammatory activation. On the other hand, PARP9 was shown to inhibit PARP14 and promote STAT1 mediated inflammatory gene expression. Overall, this implies that MARylating PARPs also participate in modulating an immune response. Consistently, in the human placental microarray, we observed an increase in the transcript levels of such MARylating and non- enzymatic PARPs (PARP4, PARP9, PARP12 and PARP14) in the inflammation-driven PE placentas. While we did not observe an increase in the transcript levels of PARP1 or other PARylating enzymes in this dataset, we did observe increased PARylation in the human placentas from the inflammation-driven PE patients, in the trophoblasts treated with TNF- α and in the LPS treated rat placentas. A number of studies also demonstrated activation of PARP1 mediated PARylation in inflammatory conditions with no reported increase in the PARP1

gene or protein levels, indicating that cellular stress or inflammation may induce increased PARP1 enzymatic activity without increasing its expression [75, 77, 78]. Apart from having an inflammatory signature, we also observed oxidative DNA damage in the human and rat inflammation-driven PE placentas. Several studies reported that PARP1-generated PAR molecules and PARP-5a/12/13/14/15 are required for the formation of stress granules during cellular stress to regulate protein translation [79-81]. Like PARP1, PARP2 - a PARylating enzyme – has similar function in DNA damage repair [33]. Thus, it is likely that several PARPs were simultaneously activated in the inflammation-driven PE placentas leading to a decline in NAD^+ levels.

Due to the known substantial contribution of PARP1 in inflammation, inhibition of PARP1 has been shown to reduce inflammation and restore cellular NAD^+ levels in several studies [69-72]. However, PARP1 inhibition or knockout in pregnant rodent models has caused fetal defects suggesting its essential role during fetal development [82-85]. Indeed, PARP1 has diverse function including chromatin remodelling, DNA repair, transcriptional regulation, and post-translational modifications, etc. [32, 33]. Thus, the harms of a global inhibition of PARP1 could outweigh the benefits, even under pathological conditions, particularly in pregnancy. Further, it is unclear if the rise in PARP activity directly results in pathological consequences or if the resulting decline in NAD^+ levels is the reason for this effect. Some studies suggest that PARP1 is required for cell survival under mild oxidative stress as it participates in DNA damage repair [86-88]. Conversely, under conditions of severe oxidative stress, excessive activation of PARP1 has been shown to induce cell death due to energy exhaustion by consuming NAD^+ [89]. As placental oxidative stress is observed in PE, it is likely that at the initial

stages of the pathogenesis, activation of PARP1 is required for the adaptation to the stress; and later its excessive consumption of NAD^+ leads to pathological consequences. As such, boosting NAD^+ levels present an effective and safe treatment option for inflammation- driven PE.

It is now well established that reduced NAD^+ content is a hallmark of defective energy metabolism [90]. This is now recapitulated in the placenta, where inflammatory PE driven placental mitochondrial dysfunction and oxidative stress can be prevented by restoring NAD^+ levels. We speculate that the benefit of NR could result from multiple pathways (Fig 5). A higher availability of NAD^+ may improve OXPHOS function by providing more fuel. Using an LPS-induced rat model of PE, we showed that LPS treatment causes NAD^+ dysregulation and that NR treatment in the face of chronic inflammation corrects such dysfunction. Investigations into the metabolomic signature of humans with mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes (MELAS) – a mitochondrial disorder affecting the nervous and musculoskeletal systems – demonstrate a central pathological role for NAD^+ dysregulation [91]. Further, *in vitro* investigations using the Hela cell line, demonstrate that normalization of the cytoplasmic and/or mitochondrial NAD^+/NADH ratio is sufficient to compensate for the proliferation defects of the cells under conditions of compromised electron transport chain function [92]. This suggests that therapeutically targeting cellular NAD^+ pools can have profound beneficial effect on cell health and function. The sirtuin family of enzymes are known to play important roles in mitochondrial biogenesis, OXPHOS function, ROS regulation and inflammation [19, 65, 93-105]. Maintaining NAD^+ levels in the face of a chronic inflammatory insult may maintain sirtuins activation and functioning, helping maintain

mitochondrial health and function under pathological conditions. In pre-clinical animal experimentation and human clinical trials, NAD^+ supplementation has demonstrated anti-inflammatory effects [93, 106-109], consistent with our findings of reduced placental inflammation with NR treatment (supplementary 4A-D). Overall, this study suggests that chronic inflammation in the placenta causes hyperactivation of PARPs which leads to a decline in NAD^+ and mitochondrial dysfunction. Importantly, therapeutically targeting the NAD^+ signalling pathways and sustaining NAD^+ levels throughout the pregnancy maintains mitochondrial function, reduces inflammation and oxidative damage, thus, preventing PE disease development (Fig 5). NAD^+ boosting has been proven to be effective in various pro-inflammatory disease models [19, 21, 28, 36, 110, 111]. Moreover, NR has been shown to be orally bioavailable and safe for human consumption [27, 106, 109, 112]. Several human studies have demonstrated that NR is effective in elevating NAD+ levels and can improve metabolic function [27, 113-118]. NR was also shown to reduce circulating inflammatory markers in humans [109], suggesting that it could be effective for use with inflammatory-driven PE patients. Given that another NAD^+ donor, NAM, has been found to be effective in hypoxia-driven PE mouse models [37, 38], boosting NAD^+ levels may have the potential to combat against all subclasses of human PE, thus making it a very promising therapeutic intervention. In the current study, we administered NR from the beginning of pregnancy -demonstrating its preventative potential. In future, pre-clinical and clinical trials could be carried out to test the effectiveness of NAD^+ boosting strategies, such as supplementing with vitamin B3 derivatives, during pregnancy or once PE has been established – establishing its treatment potential as well.

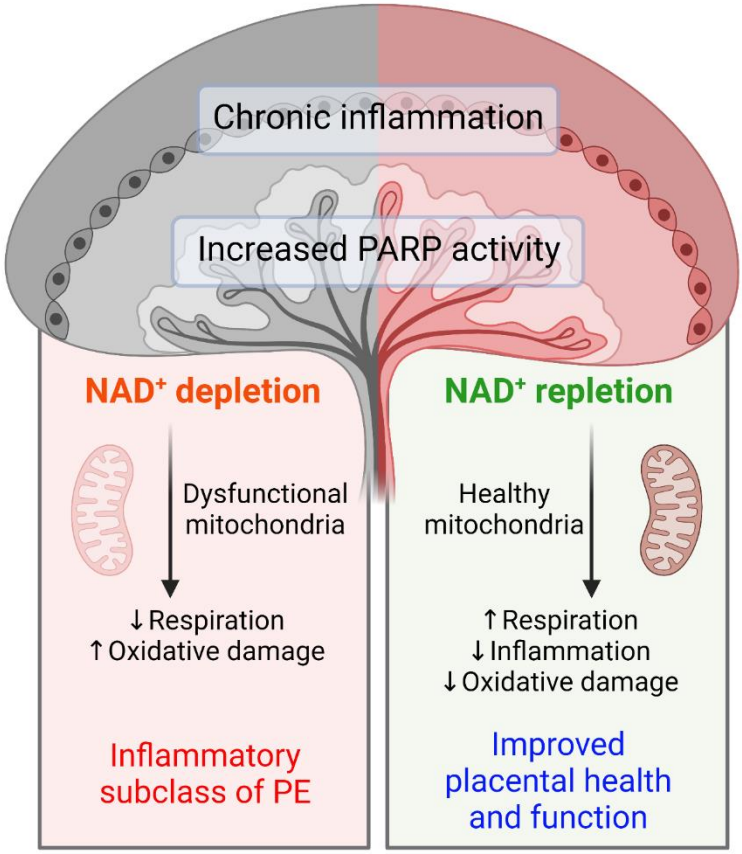

Figure 5: Effect of NAD^+ boosting on inflammatory PE. Our findings suggest that a decline in placental NAD^+ levels due to PARP hyperactivity in inflammatory PE leads to mitochondrial dysfunction and oxidative damage while NAD^+ boosting strategies, through NR treatment, improves placental mitochondrial function and resolves placental inflammation and oxidative damage, thus, preventing the development of inflammatory PE (Created with BioRender.com).

5.4 Materials and Methods

Study design

This study aimed to investigate the role of NAD^+ in inflammation-driven PE (PE3), and to test the therapeutic potential of NAD^+ boosting in an animal model of immune driven PE. We hypothesized that there is a decline in placental NAD^+ levels due to hyperactivity of NAD^+ consuming enzymes and that it leads to impaired placental mitochondrial function, collectively contributing to the development of PE. Thus, replenishing NAD^+ could be an attractive preventative strategy in this subclass of PE patients. We have used human placenta samples to profile placental NAD^+ levels, activity of NAD^+ consuming PARP enzymes and mitochondrial content/protein expression. We next performed cell culture experiments using SVneo/HTR8 trophoblast cell line to understand how NR can benefit placental cells under an inflammatory condition by treating the cells with TNF-α, a pro- inflammatory cytokine that is increased in the placenta of inflammation-driven PE patients [3]. Finally, we employed an LPS-induced rat model of inflammation-driven PE to test the potential of NR in preventing PE development. In this model, we evaluated BP, fetal/placental weights, placental morphology, inflammation, mitochondrial function, and oxidative stress. We also assessed placental transcriptome and NAD^+ metabolism to understand how boosting NAD^+ may work to prevent PE development.

Frozen Human placenta tissue

For the measurement of placental NAD^+ and mitochondrial content, a total of 128 frozen placenta samples and matched histological sections were purchased from the Research

Centre for Women's and Infants' Health BioBank (RCWIH), Mount Sinai Hospital, Toronto, Canada. Samples included term and preterm placentas from healthy controls (n=43), individuals with chronic hypertension (n=23), parental-driven PE subclass (PE1; n=16), hypoxia-driven PE subclass (PE2; n=42) and inflammation-driven PE subclass (PE3; n=17) patients. PE was defined as the onset of hypertension (systolic pressure > 140 mm Hg and/or diastolic pressure > 90 mm Hg) after 20 weeks of gestation coupled with evidence of maternal end-organ dysfunction [2]. Classification of the molecular subclass was done by microarray and confirmed by qPCR analysis, as described in Leavey et al., 2016 [3]. For comparison purpose, control healthy term and preterm placentas were combined as a singular healthy "control" group, since PE placentas included both term and preterm placentas.

Detailed methods of microarray gene expression analysis, protein PARylation and NAD(H) measurements, mitochondrial protein and oxidative stress measurements are provided in the appended supplement (Supplementary 5)

Cell culture

The HTR-8/SVneo cell line, derived from human first-trimester placental villus explant, was purchased from ATCC (CRL-3271). Cells were cultured in Gibco® 1X RPMI-1640 (Life Technologies™) medium supplemented with 10% fetal bovine serum (FBS), at 37 °C, 5% CO_2 and ~20% O_2 condition. Methods of protein PARylation and NAD(H) measurements, mitochondrial respiration assay and OXPHOS protein expression; and trophoblast functional (invasion) assay are provided in the appended supplement (Supplementary 5). Briefly, cells were treated with TNF-α (10mg/ml) to induce

inflammation and NR (250 μM) was co-treated for specified timepoints to test its potential to improve trophoblast mitochondrial and cellular function.

Animal study

All animal experiments were carried out according to University of Ottawa animal care ethics and guidelines (protocol# HS2923). Sprague Dawley rats (Charles River Laboratories International, Inc.) were kept in a room with 23°C temperature and a 12:12- hr light-dark cycle. Estrus cycle was confirmed by doing vaginal smears to allow timed mating [119]. Vaginal smears were checked the following day to identify presence of sperms to confirm pregnancy. Additionally, body weights were recorded daily to track pregnancy. The following experimental groups were included:

I. LPS (n=10): Lipopolysaccharide (Escherichia coli O55:B5, L2880-10MG, Sigma-Aldrich) was solubilized in sterile normal saline and animals were intraperitoneally (IP) injected from GD 13-18 at a daily incremental dose from 20ug-70ug/kg/day, as shown previously [51].

II. NR (n=10): NR was administered by oral gavage from GD 1-19. NR was solubilized in drinking water and sterile filtered and given at a concentration of 200 mg/kg/day.

III. LPS + NR model (n=10): NR and LPS were administered as mentioned above.

IV. Saline (n=10): Controls received oral gavage of sterile drinking water from GD 1-19 and saline IP injections from GD 13-18.

Animals were sacrificed on GD 19, fetal and placental weights and litter size were recorded and organs were collected for future analysis.

Detailed methods of pregnancy outcome, placental histomorphology, inflammation, gene expression, protein PARylation and metabolite measurements, placenta mitochondrial functionality assay and protein expression, and oxidative stress measurements are provided in the appended supplement (Supplementary 5).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (Version 9.5). Error bars in graphical presentations indicate mean ± standard deviation (SD). Depending on the treatment groups, a unpaired t-test or one-way or two-way ANOVA with a Holm-Šídák's multiple comparisons test was applied. For animal experimentations, robust regression, and outlier removal (ROUT) method (available on GraphPad Prism) was applied to exclude any outliers. A p value of < 0.05 was considered statistically significant.

Author Contributions: Conceptualization, F.J., G.V, K.J.M and S.A.B.; data collection and analysis, F.J., G.V, Y.C., A.N-A., A.E.G; writing, F.J.; review and editing, F.J., K.J.M and S.A.B.; project supervision and funding, K.J.M and S.A.B. All authors have read and agreed to the published version of the manuscript.

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References

1. Kuklina, E.V., C. Ayala, and W.M. Callaghan, *Hypertensive disorders and severe obstetric morbidity in the United States.* Obstet Gynecol, 2009. **113**(6): p. 1299-1306.
2. Backes, C.H., et al., *Maternal preeclampsia and neonatal outcomes.* J Pregnancy, 2011. **2011**: p. 214365.
3. Leavey, K., et al., *Unsupervised Placental Gene Expression Profiling Identifies Clinically Relevant Subclasses of Human Preeclampsia.* Hypertension, 2016. **68**(1): p. 137-47.
4. Leavey, K., S.A. Bainbridge, and B.J. Cox, *Large scale aggregate microarray analysis reveals three distinct molecular subclasses of human preeclampsia.* PLoS One, 2015. **10**(2): p. e0116508.
5. Powers, R.W., et al., *Low placental growth factor across pregnancy identifies a subset of women with preterm preeclampsia: type 1 versus type 2 preeclampsia?* Hypertension, 2012. **60**(1): p. 239-46.
6. Redman, C.W., I.L. Sargent, and A.C. Staff, *IFPA Senior Award Lecture: making sense of pre-eclampsia - two placental causes of preeclampsia?* Placenta, 2014. **35 Suppl**: p. S20-5.
7. von Dadelszen, P., L.A. Magee, and J.M. Roberts, *Subclassification of preeclampsia.* Hypertens Pregnancy, 2003. **22**(2): p. 143-8.
8. Jahan, F., et al., *Placental Mitochondrial Function and Dysfunction in Preeclampsia.* Int J Mol Sci, 2023. **24**(4).
9. Shi, Z., et al., *Comparative proteomics analysis suggests that placental mitochondria are involved in the development of pre-eclampsia.* PLoS One, 2013. **8**(5): p. e64351.
10. Vishnyakova, P.A., et al., *Mitochondrial role in adaptive response to stress conditions in preeclampsia.* Sci Rep, 2016. **6**: p. 32410.
11. Yu, J., et al., *Downregulation of Mitofusin 2 in Placenta Is Related to Preeclampsia.* Biomed Res Int, 2016. **2016**: p. 6323086.
12. Holland, O.J., et al., *Placental mitochondrial adaptations in preeclampsia associated with progression to term delivery.* Cell Death Dis, 2018. **9**(12): p. 1150.
13. Aye, I., et al., *Placental energy metabolism in health and disease-significance of development and implications for preeclampsia.* Am J Obstet Gynecol, 2022. **226**(2S): p. S928-S944.
14. Hebert, J.F. and L. Myatt, *Placental mitochondrial dysfunction with metabolic diseases: Therapeutic approaches.* Biochim Biophys Acta Mol Basis Dis, 2021. **1867**(1): p. 165967.
15. Holland, O., et al., *Review: Placental mitochondrial function and structure in gestational disorders.* Placenta, 2017. **54**: p. 2-9.
16. Verdin, E., *NAD(+) in aging, metabolism, and neurodegeneration.* Science, 2015. **350**(6265): p. 1208-13.
17. Yang, S.J., et al., *Nicotinamide improves glucose metabolism and affects the hepatic NAD-sirtuin pathway in a rodent model of obesity and type 2 diabetes.* J Nutr Biochem, 2014. **25**(1): p. 66-72.

18. Penberthy, W.T. and I. Tsunoda, *The importance of NAD in multiple sclerosis.* Curr Pharm Des, 2009. **15**(1): p. 64-99.
19. Imai, S. and L. Guarente, *NAD+ and sirtuins in aging and disease.* Trends Cell Biol, 2014. **24**(8): p. 464-71.
20. Cuzzocrea, S., et al., *Role of poly(ADP-ribose) glycohydrolase in the development of inflammatory bowel disease in mice.* Free Radic Biol Med, 2007. **42**(1): p. 90-105.
21. Gariani, K., et al., *Inhibiting poly ADP-ribosylation increases fatty acid oxidation and protects against fatty liver disease.* J Hepatol, 2017. **66**(1): p. 132-141.
22. Ryu, D., et al., *NAD+ repletion improves muscle function in muscular dystrophy and counters global PARylation.* Sci Transl Med, 2016. **8**(361): p. 361ra139.
23. Zheng, M., M.B. Schultz, and D.A. Sinclair, *NAD(+) in COVID-19 and viral infections.* Trends Immunol, 2022. **43**(4): p. 283-295.
24. Jahan, F. and R.A. Bagchi, *Enhancing NAD(+) Metabolome in Cardiovascular Diseases: Promises and Considerations.* Front Cardiovasc Med, 2021. **8**: p. 716989.
25. Meyer, T., et al., *NAD(+) metabolism drives astrocyte proinflammatory reprogramming in central nervous system autoimmunity.* Proc Natl Acad Sci U S A, 2022. **119**(35): p. e2211310119.
26. Zhao, Y., et al., *NAD(+) improves cognitive function and reduces neuroinflammation by ameliorating mitochondrial damage and decreasing ROS production in chronic cerebral hypoperfusion models through Sirt1/PGC-1alpha pathway.* J Neuroinflammation, 2021. **18**(1): p. 207.
27. Zhou, B., et al., *Boosting NAD level suppresses inflammatory activation of PBMCs in heart failure.* J Clin Invest, 2020. **130**(11): p. 6054-6063.
28. Gariani, K., et al., *Eliciting the mitochondrial unfolded protein response by nicotinamide adenine dinucleotide repletion reverses fatty liver disease in mice.* Hepatology, 2016. **63**(4): p. 1190-204.
29. Belenky, P., K.L. Bogan, and C. Brenner, *NAD+ metabolism in health and disease.* Trends Biochem Sci, 2007. **32**(1): p. 12-9.
30. Xie, N., et al., *NAD(+) metabolism: pathophysiologic mechanisms and therapeutic potential.* Signal Transduct Target Ther, 2020. **5**(1): p. 227.
31. Canto, C., K.J. Menzies, and J. Auwerx, *NAD(+) Metabolism and the Control of Energy Homeostasis: A Balancing Act between Mitochondria and the Nucleus.* Cell Metab, 2015. **22**(1): p. 31-53.
32. Bai, P., *Biology of Poly(ADP-Ribose) Polymerases: The Factotums of Cell Maintenance.* Mol Cell, 2015. **58**(6): p. 947-58.
33. Gupte, R., Z. Liu, and W.L. Kraus, *PARPs and ADP-ribosylation: recent advances linking molecular functions to biological outcomes.* Genes Dev, 2017. **31**(2): p. 101-126.
34. Fouquerel, E., et al., *ARTD1/PARP1 negatively regulates glycolysis by inhibiting hexokinase 1 independent of NAD+ depletion.* Cell Rep, 2014. **8**(6): p. 1819-1831.
35. Du, X., et al., *Inhibition of GAPDH activity by poly(ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells.* J Clin Invest, 2003. **112**(7): p. 1049-57.

36. Pirinen, E., et al., *Pharmacological Inhibition of poly(ADP-ribose) polymerases improves fitness and mitochondrial function in skeletal muscle.* Cell Metab, 2014. **19**(6): p. 1034-41.
37. Fushima, T., et al., *Nicotinamide ameliorates a preeclampsia-like condition in mice with reduced uterine perfusion pressure.* Am J Physiol Renal Physiol, 2017. **312**(2): p. F366-F372.
38. Li, F., et al., *Nicotinamide benefits both mothers and pups in two contrasting mouse models of preeclampsia.* Proc Natl Acad Sci U S A, 2016. **113**(47): p. 13450-13455.
39. Picard, M., et al., *Mitochondrial structure and function are disrupted by standard isolation methods.* PLoS One, 2011. **6**(3): p. e18317.
40. Larsen, S., et al., *Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects.* J Physiol, 2012. **590**(14): p. 3349-60.
41. Valavanidis, A., T. Vlachogianni, and C. Fiotakis, *8-hydroxy-2' -deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis.* J Environ Sci Health C Environ Carcinog Ecotoxicol Rev, 2009. **27**(2): p. 120-39.
42. Lyall, F., et al., *Human trophoblast invasion and spiral artery transformation: the role of PECAM-1 in normal pregnancy, preeclampsia, and fetal growth restriction.* Am J Pathol, 2001. **158**(5): p. 1713-21.
43. James, J.L., G.S. Whitley, and J.E. Cartwright, *Pre-eclampsia: fitting together the placental, immune and cardiovascular pieces.* J Pathol, 2010. **221**(4): p. 363-78.
44. Natenzon, A., et al., *Diminished trophoblast differentiation in early onset preeclampsia.* Placenta, 2022. **120**: p. 25-31.
45. Farrell, A., et al., *Faulty oxygen sensing disrupts angiomotin function in trophoblast cell migration and predisposes to preeclampsia.* JCI Insight, 2019. **4**(8).
46. Mookerjee, S.A., et al., *Quantifying intracellular rates of glycolytic and oxidative ATP production and consumption using extracellular flux measurements.* J Biol Chem, 2018. **293**(32): p. 12649-12652.
47. Moser, G., et al., *Human trophoblast invasion: new and unexpected routes and functions.* Histochem Cell Biol, 2018. **150**(4): p. 361-370.
48. Bauer, S., et al., *Tumor necrosis factor-alpha inhibits trophoblast migration through elevation of plasminogen activator inhibitor-1 in first-trimester villous explant cultures.* J Clin Endocrinol Metab, 2004. **89**(2): p. 812-22.
49. Otun, H.A., et al., *Effect of tumour necrosis factor-alpha in combination with interferon-gamma on first trimester extravillous trophoblast invasion.* J Reprod Immunol, 2011. **88**(1): p. 1-11.
50. Huber, A.V., et al., *TNFalpha-mediated induction of PAI-1 restricts invasion of HTR-8/SVneo trophoblast cells.* Placenta, 2006. **27**(2-3): p. 127-36.
51. Hu, J., J. Zhang, and B. Zhu, *Protective effect of metformin on a rat model of lipopolysaccharide-induced preeclampsia.* Fundam Clin Pharmacol, 2019. **33**(6): p. 649-658.
52. Cotechini, T., et al., *Inflammation in rat pregnancy inhibits spiral artery remodeling leading to fetal growth restriction and features of preeclampsia.* J Exp Med, 2014. **211**(1): p. 165-79.

53. Carvalho-Galvao, A., et al., *Central Inhibition of Tumor Necrosis Factor Alpha Reduces Hypertension by Attenuating Oxidative Stress in the Rostral Ventrolateral Medulla in Renovascular Hypertensive Rats.* Front Physiol, 2019. **10**: p. 491.
54. Peracoli, J.C., M.V. Rudge, and M.T. Peracoli, *Tumor necrosis factor-alpha in gestation and puerperium of women with gestational hypertension and pre-eclampsia.* Am J Reprod Immunol, 2007. **57**(3): p. 177-85.
55. Luo, X. and W.L. Kraus, *On PAR with PARP: cellular stress signaling through poly(ADP-ribose) and PARP-1.* Genes Dev, 2012. **26**(5): p. 417-32.
56. Yamashita, S., et al., *Physiological levels of poly(ADP-ribose) during the cell cycle regulate HeLa cell proliferation.* Exp Cell Res, 2022. **417**(1): p. 113163.
57. Liu, Q., et al., *Structural basis for formation and hydrolysis of the calcium messenger cyclic ADP-ribose by human CD38.* J Biol Chem, 2007. **282**(8): p. 5853-61.
58. Hogan, K.A., C.C.S. Chini, and E.N. Chini, *The Multi-faceted Ecto-enzyme CD38: Roles in Immunomodulation, Cancer, Aging, and Metabolic Diseases.* Front Immunol, 2019. **10**: p. 1187.
59. Yung, H.W., et al., *Noncanonical mitochondrial unfolded protein response impairs placental oxidative phosphorylation in early-onset preeclampsia.* Proc Natl Acad Sci U S A, 2019. **116**(36): p. 18109-18118.
60. Lugrin, J., et al., *The role of oxidative stress during inflammatory processes.* Biol Chem, 2014. **395**(2): p. 203-30.
61. Zuo, L., et al., *Inflammaging and Oxidative Stress in Human Diseases: From Molecular Mechanisms to Novel Treatments.* Int J Mol Sci, 2019. **20**(18).
62. Kowalczyk, P., et al., *Mitochondrial Oxidative Stress-A Causative Factor and Therapeutic Target in Many Diseases.* Int J Mol Sci, 2021. **22**(24).
63. Bhatti, J.S., G.K. Bhatti, and P.H. Reddy, *Mitochondrial dysfunction and oxidative stress in metabolic disorders - A step towards mitochondria based therapeutic strategies.* Biochim Biophys Acta Mol Basis Dis, 2017. **1863**(5): p. 1066-1077.
64. Valdiglesias, V., et al., *gammaH2AX as a marker of DNA double strand breaks and genomic instability in human population studies.* Mutat Res, 2013. **753**(1): p. 24-40.
65. Ansari, A., et al., *Function of the SIRT3 mitochondrial deacetylase in cellular physiology, cancer, and neurodegenerative disease.* Aging Cell, 2017. **16**(1): p. 4-16.
66. Gibson, B.A. and W.L. Kraus, *New insights into the molecular and cellular functions of poly(ADP-ribose) and PARPs.* Nat Rev Mol Cell Biol, 2012. **13**(7): p. 411-24.
67. Ryu, K.W., D.S. Kim, and W.L. Kraus, *New facets in the regulation of gene expression by ADP-ribosylation and poly(ADP-ribose) polymerases.* Chem Rev, 2015. **115**(6): p. 2453-81.
68. Xu, S., et al., *Poly(ADP-ribose) polymerase 1 (PARP1) in atherosclerosis: from molecular mechanisms to therapeutic implications.* Med Res Rev, 2014. **34**(3): p. 644-75.
69. Mukhopadhyay, P., et al., *Poly (ADP-ribose) polymerase-1 is a key mediator of liver inflammation and fibrosis.* Hepatology, 2014. **59**(5): p. 1998-2009.

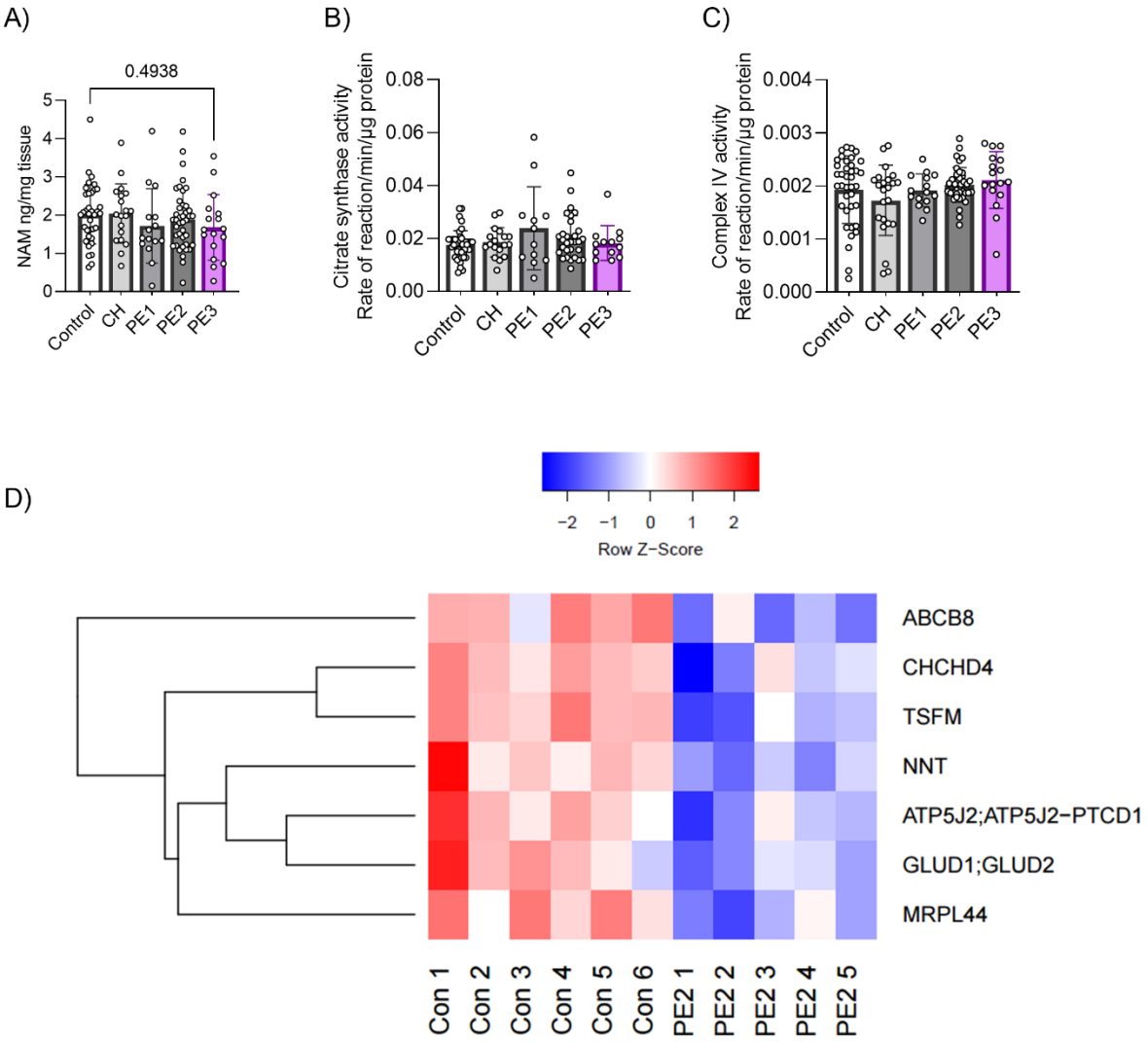
70. Pazzaglia, S. and C. Pioli, *Multifaceted Role of PARP-1 in DNA Repair and Inflammation: Pathological and Therapeutic Implications in Cancer and Non-Cancer Diseases.* Cells, 2019. **9**(1).
71. Wasyluk, W. and A. Zwolak, *PARP Inhibitors: An Innovative Approach to the Treatment of Inflammation and Metabolic Disorders in Sepsis.* J Inflamm Res, 2021. **14**: p. 1827-1844.
72. Liu, Z., et al., *PARP-1 inhibition attenuates the inflammatory response in the cartilage of a rat model of osteoarthritis.* Bone Joint Res, 2021. **10**(7): p. 401-410.
73. Bohio, A.A., et al., *c-Abl-Mediated Tyrosine Phosphorylation of PARP1 Is Crucial for Expression of Proinflammatory Genes.* J Immunol, 2019. **203**(6): p. 1521-1531.
74. Ke, Y., et al., *Erratum: PARP1 promotes gene expression at the post-transcriptional level by modulating the RNA-binding protein HuR.* Nat Commun, 2017. **8**: p. 15191.
75. Yang, Z., et al., *PARP-1 mediates LPS-induced HMGB1 release by macrophages through regulation of HMGB1 acetylation.* J Immunol, 2014. **193**(12): p. 6114-23.
76. Zhu, H. and C. Zheng, *When PARPs Meet Antiviral Innate Immunity.* Trends Microbiol, 2021. **29**(9): p. 776-778.
77. Noguchi, T., et al., *Gefitinib initiates sterile inflammation by promoting IL-1beta and HMGB1 release via two distinct mechanisms.* Cell Death Dis, 2021. **12**(1): p. 49.
78. Ke, Y., et al., *PARP1 promotes gene expression at the post-transcriptiona level by modulating the RNA-binding protein HuR.* Nat Commun, 2017. **8**: p. 14632.
79. Catara, G., et al., *PARP1-produced poly-ADP-ribose causes the PARP12 translocation to stress granules and impairment of Golgi complex functions.* Sci Rep, 2017. **7**(1): p. 14035.
80. Welsby, I., et al., *PARP12, an interferon-stimulated gene involved in the control of protein translation and inflammation.* J Biol Chem, 2014. **289**(38): p. 26642-26657.
81. Leung, A.K., et al., *Poly(ADP-ribose) regulates stress responses and microRNA activity in the cytoplasm.* Mol Cell, 2011. **42**(4): p. 489-99.
82. LaFargue, C.J., et al., *Exploring and comparing adverse events between PARP inhibitors.* Lancet Oncol, 2019. **20**(1): p. e15-e28.
83. Yang, F., C. Baumann, and R. De La Fuente, *Persistence of histone H2AX phosphorylation after meiotic chromosome synapsis and abnormal centromere cohesion in poly (ADP-ribose) polymerase (Parp-1) null oocytes.* Dev Biol, 2009. **331**(2): p. 326-38.
84. Kelleher, A.M., et al., *Deficiency of PARP-1 and PARP-2 in the mouse uterus results in decidualization failure and pregnancy loss.* Proc Natl Acad Sci U S A, 2021. **118**(40).
85. Li, J., et al., *Poly-ADP-ribose polymerase (PARP) inhibitors and ovarian function.* Biomed Pharmacother, 2023. **157**: p. 114028.
86. Ke, Y., et al., *The Role of PARPs in Inflammation-and Metabolic-Related Diseases: Molecular Mechanisms and Beyond.* Cells, 2019. **8**(9).

87. Anandhan, A., et al., *Metabolic Dysfunction in Parkinson's Disease: Bioenergetics, Redox Homeostasis and Central Carbon Metabolism.* Brain Res Bull, 2017. **133**: p. 12-30.
88. Mao, K. and G. Zhang, *The role of PARP1 in neurodegenerative diseases and aging.* FEBS J, 2022. **289**(8): p. 2013-2024.
89. Ponce, D.P., et al., *Increased susceptibility to oxidative death of lymphocytes from Alzheimer patients correlates with dementia severity.* Curr Alzheimer Res, 2014. **11**(9): p. 892-8.
90. Amjad, S., et al., *Role of NAD(+) in regulating cellular and metabolic signaling pathways.* Mol Metab, 2021. **49**: p. 101195.
91. Sharma, R., et al., *Circulating markers of NADH-reductive stress correlate with mitochondrial disease severity.* J Clin Invest, 2021. **131**(2).
92. Titov, D.V., et al., *Complementation of mitochondrial electron transport chain by manipulation of the NAD+/NADH ratio.* Science, 2016. **352**(6282): p. 231-5.
93. Kang, H., Y.K. Park, and J.Y. Lee, *Nicotinamide riboside, an NAD(+) precursor, attenuates inflammation and oxidative stress by activating sirtuin 1 in alcohol-stimulated macrophages.* Lab Invest, 2021. **101**(9): p. 1225-1237.
94. Rajendrasozhan, S., et al., *SIRT1, an antiinflammatory and antiaging protein, is decreased in lungs of patients with chronic obstructive pulmonary disease.* Am J Respir Crit Care Med, 2008. **177**(8): p. 861-70.
95. Yuan, F., et al., *SIRT2 inhibition exacerbates neuroinflammation and blood-brain barrier disruption in experimental traumatic brain injury by enhancing NF-kappaB p65 acetylation and activation.* J Neurochem, 2016. **136**(3): p. 581-93.
96. Yu, S.S., et al., *Sirtuin 6 protects cardiomyocytes from hypertrophy in vitro via inhibition of NF-kappaB-dependent transcriptional activity.* Br J Pharmacol, 2013. **168**(1): p. 117-28.
97. Tao, Y., et al., *SIRT4 Suppresses Inflammatory Responses in Human Umbilical Vein Endothelial Cells.* Cardiovasc Toxicol, 2015. **15**(3): p. 217-23.
98. Qin, K., et al., *NAD(+) dependent deacetylase Sirtuin 5 rescues the innate inflammatory response of endotoxin tolerant macrophages by promoting acetylation of p65.* J Autoimmun, 2017. **81**: p. 120-129.
99. Chen, X., et al., *High levels of SIRT1 expression enhance tumorigenesis and associate with a poor prognosis of colorectal carcinoma patients.* Sci Rep, 2014. **4**: p. 7481.
100. Dikalova, A.E., et al., *Mitochondrial Deacetylase Sirt3 Reduces Vascular Dysfunction and Hypertension While Sirt3 Depletion in Essential Hypertension Is Linked to Vascular Inflammation and Oxidative Stress.* Circ Res, 2020. **126**(4): p. 439-452.
101. Chen, K.L., et al., *SIRT7 Regulates Lipopolysaccharide-Induced Inflammatory Injury by Suppressing the NF-kappaB Signaling Pathway.* Oxid Med Cell Longev, 2019. **2019**: p. 3187972.
102. Shi, T., et al., *SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes.* J Biol Chem, 2005. **280**(14): p. 13560-7.
103. Greiten, L.E., et al., *Sirtuin 6 Protects Against Oxidative Stress and Vascular Dysfunction in Mice.* Front Physiol, 2021. **12**: p. 753501.

104. Smirnov, D., et al., *SIRT6 is a key regulator of mitochondrial function in the brain.* Cell Death Dis, 2023. **14**(1): p. 35.
105. Singh, C.K., et al., *The Role of Sirtuins in Antioxidant and Redox Signaling.* Antioxid Redox Signal, 2018. **28**(8): p. 643-661.
106. Kwon, J., et al., *The Clinical Effects of Nicotinamide Riboside on Inflammatory Parameters*. Curr Dev Nutr. 2022 Jun 14;6(Suppl 1):987. doi: 10.1093/cdn/nzac068.016. eCollection 2022 Jun.
107. Lee, H.J. and S.J. Yang, *Nicotinamide riboside regulates inflammation and mitochondrial markers in AML12 hepatocytes.* Nutr Res Pract, 2019. **13**(1): p. 3-10.
108. de Castro, J.M., et al., *Nicotinamide Riboside Neutralizes Hypothalamic Inflammation and Increases Weight Loss Without Altering Muscle Mass in Obese Rats Under Calorie Restriction: A Preliminary Investigation.* Front Nutr, 2021. **8**: p. 648893.
109. Elhassan, Y.S., et al., *Nicotinamide Riboside Augments the Aged Human Skeletal Muscle NAD(+) Metabolome and Induces Transcriptomic and Anti-inflammatory Signatures.* Cell Rep, 2019. **28**(7): p. 1717-1728 e6.
110. Zhang, H., et al., *NAD(+) repletion improves mitochondrial and stem cell function and enhances life span in mice.* Science, 2016. **352**(6292): p. 1436-43.
111. Lee, H.J., et al., *Nicotinamide Riboside Ameliorates Hepatic Metaflammation by Modulating NLRP3 Inflammasome in a Rodent Model of Type 2 Diabetes.* J Med Food, 2015. **18**(11): p. 1207-13.
112. Trammell, S.A., et al., *Nicotinamide riboside is uniquely and orally bioavailable in mice and humans.* Nat Commun, 2016. **7**: p. 12948.
113. Veenhuis, S.J.G., et al., *Nicotinamide Riboside Improves Ataxia Scores and Immunoglobulin Levels in Ataxia Telangiectasia.* Mov Disord, 2021. **36**(12): p. 2951-2957.
114. Brakedal, B., et al., *The NADPARK study: A randomized phase I trial of nicotinamide riboside supplementation in Parkinson's disease.* Cell Metab, 2022. **34**(3): p. 396-407 e6.
115. Wang, D.D., et al., *Safety and Tolerability of Nicotinamide Riboside in Heart Failure With Reduced Ejection Fraction.* JACC Basic Transl Sci, 2022. **7**(12): p. 1183-1196.
116. Remie, C.M.E., et al., *Nicotinamide riboside supplementation alters body composition and skeletal muscle acetylcarnitine concentrations in healthy obese humans.* Am J Clin Nutr, 2020. **112**(2): p. 413-426.
117. Nascimento, E.B.M., et al., *Nicotinamide Riboside Enhances In Vitro Beta-adrenergic Brown Adipose Tissue Activity in Humans.* J Clin Endocrinol Metab, 2021. **106**(5): p. 1437-1447.
118. Martens, C.R., et al., *Chronic nicotinamide riboside supplementation is well-tolerated and elevates NAD(+) in healthy middle-aged and older adults.* Nat Commun, 2018. **9**(1): p. 1286.
119. Cora, M.C., L. Kooistra, and G. Travlos, *Vaginal Cytology of the Laboratory Rat and Mouse: Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears.* Toxicol Pathol, 2015. **43**(6): p. 776-93.

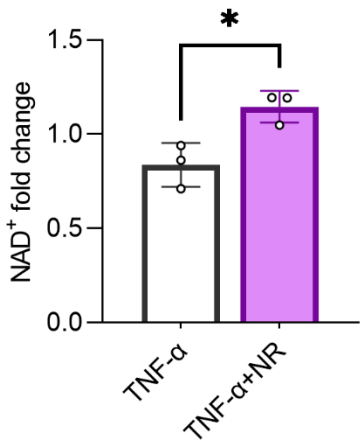
120. Lu, W., et al., *Extraction and Quantitation of Nicotinamide Adenine Dinucleotide Redox Cofactors.* Antioxid Redox Signal, 2018. **28**(3): p. 167-179.
121. Fischer, A.H., et al., *Hematoxylin and eosin staining of tissue and cell sections.* CSH Protoc, 2008. **2008**: p. pdb prot4986.
122. Frezza, C., S. Cipolat, and L. Scorrano, *Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts.* Nat Protoc, 2007. **2**(2): p. 287-95.
123. Kuznetsov, A.V., et al., *Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells.* Nat Protoc, 2008. **3**(6): p. 965-76.
124. Bankhead, P., et al., *QuPath: Open source software for digital pathology image analysis.* Sci Rep, 2017. **7**(1): p. 16878.

Supplementary 1



Supplementary Figure 1: Placental NAD^+ related metabolite-NAM levels and mitochondrial content in human PE subclasses. A) Placental NAM levels were determined by LC/MS. B-C) Placental mitochondrial content was determined by measuring activity of two markers- citrate synthase and complex IV by performing enzymatic activity assays (n=17-41 placentas/group). One-way ANOVA with Holm-Šídák's multiple comparisons test. Error bar indicates standard deviation (SD). D) Heatmap of downregulated mitochondrial proteins in hypoxia-driven PE2 placentas compared to control placentas (n=5-6 per group). Shown are only proteins that met the False Discovery Rate (FDR) threshold of 0.05. Control= control term and preterm, chronic hypertension control= (CH), Gestational parent-driven PE subclass = PE1, Hypoxia-driven PE subclass = PE2, Inflammation-driven PE subclass = PE3.

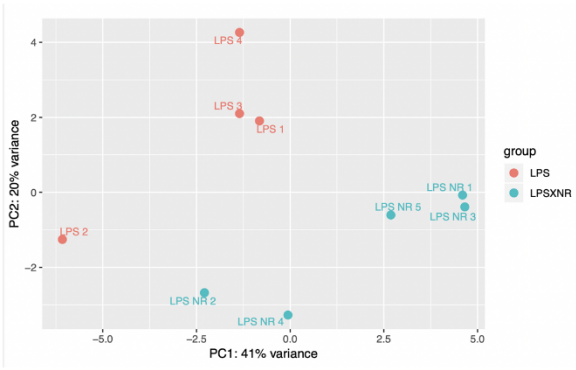
Supplementary 2



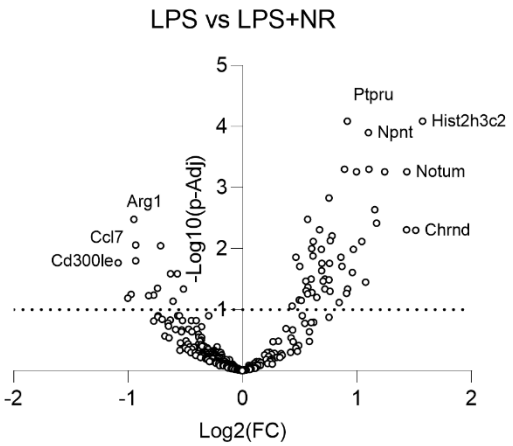
Supplementary Figure 2: NAD^+ boosting in HTR8/SVneo cells under an inflammatory condition. Total cellular NAD^+ quantification was performed on cells treated with 10ng/ml TNF-α and/or 250 μM NR for 24 hrs (n=3). One-tailed unpaired t-test, *$P < 0.05$. Error bar indicates standard deviation (SD). Error bar indicates standard deviation (SD). TNF-α= Tumor necrosis factor, NR= nicotinamide riboside.

Supplementary 3

A)



B)



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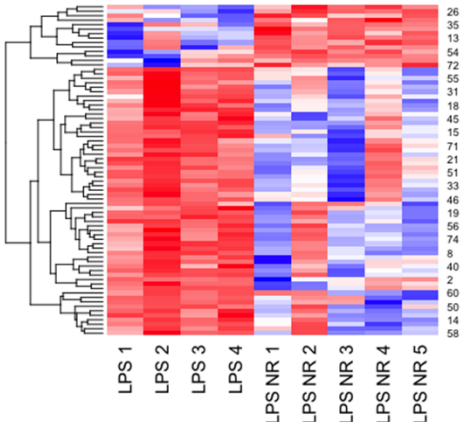

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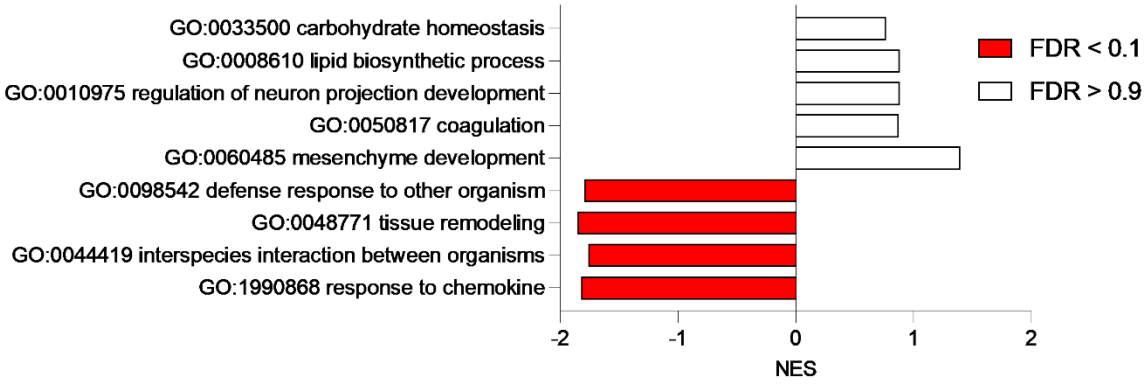
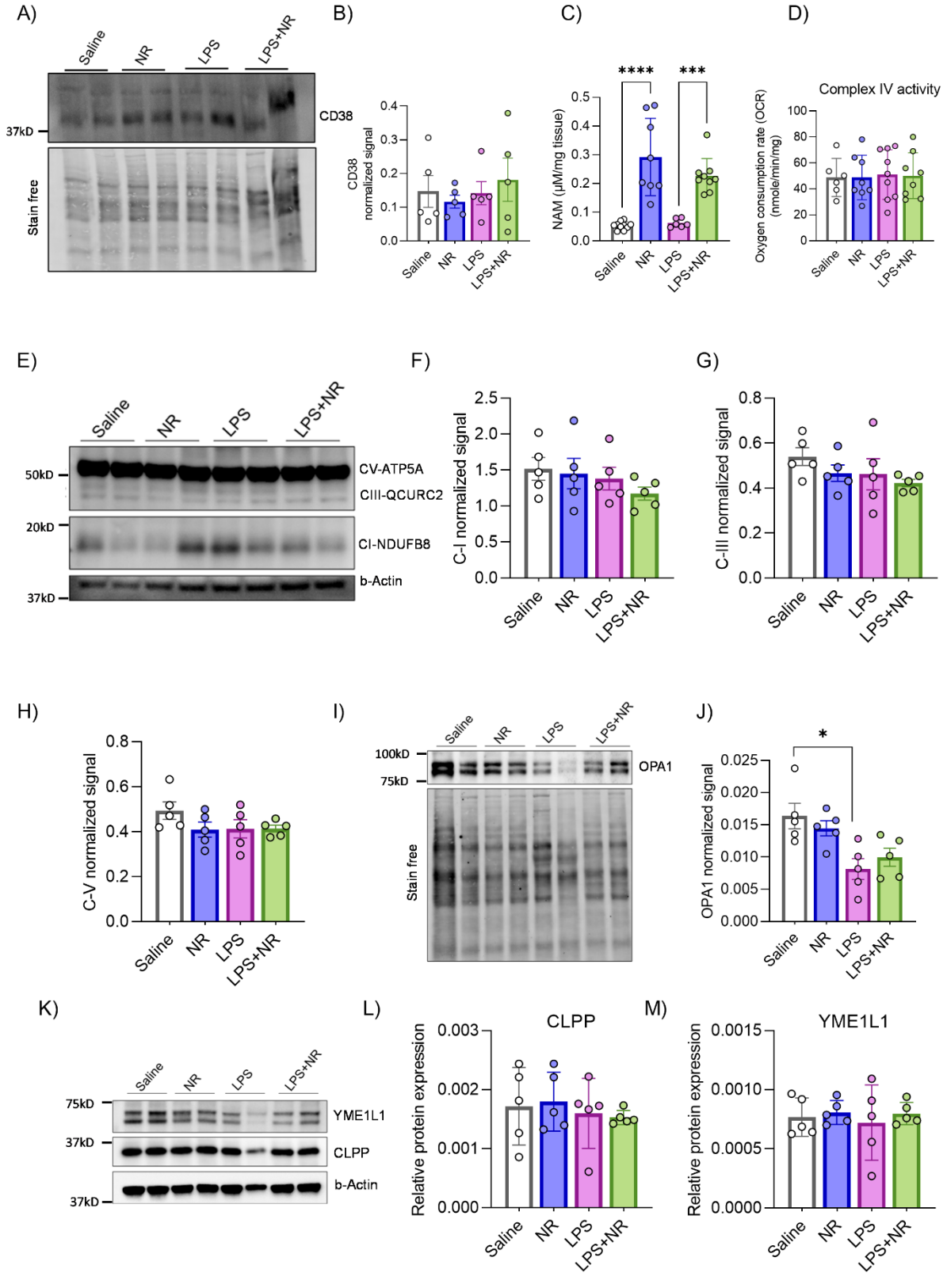


Supplementary Figure 3: Effect of NAD^+ boosting on placental transcriptome of a rat model of inflammatory PE. Principal component analysis (PCA), Valcano plot, Heatmap and GO term analysis were performed between LPS and LPS+NR treated rat placentas (n=4-5 litter/treatment, 1placenta/litter).


A)
Saline
NR
LPS
LPS+NR
37kD
CD38
Stain free
B)
CD38 normalized signal
C)
****
***
NAM (µM/mg tissue)
D)
Complex IV activity
Oxygen consumption rate (OCR) (nmole/min/mg)
E)
50kD
CV-ATP5A
CIII-QCURC2
20kD
CI-NDUFB8
b-Actin
37kD
F)
C-I normalized signal
G)
C-III normalized signal
H)
C-V normalized signal
I)
100kD
75kD
OPA1
Stain free
J)
*
OPA1 normalized signal
K)
75kD
YME1L1
37kD
CLPP
b-Actin
37kD
L)
CLPP
Relative protein expression
M)
YME1L1
Relative protein expression

Supplementary Figure 4: Effect of LPS and NAD^+ boosting on placental expression of NAD^+ consuming enzyme CD38, NAM levels and mitochondria. A-B) Placental expression of NAD^+ consuming enzyme CD38 were determined by western blot with anti-CD38 antibody (n=5 litter/treatment, 1placenta/litter). C) NAM levels were determined by LC/MS (n=6-10 litter/treatment, 1placenta/litter), D) Mitochondrial complex-IV activity was measured by Oxygraph assay (n=4-8 litter/treatment, 6-8 placentas/litter). E-M) Placental OXPHOS, OPA1, CLPP and YME1L1 protein levels were determined by western blot (n=5 litter/treatment, 1placenta/litter). Two-way ANOVA with Holm-Šídák's multiple comparisons test, *P＜0.05,**P＜0.01,***P＜0.001. Error bar indicates standard deviation (SD).

Supplementary 5

Materials and Methods

1. Human sample processing

1.1 Microarray analysis of gene expression:

A microarray dataset previously generated by our group and available at the Gene Expression Omnibus (GEO) database (GSE75010) was used to assess PE subclass-specific expression of inflammatory markers and NAD^+-consuming enzymes [3]. This microarray dataset was generated from frozen human placenta tissues that were purchased from RCWIH, Mount Sinai Hospital, Toronto, Canada. Briefly, RNA isolation was performed using Trizol and RNAeasy spin columns. Human Gene 1.0 ST Array chips (Affymetrix) was used to perform microarray at the Princess Margaret Genomics Centre (Toronto, Canada). The heatmap of expression of key genes of interest was generated in R studio.

1.2 Measurement of PARylation

Paraffin embedded placental tissue sections (5-µm), mounted on glass slides, were purchased from RCWIH, Mount Sinai Hospital, Toronto, Canada. These specimens were collected from the same cases as the frozen tissue biopsies described above (Table 1) and were used to assess protein PARylation and oxidative stress (section 1.5 below) within the placenta for each of the three PE subclasses and controls. Sections were dewaxed in xylene, and hydrated in a sequence of 100%, 95%, 80%, 70% and 50% ethanol, and water. sections were subjected to antigen retrieval using citrate buffer (0.1 M, pH 6.0) in microwave for 7 minutes and endogenous peroxidase blocking using 3% H_2O_2 in PBS for 30 minutes. To prevent nonspecific binding, protein block solution (DAKO, X0909) was utilized for 1 hour. Subsequently, the tissue sections were incubated overnight at 4°C with Anti-PAR antibody (1:250, Cell signalling 83732) diluted in PBS containing 1% BSA, overnight at 4°C. Then, slides were incubated with Alexa Fluor 595 goat anti-rabbit secondary antibody (1:4000, Invitrogen A-11012) and kept in dark for 1h at room temperature. Tissue was counterstained with DAPI (Promega, P36931). 5 snapshots of random regions of interest (20X magnification) of the placental villi (trophoblast) were selected for fluorescence intensity quantification using ImageJ software.

1.3 Measurement of NAD(H)

Flash frozen human placental samples (same cases as described above) were pulverized in liquid nitrogen to generate powdered tissue used for NAD(H) and mitochondrial protein analysis (section 1.4 below). For measurements of NAD(H), 13-30 mg of powdered placental tissue was weighed in a 1.5 ml microcentrifuge tube and

1000 µl of solvent A (40:40:20 acetonitrile:methanol:water with 0.1M formic acid) was added. The mixture was vortexed for 10 sec and kept on ice for 3 min. 87 µl of solvent B (15% NH4HCO3 in water) was added, vortexed and kept on dry ice for 20 min to neutralize. The sample was centrifuged at 16,000 g for 15 min at 4 °C and supernatant was collected for LC-MS analysis [120].

NAD, NADH and NAM were determined using validated liquid chromatography mass spectrometry/mass spectrometry (LC/MS/MS). After sample extraction, three internal standards [IS, 13C5NAD, 13C 2,6,7 NAM and 6,7-Dimethyl-2,3-di(2-pyridyl)-quinoxaline (DMDPQ)] were added to all samples [blanks, standards, quality controls (QC) and tissue extracts]. After vortexing and centrifugation, the supernatant was transferred to auto- sampler vials for injection. The chromatographic separation was carried on an LC system (Accela, Thermo). The column was a Synergi Polar-RP (5 cm x 4.6mm, 2.5 µm) column (phenomenex). Separation was performed at 30 °C with a gradient elution of acetonitrile- 0.1% (v/v) formic acid in water at a flow rate of 0.5ml/min. The auto-sampler was set at 4°C and the injection volume was 10 ul. Mass spectral analyses were accomplished on a quadrupole tandem mass spectrometer (TSQ Quantum Access MAX, Thermo) with electrospray ionization in positive mode. Multiple-reaction-monitoring (MRM) mode was used to detect all compounds. MRM transitions were m/z 646 to 136, 666 to 649, 123 to 80, 669 to 136, 127 to 83, and 313 to 246 for NAD^+, NADH, NAM, 13C5NAD,13C 2,6,7 NAM, and DMDPQ, respectively. The calibration ranges were 200-20000 ng/ml, 100- 10000 ng/ml, 5-500 ng/ml for NAD^+, NADH and NAM, respectively. The intra-day and inter- day precision were all between 80-120% and all extracted samples were stable at 4 degrees for 24 hours.

1.4 Measurement of mitochondrial proteins

For measurements of mitochondrial proteins, 12.5 mg of powdered placenta sample was added to 0.75 mL of cold lysis buffer (1% SDS in 50 mM triethylammonium bicarbonate (TEAB), Thermo Scientific Product No. 90114), and homogenized by passing 20 times through 21 G needle. Lysate was centrifuged at 20,000 x g for 10 minutes at 4C. Supernatant was separated, protein concentration was measured using BCA Protein Assay Kit (Thermo Scientific Product No. 23227), and 100 ug of sample was transferred into a new tube and adjusted to a final volume of 100 uL with 100 mM TEAB. 10 mM Reduction buffer (from freshly prepared 1M dithiothreitol in water) was added and kept at room temperature for 1 hour. 20 mM Alkylation buffer (from 1M iodoacetamide in water) was added and kept at room temperature for 45 min in the dark. Six volumes of pre-chilled protein precipitation buffer (50% acetone + 50% ethanol + 0.1% acetic acid) was added, vortexed and allowed to precipitate overnight at -20C. The following day, samples were centrifuged in a microfuge for 10 minutes at 10,000 x g. The supernatant was discarded carefully, and the remaining precipitation buffer was evaporated for 20 minutes at room temperature without over drying. 100µg of precipitated protein pellets was resuspended with 100µL of 100mM TEAB. Trypsin (Trypsin Gold, Promega) was used to digest the proteins (1:100 trypsin to protein by weight) and kept at 37°C for overnight. Peptide labeling was performed using TMT10plex Isobaric Label Reagent Set plus TMT11-131C (A34808, Thermo Scientific), following manufacturer's instructions.

Following reconstitution in 20uL 2% acetonitrile and 0.1% formic acid in water, 2uL was injected for analysis. The system used consisted of an Ultimate 3000 coupled with an

Exploris 480 mass spectrometer (ThermoFisher Scientific, San Jose, CA) equipped with a Nanospray Flex interface operated in positive ion mode. The mobile phases consisted of 0.1%(v/v) FA in water as buffer A and 0.1% (v/v) FA in 80% acetonitrile as buffer B. The analysis was done on a column of 75µm × 200 mm, packed also in-house with reverse-phase Magic C18AQ resins (3µm; 120-Å pore size; Dr. Maisch GmbH, Ammerbuch, Germany).

Briefly, the sample was loaded on the column using 98% buffer A at a flow rate of 1µL/min for 10min. Then, a gradient from 5% to 35% buffer B was performed in 60 min at a flow rate of 300nL/min. The MS method was set up following the TMT MS2 template in Xcalibur 4.3.73.11. Specifically, it consists of one full MS scan from 350 to 1200 m/z, with a resolution of 120 000, defined at m/z 200m, followed by data-dependent MS/MS scan within 2 seconds, with dynamic exclusion of 1, exclusion duration of 45 s, and mass tolerance of 10 ppm, at an intensity threshold of 5E3. The isolation window was set to 0.7 Da, and the HCD collision energy was 36. The resolution of MS2 was set to 45,000, with the first mass as 110 to include all the TMT tags. To improve the mass accuracy, all the measurements in the Orbitrap mass analyzer were performed with internal recalibration (“Lock Mass” at 445.120025). On the Orbitrap, the charge state rejection function was enabled, with only 2~5 charges included. The raw data were processed and analyzed using MaxQuant (version 1.6.6.0). A target-decoy database search was carried out for *Rattus norvegicus* UniProtKB/Swiss-Prot and UniProtKB/TrEMBL protein sequence databases including commonly observed contaminants and their reversed sequences. A precursor ion mass tolerance of 0.5 Da was used to carry out this database

search. TMT reporter intensities were corrected using the error correction factors provided with the kit.

1.5 Measurement of oxidative stress

Placental tissue sections (5 um) were deparaffinized and subjected to antigen retrieval as mentioned in 1.2. DNA was denatured by treating the slides with 2N HCl for 5 minutes at room temperature, followed by a neutralization step in 1M Tris-base for 5 minutes at room temperature. Non-specific binding sites were blocked by incubating the sections in 10% normal goat serum in PBS, 1h at room temperature. Next, tissues were incubated with 8-oxo-dG antibody (1:250, R&D Systems 4354-MC-050) diluted in PBS containing 0.1% BSA, overnight at 4°C. Slides were incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (1:4000, Invitrogen A11029) in PBS containing 0.1% BSA in dark for 1h at room temperature. Tissue was counterstained with 7AAD (1:50, Invitrogen A1310) diluted in water for 30 minutes at room temperature in the dark. Quantification was performed as described in 1.2.

2. Cell culture measurements

2.1 Measurement of PARylation

The HTR-8/SVneo cells were cultured in Gibco® 1X RPMI-1640 (Life Technologies™) medium supplemented with 10% fetal bovine serum (FBS), at 37 °C, 5% CO_2 and ~20% O_2 condition. 50,000 cells were plated on a 12 well-plate and allowed to grow overnight. The following day the cells were treated with TNF-α (10mg/ml), with and without NR (150 µM- 1 mM), for 24 hrs. A supplemented radioimmunoprecipitation (RIPA) buffer was prepared, which included Roche Complete Protease Inhibitor Cocktail

(05892970001, Roche Canada, Basel, Switzerland), Roche PHOStop Phosphatase Inhibitor Cocktail (4906837001 Roche Canada, Basel, Switzerland), 5 mM of sodium butyrate, 100 µM of fresh tannic acid, and 1 µM of olaparib. Cells were then washed with ice-cold PBS and RIPA lysis buffer was added to each dish. Lysate was collected by scraping with a cell scraper (Thermo Scientific™). The lysate was vortexed and kept in a rotor for 30 min for slow mixing. The lysate was then centrifuged at 20,000 relative centrifugal force (r.c.f.) at 4°C for 15 minutes, and the supernatant was collected.

The protein concentration was determined using the DC protein assay (Bio-Rad Laboratories, Hercules, California, USA), with Bovine serum albumin (BSA) standards prepared in RIPA buffer. The resulting supernatant was then transferred to new tubes and stored in a -80°C freezer. The samples were prepared with 4x Laemmli Buffer (Bio-Rad Laboratories, Hercules, California, USA Cat# 1610737) augmented with 10% β-mercaptoethanol (Fisher Bioreagents, BP176-100) and boiled for 5 minutes.

Next, western blotting was performed using 8%, 10%, or 12% SDS-PAGE gels, depending on the size of the protein of interest. The TGX Stain-free FastCast Acrylamide Kit (Bio-Rad Laboratories, Hercules, California, USA Cat# 1610183) with 10% Ammonium persulfate (APS) was used to make the gel. Following SDS-PAGE, the Stain Free gels were activated and then the proteins were 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). To detect the total protein, the blot was imaged again under stain-free blot setting with automatic exposure time. Then the blot was blocked 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. The membranes were then incubated with primary anti-PAR antibody (Millipore Sigma, AM80-100UG) diluted in TBS-T buffer overnight at 4°C with gentle rocking. After three washes each for 10 minutes with TBS-T, the membrane was incubated for one hour with the matching IgG, horseradish peroxidase (HRP)-conjugated secondary antibody, rocking at room temperature. After three 10 min washes in TBS-T, detection was carried out 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. Later, images were retrieved, and the quantification of blots was performed using either ImageLab or FIJI software.

2.2 Measurement of NAD(H)

250,000 HTR-8/SVneo cells were plated on a 6 cm^3 plate and incubated overnight in serum starved RPMI 1640 medium (Gibco® 1X RPMI-1640, Life Technologies™) at 37 °C, 5% CO2 and ~20% O_2 condition. The following day, 10% FBS containing RPMI 1640 medium was added, and cells were treated with 10 ng/ml TNF-α, with and without NR 250 μM, and incubated for 24 hrs. NAD(H) levels were quantified using a Biovision NAD^+/NADH kit (K337) according to manufacturer's instruction.

2.3 Measurement of mitochondrial respiration and protein content

Mitochondrial respiration or oxygen consumption rate (OCR) was measured by performing XFe96 seahorse assay. 2,500 cell/well was plated in seahorse cell culture plate in 180 ul of RPMI 1640 media with 10% FBS and incubated at 37°C, 5% CO_2 for

24 hrs. 12 hours prior to running the assay cartridge was hydrated. 200 uL of XF Calibrant (pH 7.4) was added to each well of the calibration plate and the cartridge was placed on the machine and sealed. The cartridge and calibration plate were placed in a 37°C incubator (no CO_2). 100 mL of seahorse media was prepared freshly before starting the assay. Media constituents: 8.3 g/L DMEM powder (no H2CO3), 2 g/L D-glucose, 1 mM pyruvate, 0.3 g/L glutamine and made in 90ml of sterile molecular grade water. pH was set at 7.4 and the media was topped up to 100 ml with molecular grade water and filter sterilized. Cells were washed with seahorse media 2x. The seahorse plate was placed in the 37°C incubator (no CO_2) for 45 minutes to allow the plate to equilibrate. All seahorse drugs were prepared in seahorse media and loaded in the cartridge. Calibration was performed, followed by measurements of the basal respiration. Oligomycin (1 μM) was injected to inhibit mitochondrial complex V to record non-phosphorylating OCR. FCCP (0.5 μM), a mitochondrial uncoupler was injected to record maximal OCR. Antimycin A (1 μM) was injected to determine non-mitochondrial respiration to subtract from the recorded basal and maximal respiration. Monensin (200 μM) was injected to record glycolytic capacity of the cells by recording extracellular acidification rates. Results were normalized by measuring protein concentration of each well via Bradford assay. Briefly, seahorse media was removed from plate using a multichannel pipette and cells were washed with 100uL PBS. 30 uL of the lysis buffer (1mM Tris-HCL- pH 7.4, 1mM EDTA, 0.5% triton 100x) was added to each well using a multi-channel pipette and mixed gently, with 5 uL from each well mixed with 195 uL of 1:5 Bradford reagent (Biorad) for protein quantification. Absorbance was read at 595nm

using a spectrophotometer. The measurement was compared with BSA protein standards to get relative quantification.

Mitochondrial OXPHOS protein expression was assessed by western blotting. Briefly, HTR-8/SVneo cells were cultured as per conditions described in 2.1 [TNF-α (10mg/ml) ± NR (250 μM) for 24 hrs]. Western blotting protocol was similar to that described in 2.1, using the OXPHOS antibody cocktail (ab110413).

2.4 Measurement of invasive capacity

250,000 HTR8/Svneo cells were plated on 6cm^3 plates. After overnight incubation, cells were treated with TNF-α (10ng/ml) ± NR (250μM) in 10% FBS containing RMPI1640 media for 48hrs. Media was changed at 24hrs, with replenishment of TNF-α (and/or NR treatment. Matrigel coated invasion inserts were used in a boyden chamber invasion assay. Briefly, a 24 well-plate containing Matrigel inserts were removed from freezer and warmed to room temperature (30 minutes). 750ul of serum free warm RPMI 1640 media was added to the bottom of each well, with an additional 500ul media added on top of each insert and incubated at 37° C for 2hrs to rehydrate the Matrigel. HTR8/Svneo cells were then trypsinized, neutralized, and transferred to top portion of the chamber, onto of the Matrigel (50,000 cells/well) in serum depleted RPMI1640 media. Serum enriched media was placed in the bottom of the chamber, promoting cellular migration through the Matrigel. The cells were incubated in the chamber overnight (with all the treatments) at 37° C, at 5% CO_2 and ~20% O_2 condition.

After a 24 hrs incubation, media was removed from top and bottom of the chamber, with the insert removed and washed with PBS (2x). Then, 700uL of 10% Formalin was

added to the wells, fixing the adherent cells to the insert at room temperature for 10 minutes. Inserts were removed from the fixative, and wash 2x with PBS. Inserts were then placed in 100% methanol for 20 min at room temperature to permeabilize the cells. Inserts were then washed 2x with PBS, and adherent cells stained with hematoxylin for 10 minutes at room temperature. Inserts were washed once more with PBS, followed by tap water twice. Using a cotton swab, any cells remaining in the top chamber (non-invaded cells) were removed. The insert was allowed to air dry, following which the membrane was cut out and mounted on a glass slide.

3. Animal model measurements

3.1 Measurements of pregnancy outcomes

Maternal mean arterial blood pressure (MAP) was assessed across the last portion of pregnancy (GD14-19) for each of the four different treatment groups (control, NR alone, LPS alone, LPS + NR; n=6-8/group). Measurements were captured using the CODA® high-throughput non-invasive tail cuff measurement system (Kent Scientific Corporation). Animals were first acclimatized to the system for 4-5 days. Prior to recording, animals were stabilized for 5-10min, and body temperature was checked. Then BP was recorded for 20min on each testing day.

At GD19, animals were sacrificed. Litter size, number of resorptions, fetal weights and placental weights were collected. Placentas were collected and flash frozen for RNA, metabolite, and protein measurements. One placenta per litter were fixed in 4% PFA for histopathology.

3.2 Measurement of placenta histomorphology

PFA-fixed mid-line placenta tissues (n=1 placenta/litter; n= 10 litters/ group) were paraffin embedded, sectioned at 5 uM thickness and placed on glass slides. Sections were deparaffinized, rehydrated and stained with H&E using standard protocols [121]. Whole- slide image was captured using Axio Scan.Z1 microscope at 20X magnification. Measurements of total placenta mid-line cross-sectional area, as well as cross-sectional areas of each of the individual regions of the placenta (chorionic plate, labyrinth zone, junctional zone) were analyzed using ZEN 3.1 blue edition.

3.3 Measurement of placenta gene expression

RNA was isolated from frozen placenta tissues (n=1 placenta/litter; n=4-5 litters/group) using standard Trizol® methods. Purified RNA was submitted to StemCore Laboratories at the Ottawa Hospital Research Institute for RNA-Sequencing as follows. RNA quantification and quality was assessed using a Qubit HS RNA assay (ThermoFisher Scientific, Q32852) and the Fragment Analyzer Standard Sensitivity RNA assay (Agilent, DNF-471-0500). All samples had an RNA Quality Number (RQN) above 8.0. DNA library was prepared using the Illumina Stranded mRNA Preparation Kit (Illumina). Libraries were quantified using the Qubit Double Stranded DNA HS kit (ThermoFisher Scientific, Q33230) and integrity confirmed with the High Sensitivity NGS assay on an AATI Fragment Analyzer (Agilent). DNA libraries were normalized, pooled, and diluted. 75-basepair single-end sequencing reads were obtained from pooled DNA libraries using the Illumina NS500 75 cycle high output kit on an Illumina NextSeq 500 Sequencer.

Sequencing results were separated by library barcoding, and barcodes removed using Illumina BaseSpace. Subsequently, data was imported into the Galaxy Platform maintained by GenAP (http://www.genap.ca) and hosted by the Digital Research Alliance of Canada. Quality of sequencing was assessed using the FastQC function. Sequences were aligned to the rat genome (mRatBN7.2) and read quantification was performed using the HISAT2 and feature Counts functions, respectively. Count normalization, differential gene expression, and principal component analysis between control and LPS treated group was performed using DESeq2 and an FDR of 10%. Using only the differentially expressed genes with LPS treatment, to determine genes rescued with NR treatment, a subsequent analysis using DESeq2 identified genes that changed between LPS and LPS with NR groups with an FDR of 10% was performed.

Z-scores were calculated from normalized counts of differentially expressed genes and heatmaps generated in R studio. Gene Set Enrichment Analysis was performed using WebGestalt (http://www.webgestalt.org/) and the Reactome and Gene Ontology Biological Process databases.

3.4 Measurement of placenta TNF-α protein content

Placenta TNF-α protein expression was measured using Quantikine® Rat TNF-α Immunoassay immunosorbent assay (R&D Systems, Minneapolis, MN). Frozen placenta tissue (n=1 placenta/litter; n=7-9 litters/group) was pounded in liquid N_2 and weight about 40mg for homogenising 5mM EDTA/PBS extraction buffer with protease inhibitor cocktail (complete™ Roche 11836170001). TNF-α ELISA was then carried out according to manufacturer's protocol.

3.5 Measurement of protein PARylation

Flash frozen placenta (n=1 placenta/litter; n=10 litters/group) was pulverized, and 15-25 mg of tissue was used. RIPA buffer was added to the tissue and homogenized using 21G syringe. The homogenate was vortexed for 30 min to ensure complete homogenization. The homogenate was centrifuged at 20,000 relative centrifugal force (rcf) for 15 minutes at 4°C, and the supernatant was transferred to a 1.5 mL microcentrifuge tube. Western blotting was performed as described in 2.1 using anti-PAR antibody (Millipore Sigma, AM80-100UG).

3.6 Measurement of NAD(H)

30-40mg of powdered placenta tissue (n=1 placenta/litter; n=8-10 litters/group) was added to 300ul of extraction buffer. To prepare a homogeneous solution, the solution was drawn up and down through a 21-gauge syringe needle. Quantification was performed using the Biovision NAD^+/NADH kit (K337), according to the manufacturer's instructions.

3.7 Measurement of NAM and ADPR

Placental ADPR and NAM levels were quantified at the metabolomics core facility of University of Ottawa. Approximately 40 mg (+/- 10%) of powdered placental tissue (generated from n=1 placenta/litter; n=6-10 litters/group) was taken and added to 1.8 ml pre-chilled (-80°C) 80% methanol. Four 2.8 mm ceramic beads were added to each tube and the samples were homogenized at 2,000 rpm for 45 seconds using a MagNa Lyser bead homogenizer for up to 3 cycles. To keep samples, cool between cycles, the tubes were kept on dry ice for 1 min. Following homogenization, 1.8 ml of pre-chilled

dichloromethane (-20°C) and 900 µl of ice-cold molecular grade water were added to the homogenate. For lipid removal, the mixture was vortexed and kept on ice for 10 minutes for partitioning. Samples were then centrifuged at 4,000 rpm at 1°C for 10 min and the upper aqueous phase was carefully collected and dried at 4°C using a refrigerated centrifugal concentrator (Labconco Refrigerated CentriVap Benchtop Vacuum Concentrator).

For quantification, 0.5 ug/ml of deuterated d5-Trp (Tryptophan) and 0.05 ug/ml of d4-NAM were used as Internal Standard (IS) in 75% acetonitrile to reconstitute the study samples. The mix was further tested in samples to check carryover or matrix effect on the endogenous metabolite level. Samples were reconstituted to a final volume of 50 µL. Samples were used undiluted for ADPR measurements, and 10X diluted (in 75% acetonitrile) for NAM measurements. Mass spectral analyses were accomplished on a Quadrupole time-of-flight (QTOF) mass spectrometer (MS) (Agilent 6545B), with electrospray ionization in positive mode connected to an ultra-high performance liquid chromatography system (Agilent 1290 InfintyII). 2 µL of samples was injected into the system., and Agilent Q-TOF Quantitative Analysis software (Agilent technologies inc. Santa Clara, CA, USA) was utilized to process and analyze the data. The calibration curve was optimized for each metabolite. Following peak detection, peak area was integrated using the Agile2 integrator. A >10 signal/noise ratio and accuracy of 80%-120% was used. Metabolite concentrations were normalized by the IS signal and adjusted to account for dilution factors. Metabolite concentrations were then normalized by the tissue weight.

3.8 Measurement of mitochondrial respiration and protein content

Freshly isolated mitochondria were used to measure mitochondrial respiration rates using a high-resolution oxygraph assay. Fresh placenta tissue (n=6-8 placentas/litter; n=4-8 litters/group) was collected to isolate mitochondria according to a modified version of a previously published protocol [122]. Briefly, placentas were washed in ice-cold PBS and transferred to a beaker containing buffer A [300mM sucrose mM, 10mM Tris-HCl, 1mM EGTA-Tris Base and 0.1% bovine serum albumin (BSA, fatty acid-free); pH 7,2, kept on ice]. Placentas were cut into smaller pieces and washed with buffer A twice to eliminate residual blood. Placentas were minced and transferred to a Potter-Elvehjem homogeniser (30ml) and homogenised at 500rpm for 6 strokes. The homogenate was centrifuged for 10 min at 1,000xg at 4 °C. The supernatant was transferred to a clean tube and

centrifuged again with the same settings. Next, the supernatant was collected using a syringe to eliminate fat layer. The supernatant was collected in a clean tube and centrifuged for 10 min at 8,000xg at 4 °C. The supernatant was removed, and the pellet was resuspended in 2ml of buffer B (300mM sucrose, 10mM Tris-HCl and 0.05mM EGTA- Tris Base; pH 7) and centrifuged for 10 min at 8,000xg at 4 °C. This step was repeated. 200µl of MiR05 respiration buffer (110 mM D-sucrose, 60 mM lactobionic acid, 20 mM taurine, 20 mM HEPES, 10 mM KH_2PO_4, 3 mM $MgCl_2$, 0.5 mM EGTA, 1 g/L fatty-acid free BSA; pH 7.1 at 23 °C) was added to the pellet and gently mixed to make a mitochondrial suspension. Mitochondrial protein content was measured by performing detergent compatible colorimetric (DC) protein assay (Biorad).

Mitochondrial respiration rates were determined by using an Oxygraph assay, according to a published protocol [123]. Briefly, mitochondria (2 mg/ml in MiR05 respiration buffer) were added to the chamber and baseline traces were recorded. Substrates and inhibitors of mitochondrial respiration were injected in the following series: i) 5mM glutamate and 2.5mM malate, a Complex I substrate; ii) 1mM ADP, to measure Complex-I driven state-III respiration; iii) 1mM amytal to inhibit Complex I; iv) 5mM succinate to measure Complex-II driven state-III respiration; v) 5μM antimycin A to inhibit Complex III to prevent electron transfer to complex IV. This allows to measure complex-IV mediated respiration by adding substrate exogenously; vi) 5/0.3 mM Tetramethyl-p- phenylenediamine/ascorbate (TMPD)/Ascorbate) to measure Complex-IV driven state-III respiration, and vii) Finally, Potassium cyanide (KCN) to inhibit complex IV.

Mitochondrial protein expression was measured in flash frozen placenta tissues (n=1 placenta/litter; n=10 litters/group). Samples were prepared as described in 3.5 and western blotting was performed according to methods described in 2.1, using anti-OXPHOS (Abcam, ab110413), Anti-OPA1 antibody (Abcam, ab42364), YME1L1 (Proteintech, 11510-1-AP) and anti-CLPP (Abcam, ab124822) antibodies.

3.9 Measurement of placenta oxidative stress.

Measurement of placental p-H2A.X protein expression, a marker of double stranded DNA-breaks and oxidative stress, were assessed using frozen placenta tissues (n=1 placenta/litter; n=10 litters/group) by western blot. Samples were prepared as described in 3.4 and western blotting was performed according to 2.1. using p-H2A.X antibody

(Cell Signaling #2577). In parallel, paraffin embedded placenta tissue sections from the same litters (n=1 placenta/litter; n=5 litters/group) underwent deparaffinized, antigen retrieval and blocking for non-specific binding as described in section 1.5. Tissue sections were incubated overnight at 4°C with pH2A.X antibody (1:300, Cell Signaling #2577) diluted in PBS containing 1% BSA. Slides were then incubated with secondary antibody for 1 hour at room temperature, followed by incubation with streptavidin-HRP for 30 minutes, using the LSAB2 system-HRP (DAKO, K0675). The reaction was developed using 3,3′- Diaminobenzidine (DAB) (Sigma Aldrich D5637) plus H2O2. The tissues were counterstained with Harris hematoxylin and mounted in entellan. Whole slide images were captured using a Zeiss Axio Scan Z.1 scanner at 20X magnification and analyzed using QuPath software [124]. The decidua, junctional zone, and labyrinth were delimited to perform layer-specific automated positive cell counting. The results were expressed as pH2A.X positive cells per mm

Chapter 6 - General Discussion

6.1 Integration of major findings and interpretation

In the present thesis, using complementary datasets generated through detailed profiling of human placenta biopsies, the use of an *in vitro* human trophoblast culture model and the use of a rodent model of inflammatory PE, we have shown for the first time that NAD^+ levels are critical for maintaining placental mitochondrial function and that compromised NAD^+-signalling is linked to placental dysfunction in the context of inflammation-mediated PE. Specifically, we demonstrated that chronic inflammatory insults deplete placental NAD^+ content, via the activation of PARylating PARP enzymes. Further, this thesis demonstrates for the first time the therapeutic potential of targeting

placental NAD^+ signalling, via NAD^+ supplementation, to prevent placental dysfunction and the clinical manifestations of PE in the face of chronic inflammatory insults. Collectively, this body of work has expanded our current understanding of the distinct molecular mechanisms responsible for establishing placental dysfunction in cases of PE, with considerable gains in knowledge related to the inflammatory subclass of PE – a subclass that has been largely understudied to-date. Further, this body of work establishes a solid rationale for the exploration of NAD^+ targeted therapies in the context of PE – a common and debilitating obstetrical condition that currently has no effective treatment or cure. The major findings presented in this thesis, along with the impact of those findings, are summarized below:

i. *Placentas from human cases of inflammation-driven PE demonstrate dysregulated NAD^+ signaling*: Analysis of a pre-existing gene expression dataset from cases of PE that spanned all three subclasses, demonstrated an over-expression of pro-inflammatory signalling molecules (i.e. TNF-α) accompanied by a number of PARP transcripts uniquely in samples from the inflammation-driven PE subclass. Matched histological placenta specimens confirmed the presence of heightened PARP activation in these placentas, with increased immunoreactivity for the presence of PARylated proteins. These findings were coupled to a decrease in placental NAD^+ content, evidence of altered mitochondrial proteome content and oxidative damage. This NAD^+-centric disease framework has been widely described in other inflammatory disease models of non-pregnant populations [60, 84-89], however this is the first time it

has been identified and described in the context of an inflammatory obstetrical condition.

ii. *Improved human trophoblast function with NAD^+ boosting: In vitro*, under pro-inflammatory conditions induced by exogenous TNF-α treatment, human trophoblast cells likewise demonstrate heightened PARP activation, with increased protein PARylation, depleted NAD^+ stores and reduced mitochondrial respiration. However, with NAD^+ supplementation, all these NAD^+-signalling measurements returned to normal. Most importantly, the functional deficits of decreased cellular invasion, observed following pro-inflammatory stimulation, were rescued by NAD^+ supplementation, suggesting a therapeutic benefit to placental function with NAD^+ targeting strategies.

iii. *An LPS-induced inflammatory rat model most closely replicates inflammation-mediated PE in humans:* Rodents (both mice and rats) do not naturally get PE, yet several experimental protocols have been developed to illicit PE-like symptoms in a variety of rodent models. Most of these models are well suited for the study of gestational parent-driven or hypoxia-driven PE (subclasses 1 and 2), with only a few models generated using an inflammation-driven approach (subclass 3). However, to date, no comprehensive validation of these inflammatory PE models has been completed, and as such there is no consensus on which model best replicates the inflammatory PE condition observed in humans. In a direct comparison of three of the most commonly used rat models of inflammation-driven PE (TNF-α-infusion, Poly I:C and LPS models), it was determined that only LPS administration was effective in inducing a

comprehensive model of human immune-mediated PE, with evidence of maternal hypertension, placental inflammation and profound placental dysfunction with ensuing fetal growth restriction. Of central importance to the current thesis, this model likewise demonstrates evidence of dysregulated placental NAD^+ metabolism and mitochondrial dysfunction, and as such serves as a strong model to study the establishment of placental disease in this unique PE subclass.

iv. *NAD^+ supplementation during pregnancy prevents the development of placental dysfunction and clinical signs of PE in a rodent model of inflammation-driven PE:* With a validated rodent model of inflammation-driven PE in hand, studies focused on therapeutic prevention of PE establishment via NAD^+ supplementation were carried out. Arguably the most impactful findings from this thesis demonstrate that NAD^+ supplementation with NR can prevent the clinical manifestation of PE in LPS-treated dams and prevent the development of fetal growth restriction in the inflammation-exposed pups. In depth analysis of these treated placentas indicate that NAD^+ supplementation can modulate the inflammatory response within the placenta, improving mitochondrial function, supressing oxidative damage and increasing its overall size – collectively preventing the establishment of placental dysfunction to the benefit of fetal growth profiles. Such benefits of NAD^+ boosting are consistent with the literature on animal models of other non-pregnant inflammatory diseases, including ageing, neurodegeneration and muscular dystrophy [60, 84, 85, 88, 89, 252]. In these well described models, NAD^+ boosting is proposed to improve ETC

function, NAD^+/NADH redox homeostasis, activate mitochondrial antioxidants and other NAD^+ dependent enzymes [54, 88, 91]. Further, the anti-inflammatory effects of NAD^+ have been well described, indirectly as a secondary effect of reduced oxidative stress or improvement in cellular function, or more directly via upregulation of anti-inflammatory protein expression [87, 91, 252].

6.2 Clinical Implications of findings

In pre-clinical studies in non-pregnant populations, boosting NAD^+ has been demonstrated as an effective therapy to improve mitochondrial function in the context of several disease pathologies [60, 84-91, 247, 253-261]. In human clinical trials, NR has been shown to successfully increase systemic levels of NAD^+ and its related metabolites, without causing any adverse effect [257, 261, 262]. Collectively, these studies have demonstrated that therapeutically elevating NAD^+ levels improves metabolic health [261, 262], although there has been a degree of variability in published findings to date - likely the result of methodological considerations including small sample size, differences in dosage used and heterogeneity among the study participants. Healthy middle-aged individuals (men and women) who received 1,000mg NR/day for 3-6 weeks demonstrated lower systemic inflammation and improved cardiovascular function [263] [264], and a similar treatment regimen in a population of obese and overweight individuals (men and women) resulted in an improvement in body composition and metabolic rate [265, 266]. In patients with heart failure, two studies have demonstrated that administration of 2,000mg NR/day for 9 days to 12 weeks improved mitochondrial function and reduced inflammation in the peripheral blood mononuclear cells (PBMCs) [257, 267]. In patients with Parkinson's, administration of

1,000mg NR/day for 30 days, improved cerebral metabolic function [268] and in patients with Ataxia administration of 25mg NR/day for 16 weeks improved physical function [269]. A recent study demonstrated that NR administration at incremental doses of 250mg-1000mg/day for 5 months, enhanced muscle mitochondrial biogenesis and stem cell differentiation, and improved gut microbiome composition [270]. Together, these clinical trials have demonstrated that NAD^+ supplementation via NR treatment is safe, well tolerated and appears to improve systemic metabolic health in non-pregnant populations.

To date there have been no human clinical trials testing the safety or efficacy of NR treatment in pregnant populations. There have been two other pre-clinical studies in rodent models, examining the effects of maternal NR treatment, either during pregnancy or during lactation – both demonstrating beneficial effects.

- In diet-restricted hypoglycemic pregnant mice, NR administration improved blood glucose levels through enriching TCA cycle metabolites to induce gluconeogenesis. Thus, NR treatment normalised maternal hypoglycemia and improved fetal weight, demonstrating its potential as an intervention for glycemic control during pregnancy [271].
- In rodents, maternal liver NAD^+ metabolome has been shown to decline at postpartum. Replenishing maternal liver NAD^+ metabolome by NR administration during lactation benefits both the mother and offspring. NR treatment improved maternal postpartum weight loss, increased lactation, and enriched nutrient value of mother's milk. Subsequently pups weaned by NR treated mothers showed

improved glycemic control, physical performance, memory formation and had increased neurogenesis at adulthood [272].

Collectively, these described findings, coupled to the findings of the current thesis and the clinical trials carried out in non-pregnant population, make a compelling case for the safety and therapeutic potential of NR treatment in pregnant populations.

6.3 Future Directions

The central role of NAD^+ signalling in the maintenance of metabolic health has been well characterized in all vital organ systems – *except* the human placenta. In fact, the work presented in the current thesis is some of the first carried out to date, identifying dysregulation of these metabolic pathways as central to the establishment of a common obstetrical disorder. As such, there is much left to be done to fully understand the role of placental and fetal NAD^+ signalling in the context of both healthy and pathological pregnancies. Specifically, future work focused on teasing apart which PARP enzymes become highly activated in the placenta in context of chronic inflammation, and what functions aside from excess protein PARylation they may be taking part in is needed. Further, the specific molecular mechanisms through which PARP-mediated NAD^+ depletion compromises mitochondrial function are warranted, with specific attention given to the expression and activity of different SIRT proteins. Of particular interest, the specific mechanisms through which NAD^+ supplementation may directly or indirectly improve placental function (i.e. improved mitochondrial dynamics, antioxidant function, anti-inflammatory properties) will be important to characterize, strengthening the case to launch clinical trials of preventative NR supplementation in pregnant individuals at high risk for the development of PE. Additional pre-clinical studies using the rodent model of

inflammation-mediated PE are also warranted. In the current thesis, NR treatment was initiated prior to onset of LPS injection, and therefore prior to the establishment of disease – mimicking a situation in which an individual would take a preventative treatment of NR as soon as they planned to become pregnant or found out they were pregnant. In future pre-clinical studies, initiating NR treatment after the LPS injections have begun would allow for an assessment of the therapeutic efficacy of NR treatment to reduce PE disease severity once the disease has already been established. These studies are important to understand how NR treatment might be used clinically in human pregnant populations. Finally, rigorous safety studies need to be performed in pre-clinical pregnancy models to determine safe and optimal dosing strategies for NR treatment, including an examination on long-term health effects on the offspring. In future, prenatal administration of NAD^+ precursors could be tested in women who are at high risk for developing PE and in women who are diagnosed with PE.

6.4 Conclusions

Despite accounting for almost 20% of the PE patient population, our understanding of the inflammation-driven subclass of PE is limited. The results of the current thesis have established a role for dysregulated NAD^+ signalling in the establishment of placental dysfunction and adverse pregnancy outcomes in this PE subclass, significantly enhancing our current understanding of the pathophysiology in this unique patient population. Importantly, therapeutic boosting of placental NAD^+ stores during pregnancy protects placental health and function and demonstrates tremendous promise as an effective intervention aimed at preventing the establishment and/or progression of inflammation-mediated PE. In this thesis, we also evaluated three rat models of

inflammation-driven PE and established that LPS model is the closest to mimic this subclass of human PE. Finally, as there was no validated animal model to study this disease subclass, the findings of this thesis will also help by identifying daily LPS injections from GD 13-18 as the most suitable model for future mechanistic or interventional investigations in this field.

References

1. Bauer, M.K., et al., *Fetal growth and placental function.* Mol Cell Endocrinol, 1998. **140**(1-2): p. 115-20.
2. Gude, N.M., et al., *Growth and function of the normal human placenta.* Thromb Res, 2004. **114**(5-6): p. 397-407.
3. Richard, A.P., Steven, H. A., David, H. R., William, E. B. & William, W. F., *Placental Development.* Elsevier, 2017: p. 101–113.
4. Boyd, J.D.H., W. J., *The Human Placenta.* 1970.
5. Enders, A.C. and B.F. King, *Formation and differentiation of extraembryonic mesoderm in the rhesus monkey.* Am J Anat, 1988. **181**(4): p. 327-40.
6. Knofler, M., et al., *Human placenta and trophoblast development: key molecular mechanisms and model systems.* Cell Mol Life Sci, 2019. **76**(18): p. 3479-3496.
7. Pijnenborg, R., L. Vercruysse, and M. Hanssens, *The uterine spiral arteries in human pregnancy: facts and controversies.* Placenta, 2006. **27**(9-10): p. 939-58.
8. Li, Q., et al., *Current understanding in deciphering trophoblast cell differentiation during human placentation.* Biol Reprod, 2022. **107**(1): p. 317-326.
9. Griffiths, S.K. and J.P. Campbell, *Placental structure, function and drug transfer.* Continuing Education in Anaesthesia Critical Care & Pain, 2014. **15**(2): p. 84-89.
10. Burton, G.J., et al., *Uterine glands provide histiotrophic nutrition for the human fetus during the first trimester of pregnancy.* J Clin Endocrinol Metab, 2002. **87**(6): p. 2954-9.
11. Battaglia, F.C.M., G., *An Introduction to Fetal Physiology.* Academic Press, 1986.
12. Moffett, A. and F. Colucci, *Uterine NK cells: active regulators at the maternal-fetal interface.* J Clin Invest, 2014. **124**(5): p. 1872-9.
13. Bordoni, E.J.H.B., *Embryology: Placenta*

Treasure Island (FL): StatPearls Publishing, 2023.

14. Marin, J.J., R.I. Macias, and M.A. Serrano, *The hepatobiliary-like excretory function of the placenta. A review.* Placenta, 2003. **24**(5): p. 431-8.
15. Pasanen, M., *The expression and regulation of drug metabolism in human placenta.* Adv Drug Deliv Rev, 1999. **38**(1): p. 81-97.
16. Delorme-Axford, E., Y. Sadovsky, and C.B. Coyne, *The Placenta as a Barrier to Viral Infections.* Annu Rev Virol, 2014. **1**(1): p. 133-46.
17. Rabelo, K., et al., *Zika Induces Human Placental Damage and Inflammation.* Front Immunol, 2020. **11**: p. 2146.
18. Moffett, A. and Y.W. Loke, *The immunological paradox of pregnancy: a reappraisal.* Placenta, 2004. **25**(1): p. 1-8.
19. Couzinet, B., et al., *Progesterone stimulates luteinizing hormone secretion by acting directly on the pituitary.* J Clin Endocrinol Metab, 1992. **74**(2): p. 374-8.
20. Page, K., *The Physiology of the Human Placenta.* University College, 1993.
21. Malassine, A. and L. Cronier, *Hormones and human trophoblast differentiation: a review.* Endocrine, 2002. **19**(1): p. 3-11.

22. Handwerger, S. and M. Freemark, *The roles of placental growth hormone and placental lactogen in the regulation of human fetal growth and development.* J Pediatr Endocrinol Metab, 2000. **13**(4): p. 343-56.
23. Constancia, M., et al., *Placental-specific IGF-II is a major modulator of placental and fetal growth.* Nature, 2002. **417**(6892): p. 945-8.
24. Constancia, M., et al., *Adaptation of nutrient supply to fetal demand in the mouse involves interaction between the Igf2 gene and placental transporter systems.* Proc Natl Acad Sci U S A, 2005. **102**(52): p. 19219-24.
25. Sferruzzi-Perri, A.N., et al., *An obesogenic diet during mouse pregnancy modifies maternal nutrient partitioning and the fetal growth trajectory.* FASEB J, 2013. **27**(10): p. 3928-37.
26. Michelsen, T.M., et al., *Uteroplacental Glucose Uptake and Fetal Glucose Consumption: A Quantitative Study in Human Pregnancies.* J Clin Endocrinol Metab, 2019. **104**(3): p. 873-882.
27. Hebert, J.F. and L. Myatt, *Placental mitochondrial dysfunction with metabolic diseases: Therapeutic approaches.* Biochim Biophys Acta Mol Basis Dis, 2021. **1867**(1): p. 165967.
28. Kolahi, K.S., A.M. Valent, and K.L. Thornburg, *Cytotrophoblast, Not Syncytiotrophoblast, Dominates Glycolysis and Oxidative Phosphorylation in Human Term Placenta.* Sci Rep, 2017. **7**: p. 42941.
29. Kolahi, K., et al., *Real-Time Tracking of BODIPY-C12 Long-Chain Fatty Acid in Human Term Placenta Reveals Unique Lipid Dynamics in Cytotrophoblast Cells.* PLoS One, 2016. **11**(4): p. e0153522.
30. Mori, M., et al., *The cytotrophoblast layer of human chorionic villi becomes thinner but maintains its structural integrity during gestation.* Biol Reprod, 2007. **76**(1): p. 164-72.
31. Brosens, I., et al., *The "Great Obstetrical Syndromes" are associated with disorders of deep placentation.* Am J Obstet Gynecol, 2011. **204**(3): p. 193-201.
32. Osungbade, K.O. and O.K. Ige, *Public health perspectives of preeclampsia in developing countries: implication for health system strengthening.* J Pregnancy, 2011. **2011**: p. 481095.
33. Lee, A.C., et al., *Estimates of burden and consequences of infants born small for gestational age in low and middle income countries with INTERGROWTH-21(st) standard: analysis of CHERG datasets.* BMJ, 2017. **358**: p. j3677.
34. Steegers, E.A., et al., *Pre-eclampsia.* Lancet, 2010. **376**(9741): p. 631-44.
35. *Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy.* Obstet Gynecol, 2013. **122**(5): p. 1122-1131.
36. Khan, K.S., et al., *WHO analysis of causes of maternal death: a systematic review.* Lancet, 2006. **367**(9516): p. 1066-1074.
37. Wallis, A.B., et al., *Secular trends in the rates of preeclampsia, eclampsia, and gestational hypertension, United States, 1987-2004.* Am J Hypertens, 2008. **21**(5): p. 521-6.
38. Stevens, W., et al., *Short-term costs of preeclampsia to the United States health care system.* Am J Obstet Gynecol, 2017. **217**(3): p. 237-248 e16.

39. *Maternal hypertension in Canada.* The Public Health Agency of Canada. **Source: Canadian Institute for Health Information, Discharge Abstract Database (DAD)**.
40. Liu, A., et al., *Utilization of health care services of pregnant women complicated by preeclampsia in Ontario.* Hypertens Pregnancy, 2009. **28**(1): p. 76-84.
41. Koga, K., et al., *Elevated serum soluble fms-like tyrosine kinase 1 (sFlt1) level in women with hydatidiform mole.* Fertil Steril, 2010. **94**(1): p. 305-8.
42. Tranquilli, A.L., et al., *The definition of severe and early-onset preeclampsia. Statements from the International Society for the Study of Hypertension in Pregnancy (ISSHP).* Pregnancy Hypertens, 2013. **3**(1): p. 44-7.
43. Xiong, X. and W.D. Fraser, *Impact of pregnancy-induced hypertension on birthweight by gestational age.* Paediatr Perinat Epidemiol, 2004. **18**(3): p. 186-91.
44. Redman, C.W. and A.C. Staff, *Preeclampsia, biomarkers, syncytiotrophoblast stress, and placental capacity.* Am J Obstet Gynecol, 2015. **213**(4 Suppl): p. S9 e1, S9-11.
45. Redman, E.K., et al., *Clinical Course, Associated Factors, and Blood Pressure Profile of Delayed-Onset Postpartum Preeclampsia.* Obstet Gynecol, 2019. **134**(5): p. 995-1001.
46. McElrath, T.F., et al., *Circulating microparticle proteins obtained in the late first trimester predict spontaneous preterm birth at less than 35 weeks' gestation: a panel validation with specific characterization by parity.* Am J Obstet Gynecol, 2019. **220**(5): p. 488 e1-488 e11.
47. Powers, R.W., et al., *Low placental growth factor across pregnancy identifies a subset of women with preterm preeclampsia: type 1 versus type 2 preeclampsia?* Hypertension, 2012. **60**(1): p. 239-46.
48. Leavey, K., et al., *Unsupervised Placental Gene Expression Profiling Identifies Clinically Relevant Subclasses of Human Preeclampsia.* Hypertension, 2016. **68**(1): p. 137-47.
49. Benton, S.J., et al., *The clinical heterogeneity of preeclampsia is related to both placental gene expression and placental histopathology.* Am J Obstet Gynecol, 2018. **219**(6): p. 604 e1-604 e25.
50. Leavey, K., S.A. Bainbridge, and B.J. Cox, *Large scale aggregate microarray analysis reveals three distinct molecular subclasses of human preeclampsia.* PLoS One, 2015. **10**(2): p. e0116508.
51. Jahan, F., et al., *Placental Mitochondrial Function and Dysfunction in Preeclampsia.* Int J Mol Sci, 2023. **24**(4).
52. Holland, O., et al., *Review: Placental mitochondrial function and structure in gestational disorders.* Placenta, 2017. **54**: p. 2-9.
53. Katsyuba, E., et al., *NAD(+) homeostasis in health and disease.* Nat Metab, 2020. **2**(1): p. 9-31.
54. Canto, C., K.J. Menzies, and J. Auwerx, *NAD(+) Metabolism and the Control of Energy Homeostasis: A Balancing Act between Mitochondria and the Nucleus.* Cell Metab, 2015. **22**(1): p. 31-53.

55. Gupte, R., Z. Liu, and W.L. Kraus, *PARPs and ADP-ribosylation: recent advances linking molecular functions to biological outcomes.* Genes Dev, 2017. **31**(2): p. 101-126.
56. Ansari, A., et al., *Function of the SIRT3 mitochondrial deacetylase in cellular physiology, cancer, and neurodegenerative disease.* Aging Cell, 2017. **16**(1): p. 4-16.
57. Shi, T., et al., *SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes.* J Biol Chem, 2005. **280**(14): p. 13560-7.
58. Greiten, L.E., et al., *Sirtuin 6 Protects Against Oxidative Stress and Vascular Dysfunction in Mice.* Front Physiol, 2021. **12**: p. 753501.
59. Smirnov, D., et al., *SIRT6 is a key regulator of mitochondrial function in the brain.* Cell Death Dis, 2023. **14**(1): p. 35.
60. Imai, S. and L. Guarente, *NAD+ and sirtuins in aging and disease.* Trends Cell Biol, 2014. **24**(8): p. 464-71.
61. Singh, C.K., et al., *The Role of Sirtuins in Antioxidant and Redox Signaling.* Antioxid Redox Signal, 2018. **28**(8): p. 643-661.
62. Zhang, L.Q., et al., *Metabolic and molecular insights into an essential role of nicotinamide phosphoribosyltransferase.* Cell Death Dis, 2017. **8**(3): p. e2705.
63. Muraoka, H., et al., *Role of Nampt-Sirt6 Axis in Renal Proximal Tubules in Extracellular Matrix Deposition in Diabetic Nephropathy.* Cell Rep, 2019. **27**(1): p. 199-212 e5.
64. de Guia, R.M., et al., *Ablation of Nampt in AgRP neurons leads to neurodegeneration and impairs fasting- and ghrelin-mediated food intake.* FASEB J, 2021. **35**(5): p. e21450.
65. Wang, X., et al., *Deletion of Nampt in Projection Neurons of Adult Mice Leads to Motor Dysfunction, Neurodegeneration, and Death.* Cell Rep, 2017. **20**(9): p. 2184-2200.
66. Basse, A.L., et al., *Nampt controls skeletal muscle development by maintaining Ca(2+) homeostasis and mitochondrial integrity.* Mol Metab, 2021. **53**: p. 101271.
67. Ke, Y., et al., *The Role of PARPs in Inflammation-and Metabolic-Related Diseases: Molecular Mechanisms and Beyond.* Cells, 2019. **8**(9).
68. Richard, I.A., et al., *Beyond PARP1: The Potential of Other Members of the Poly (ADP-Ribose) Polymerase Family in DNA Repair and Cancer Therapeutics.* Front Cell Dev Biol, 2021. **9**: p. 801200.
69. Zarkovic, G., et al., *Characterization of DNA ADP-ribosyltransferase activities of PARP2 and PARP3: new insights into DNA ADP-ribosylation.* Nucleic Acids Res, 2018. **46**(5): p. 2417-2431.
70. Messner, S. and M.O. Hottiger, *Histone ADP-ribosylation in DNA repair, replication and transcription.* Trends Cell Biol, 2011. **21**(9): p. 534-42.
71. Krishnakumar, R. and W.L. Kraus, *The PARP side of the nucleus: molecular actions, physiological outcomes, and clinical targets.* Mol Cell, 2010. **39**(1): p. 8-24.
72. Zhu, H., et al., *The Critical Role of PARPs in Regulating Innate Immune Responses.* Front Immunol, 2021. **12**: p. 712556.

73. Szabo, C., et al., *Inhibition of poly (ADP-ribose) synthetase attenuates neutrophil recruitment and exerts antiinflammatory effects.* J Exp Med, 1997. **186**(7): p. 1041-9.
74. Hauschildt, S., et al., *Role of ADP-ribosylation in activated monocytes/macrophages.* Adv Exp Med Biol, 1997. **419**: p. 249-52.
75. Bohio, A.A., et al., *c-Abl-Mediated Tyrosine Phosphorylation of PARP1 Is Crucial for Expression of Proinflammatory Genes.* J Immunol, 2019. **203**(6): p. 1521-1531.
76. Hassa, P.O. and M.O. Hottiger, *A role of poly (ADP-ribose) polymerase in NF-kappaB transcriptional activation.* Biol Chem, 1999. **380**(7-8): p. 953-9.
77. Ke, Y., et al., *Erratum: PARP1 promotes gene expression at the post-transcriptional level by modulating the RNA-binding protein HuR.* Nat Commun, 2017. **8**: p. 15191.
78. Catara, G., et al., *PARP1-produced poly-ADP-ribose causes the PARP12 translocation to stress granules and impairment of Golgi complex functions.* Sci Rep, 2017. **7**(1): p. 14035.
79. Herman, A.B., et al., *Regulation of Stress Granule Formation by Inflammation, Vascular Injury, and Atherosclerosis.* Arterioscler Thromb Vasc Biol, 2019. **39**(10): p. 2014-2027.
80. Kedersha, N., P. Ivanov, and P. Anderson, *Stress granules and cell signaling: more than just a passing phase?* Trends Biochem Sci, 2013. **38**(10): p. 494-506.
81. Caprara, G., et al., *PARP14 Controls the Nuclear Accumulation of a Subset of Type I IFN-Inducible Proteins.* J Immunol, 2018. **200**(7): p. 2439-2454.
82. Fehr, A.R., et al., *The impact of PARPs and ADP-ribosylation on inflammation and host-pathogen interactions.* Genes Dev, 2020. **34**(5-6): p. 341-359.
83. Iwata, H., et al., *PARP9 and PARP14 cross-regulate macrophage activation via STAT1 ADP-ribosylation.* Nat Commun, 2016. **7**: p. 12849.
84. Gariani, K., et al., *Inhibiting poly ADP-ribosylation increases fatty acid oxidation and protects against fatty liver disease.* J Hepatol, 2017. **66**(1): p. 132-141.
85. Ryu, D., et al., *NAD+ repletion improves muscle function in muscular dystrophy and counters global PARylation.* Sci Transl Med, 2016. **8**(361): p. 361ra139.
86. Cuzzocrea, S., et al., *Role of poly(ADP-ribose) glycohydrolase in the development of inflammatory bowel disease in mice.* Free Radic Biol Med, 2007. **42**(1): p. 90-105.
87. Penberthy, W.T. and I. Tsunoda, *The importance of NAD in multiple sclerosis.* Curr Pharm Des, 2009. **15**(1): p. 64-99.
88. Verdin, E., *NAD(+) in aging, metabolism, and neurodegeneration.* Science, 2015. **350**(6265): p. 1208-13.
89. Yang, S.J., et al., *Nicotinamide improves glucose metabolism and affects the hepatic NAD-sirtuin pathway in a rodent model of obesity and type 2 diabetes.* J Nutr Biochem, 2014. **25**(1): p. 66-72.
90. Jahan, F. and R.A. Bagchi, *Enhancing NAD(+) Metabolome in Cardiovascular Diseases: Promises and Considerations.* Front Cardiovasc Med, 2021. **8**: p. 716989.
91. Xie, N., et al., *NAD(+) metabolism: pathophysiologic mechanisms and therapeutic potential.* Signal Transduct Target Ther, 2020. **5**(1): p. 227.

92. Sun, H., et al., *The combined treatment of NAD(+) and atorvastatin ameliorates the development of experimental autoimmune encephalomyelitis in C57BL/6 mice.* J Neuroimmunol, 2020. **350**: p. 577429.
93. Wu, J., et al., *Boosting NAD+ blunts TLR4-induced type I IFN in control and systemic lupus erythematosus monocytes.* J Clin Invest, 2022. **132**(5).
94. Perez-Sanchez, C., et al., *Preclinical characterization of pharmacological NAD(+) boosting as a promising therapeutic approach in Rheumatoid Arthritis.* Arthritis Rheumatol, 2023.
95. Doke, T., et al., *NAD(+) precursor supplementation prevents mtRNA/RIG-I-dependent inflammation during kidney injury.* Nat Metab, 2023. **5**(3): p. 414-430.
96. Rana, S., et al., *Preeclampsia: Pathophysiology, Challenges, and Perspectives.* Circ Res, 2019. **124**(7): p. 1094-1112.
97. Aye, I., et al., *Placental energy metabolism in health and disease-significance of development and implications for preeclampsia.* Am J Obstet Gynecol, 2022. **226**(2S): p. S928-S944.
98. Spinelli, J.B. and M.C. Haigis, *The multifaceted contributions of mitochondria to cellular metabolism.* Nat Cell Biol, 2018. **20**(7): p. 745-754.
99. Burton, G.J., H.W. Yung, and A.J. Murray, *Mitochondrial - Endoplasmic reticulum interactions in the trophoblast: Stress and senescence.* Placenta, 2017. **52**: p. 146-155.
100. Hay, W.W., Jr., *Energy and substrate requirements of the placenta and fetus.* Proc Nutr Soc, 1991. **50**(2): p. 321-36.
101. Fisher, J.J., et al., *Placental mitochondria and reactive oxygen species in the physiology and pathophysiology of pregnancy.* Clin Exp Pharmacol Physiol, 2020. **47**(1): p. 176-184.
102. Bartho, L.A., et al., *Mitochondrial transformations in the aging human placenta.* Am J Physiol Endocrinol Metab, 2020. **319**(6): p. E981-E994.
103. Correia, Y., et al., *Placental mitochondrial function as a driver of angiogenesis and placental dysfunction.* Biol Chem, 2021. **402**(8): p. 887-909.
104. Burton, G.J. and A.L. Fowden, *The placenta: a multifaceted, transient organ.* Philos Trans R Soc Lond B Biol Sci, 2015. **370**(1663): p. 20140066.
105. Jauniaux, E., et al., *Polyol concentrations in the fluid compartments of the human conceptus during the first trimester of pregnancy: maintenance of redox potential in a low oxygen environment.* J Clin Endocrinol Metab, 2005. **90**(2): p. 1171-5.
106. Bloxam, D.L. and P.M. Bobinski, *Energy metabolism and glycolysis in the human placenta during ischaemia and in normal labour.* Placenta, 1984. **5**(5): p. 381-94.
107. Holland, O.J., et al., *Changes in mitochondrial respiration in the human placenta over gestation.* Placenta, 2017. **57**: p. 102-112.
108. Jauniaux, E., et al., *Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure.* Am J Pathol, 2000. **157**(6): p. 2111-22.
109. Drose, S. and U. Brandt, *Molecular mechanisms of superoxide production by the mitochondrial respiratory chain.* Adv Exp Med Biol, 2012. **748**: p. 145-69.
110. Mannaerts, D., et al., *Oxidative stress in healthy pregnancy and preeclampsia is linked to chronic inflammation, iron status and vascular function.* PLoS One, 2018. **13**(9): p. e0202919.

111. Bardin, N., P. Murthi, and N. Alfaidy, *Normal and pathological placental angiogenesis.* Biomed Res Int, 2015. **2015**: p. 354359.
112. Basu, J., et al., *Placental Oxidative Status throughout Normal Gestation in Women with Uncomplicated Pregnancies.* Obstet Gynecol Int, 2015. **2015**: p. 276095.
113. Sferruzzi-Perri, A.N., et al., *Placental mitochondria adapt developmentally and in response to hypoxia to support fetal growth.* Proc Natl Acad Sci U S A, 2019. **116**(5): p. 1621-1626.
114. Sesso, A., et al., *Mitochondrial swelling and incipient outer membrane rupture in preapoptotic and apoptotic cells.* Anat Rec (Hoboken), 2012. **295**(10): p. 1647-59.
115. Huppertz, B., et al., *Apoptosis cascade progresses during turnover of human trophoblast: analysis of villous cytotrophoblast and syncytial fragments in vitro.* Lab Invest, 1999. **79**(12): p. 1687-702.
116. Levy, R. and D.M. Nelson, *To be, or not to be, that is the question. Apoptosis in human trophoblast.* Placenta, 2000. **21**(1): p. 1-13.
117. De los Rios Castillo, D., et al., *Atypical cristae morphology of human syncytiotrophoblast mitochondria: role for complex V.* J Biol Chem, 2011. **286**(27): p. 23911-9.
118. Martinez, F., M. Kiriakidou, and J.F. Strauss, 3rd, *Structural and functional changes in mitochondria associated with trophoblast differentiation: methods to isolate enriched preparations of syncytiotrophoblast mitochondria.* Endocrinology, 1997. **138**(5): p. 2172-83.
119. Martinez, F., et al., *Multiple functions of syncytiotrophoblast mitochondria.* Steroids, 2015. **103**: p. 11-22.
120. Watson, A.L., et al., *Susceptibility of human placental syncytiotrophoblastic mitochondria to oxygen-mediated damage in relation to gestational age.* J Clin Endocrinol Metab, 1998. **83**(5): p. 1697-705.
121. Seok, J., et al., *Mitochondrial Dynamics in Placenta-Derived Mesenchymal Stem Cells Regulate the Invasion Activity of Trophoblast.* Int J Mol Sci, 2020. **21**(22).
122. Seok, J., et al., *Human placenta-derived mesenchymal stem cells induce trophoblast invasion via dynamic effects on mitochondrial function.* J Cell Physiol, 2021. **236**(9): p. 6678-6690.
123. LeBleu, V.S., et al., *PGC-1alpha mediates mitochondrial biogenesis and oxidative phosphorylation in cancer cells to promote metastasis.* Nat Cell Biol, 2014. **16**(10): p. 992-1003, 1-15.
124. Hogan, M.C., et al., *Maternal mortality for 181 countries, 1980-2008: a systematic analysis of progress towards Millennium Development Goal 5.* Lancet, 2010. **375**(9726): p. 1609-23.
125. *ACOG Practice Bulletin No. 202: Gestational Hypertension and Preeclampsia.* Obstet Gynecol, 2019. **133**(1): p. 1.
126. Weiler, J., S. Tong, and K.R. Palmer, *Is fetal growth restriction associated with a more severe maternal phenotype in the setting of early onset pre-eclampsia? A retrospective study.* PLoS One, 2011. **6**(10): p. e26937.

127. Mastrolia, S.A., et al., *Single vs. Recurrent Episodes of Preeclampsia-population–based Epidemiological and Clinical Characteristics.* Maternal-Fetal Medicine, 2021. **3**(3): p. 190-196.
128. Hutchinson, E.S., et al., *Utero-placental haemodynamics in the pathogenesis of pre-eclampsia.* Placenta, 2009. **30**(7): p. 634-41.
129. Roberts, J.M. and C.A. Hubel, *The two stage model of preeclampsia: variations on the theme.* Placenta, 2009. **30 Suppl A**(Suppl A): p. S32-7.
130. Lyall, F., S.C. Robson, and J.N. Bulmer, *Spiral artery remodeling and trophoblast invasion in preeclampsia and fetal growth restriction: relationship to clinical outcome.* Hypertension, 2013. **62**(6): p. 1046-54.
131. Aardema, M.W., et al., *Uterine artery Doppler flow and uteroplacental vascular pathology in normal pregnancies and pregnancies complicated by pre-eclampsia and small for gestational age fetuses.* Placenta, 2001. **22**(5): p. 405-11.
132. Khong, T.Y., et al., *Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants.* Br J Obstet Gynaecol, 1986. **93**(10): p. 1049-59.
133. Espinoza, J., et al., *Normal and abnormal transformation of the spiral arteries during pregnancy.* J Perinat Med, 2006. **34**(6): p. 447-58.
134. Jones, C.J. and H. Fox, *An ultrastructural and ultrahistochemical study of the human placenta in maternal pre-eclampsia.* Placenta, 1980. **1**(1): p. 61-76.
135. Longtine, M.S., et al., *Villous trophoblast apoptosis is elevated and restricted to cytotrophoblasts in pregnancies complicated by preeclampsia, IUGR, or preeclampsia with IUGR.* Placenta, 2012. **33**(5): p. 352-9.
136. Redman, C.W., I.L. Sargent, and A.C. Staff, *IFPA Senior Award Lecture: making sense of pre-eclampsia - two placental causes of preeclampsia?* Placenta, 2014. **35 Suppl**: p. S20-5.
137. Tannetta, D., et al., *Update of syncytiotrophoblast derived extracellular vesicles in normal pregnancy and preeclampsia.* J Reprod Immunol, 2017. **119**: p. 98-106.
138. Nikuei, P., et al., *Accuracy of Soluble Endoglin for Diagnosis of Preeclampsia and its Severity.* Iran Biomed J, 2017. **21**(5): p. 312-30.
139. von Dadelszen, P., L.A. Magee, and J.M. Roberts, *Subclassification of preeclampsia.* Hypertens Pregnancy, 2003. **22**(2): p. 143-8.
140. McCartney, C.P., *Pathological Anatomy of Acute Hypertension of Pregnancy.* Circulation, 1964. **30**: p. SUPPL 2:37-42.
141. Wilson, S.L., et al., *Mining DNA methylation alterations towards a classification of placental pathologies.* Hum Mol Genet, 2018. **27**(1): p. 135-146.
142. Leavey, K., D. Grynspan, and B.J. Cox, *Both "canonical" and "immunological" preeclampsia subtypes demonstrate changes in placental immune cell composition.* Placenta, 2019. **83**: p. 53-56.
143. Roberts, J.M., et al., *Subtypes of Preeclampsia: Recognition and Determining Clinical Usefulness.* Hypertension, 2021. **77**(5): p. 1430-1441.
144. Conrad, K.P. and D.F. Benyo, *Placental cytokines and the pathogenesis of preeclampsia.* Am J Reprod Immunol, 1997. **37**(3): p. 240-9.
145. Harmon, A.C., et al., *The role of inflammation in the pathology of preeclampsia.* Clin Sci (Lond), 2016. **130**(6): p. 409-19.

146. Hiby, S.E., et al., *Combinations of maternal KIR and fetal HLA-C genes influence the risk of preeclampsia and reproductive success.* J Exp Med, 2004. **200**(8): p. 957-65.
147. Triche, E.W., et al., *Maternal-fetal HLA sharing and preeclampsia: variation in effects by seminal fluid exposure in a case-control study of nulliparous women in Iowa.* J Reprod Immunol, 2014. **101-102**: p. 111-119.
148. Kenny, L.C. and D.B. Kell, *Immunological Tolerance, Pregnancy, and Preeclampsia: The Roles of Semen Microbes and the Father.* Front Med (Lausanne), 2017. **4**: p. 239.
149. Myatt, L. and J.M. Roberts, *Preeclampsia: Syndrome or Disease?* Curr Hypertens Rep, 2015. **17**(11): p. 83.
150. Vangrieken, P., et al., *Placental Mitochondrial Abnormalities in Preeclampsia.* Reprod Sci, 2021. **28**(8): p. 2186-2199.
151. Holland, O.J., et al., *Placental mitochondrial adaptations in preeclampsia associated with progression to term delivery.* Cell Death Dis, 2018. **9**(12): p. 1150.
152. Pandey, D., et al., *Mitochondrial DNA copy number variation - A potential biomarker for early onset preeclampsia.* Pregnancy Hypertens, 2021. **23**: p. 1-4.
153. Cushen, S.C., et al., *Reduced Maternal Circulating Cell-Free Mitochondrial DNA Is Associated With the Development of Preeclampsia.* J Am Heart Assoc, 2022. **11**(2): p. e021726.
154. Marschalek, J., et al., *Maternal serum mitochondrial DNA (mtDNA) levels are elevated in preeclampsia - A matched case-control study.* Pregnancy Hypertens, 2018. **14**: p. 195-199.
155. Qiu, C., et al., *A case-control study of maternal blood mitochondrial DNA copy number and preeclampsia risk.* Int J Mol Epidemiol Genet, 2012. **3**(3): p. 237-44.
156. McCarthy, C. and L.C. Kenny, *Therapeutically targeting mitochondrial redox signalling alleviates endothelial dysfunction in preeclampsia.* Sci Rep, 2016. **6**: p. 32683.
157. Westrate, L.M., et al., *Mitochondrial morphological features are associated with fission and fusion events.* PLoS One, 2014. **9**(4): p. e95265.
158. Youle, R.J. and A.M. van der Bliek, *Mitochondrial fission, fusion, and stress.* Science, 2012. **337**(6098): p. 1062-5.
159. Yu, J., et al., *Downregulation of Mitofusin 2 in Placenta Is Related to Preeclampsia.* Biomed Res Int, 2016. **2016**: p. 6323086.
160. Ryan, J.J., et al., *PGC1alpha-mediated mitofusin-2 deficiency in female rats and humans with pulmonary arterial hypertension.* Am J Respir Crit Care Med, 2013. **187**(8): p. 865-78.
161. Salgado, S.S. and M.K.R. Salgado, *Structural changes in pre-eclamptic and eclamptic placentas--an ultrastructural study.* J Coll Physicians Surg Pak, 2011. **21**(8): p. 482-6.
162. Ausman, J., et al., *Ceramide-induced BOK promotes mitochondrial fission in preeclampsia.* Cell Death Dis, 2018. **9**(3): p. 298.
163. Acin-Perez, R., et al., *A novel approach to measure mitochondrial respiration in frozen biological samples.* EMBO J, 2020. **39**(13): p. e104073.

164. Yung, H.W., et al., *Noncanonical mitochondrial unfolded protein response impairs placental oxidative phosphorylation in early-onset preeclampsia.* Proc Natl Acad Sci U S A, 2019. **116**(36): p. 18109-18118.
165. Muralimanoharan, S., et al., *MIR-210 modulates mitochondrial respiration in placenta with preeclampsia.* Placenta, 2012. **33**(10): p. 816-23.
166. Vishnyakova, P.A., et al., *Mitochondrial role in adaptive response to stress conditions in preeclampsia.* Sci Rep, 2016. **6**: p. 32410.
167. Cadenas, E. and K.J. Davies, *Mitochondrial free radical generation, oxidative stress, and aging.* Free Radic Biol Med, 2000. **29**(3-4): p. 222-30.
168. Quinlan, C.L., et al., *The 2-oxoacid dehydrogenase complexes in mitochondria can produce superoxide/hydrogen peroxide at much higher rates than complex I.* J Biol Chem, 2014. **289**(12): p. 8312-25.
169. Guzy, R.D., et al., *Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing.* Cell Metab, 2005. **1**(6): p. 401-8.
170. Hernansanz-Agustin, P., et al., *Mitochondrial complex I deactivation is related to superoxide production in acute hypoxia.* Redox Biol, 2017. **12**: p. 1040-1051.
171. Gupta, S., A. Agarwal, and R.K. Sharma, *The role of placental oxidative stress and lipid peroxidation in preeclampsia.* Obstet Gynecol Surv, 2005. **60**(12): p. 807-16.
172. Wiktor, H., et al., *Oxidative DNA damage in placentas from normal and pre-eclamptic pregnancies.* Virchows Arch, 2004. **445**(1): p. 74-8.
173. Llurba, E., et al., *A comprehensive study of oxidative stress and antioxidant status in preeclampsia and normal pregnancy.* Free Radic Biol Med, 2004. **37**(4): p. 557-70.
174. Aris, A., et al., *Potential biomarkers of preeclampsia: inverse correlation between hydrogen peroxide and nitric oxide early in maternal circulation and at term in placenta of women with preeclampsia.* Placenta, 2009. **30**(4): p. 342-7.
175. Wang, Y. and S.W. Walsh, *Placental mitochondria as a source of oxidative stress in pre-eclampsia.* Placenta, 1998. **19**(8): p. 581-6.
176. Rafeeinia, A., et al., *Serum copper, zinc and lipid peroxidation in pregnant women with preeclampsia in gorgan.* Open Biochem J, 2014. **8**: p. 83-8.
177. Shaker, O.G. and N.A. Sadik, *Pathogenesis of preeclampsia: Implications of apoptotic markers and oxidative stress.* Hum Exp Toxicol, 2013. **32**(11): p. 1170-8.
178. Padmini, E., S. Lavanya, and V. Uthra, *Preeclamptic placental stress and over expression of mitochondrial HSP70.* Clin Chem Lab Med, 2009. **47**(9): p. 1073-80.
179. Wiktor, H. and M. Kankofer, *Superoxide dismutase activity in normal and preeclamptic placentas.* Ginekol Pol, 1998. **69**(12): p. 915-8.
180. Wu, F., F.J. Tian, and Y. Lin, *Oxidative Stress in Placenta: Health and Diseases.* Biomed Res Int, 2015. **2015**: p. 293271.
181. Brunelle, J.K. and A. Letai, *Control of mitochondrial apoptosis by the Bcl-2 family.* J Cell Sci, 2009. **122**(Pt 4): p. 437-41.
182. Allaire, A.D., et al., *Placental apoptosis in preeclampsia.* Obstet Gynecol, 2000. **96**(2): p. 271-6.

183. Leung, D.N., et al., *Increased placental apoptosis in pregnancies complicated by preeclampsia.* Am J Obstet Gynecol, 2001. **184**(6): p. 1249-50.
184. Fogarty, N.M., A.C. Ferguson-Smith, and G.J. Burton, *Syncytial knots (Tenney-Parker changes) in the human placenta: evidence of loss of transcriptional activity and oxidative damage.* Am J Pathol, 2013. **183**(1): p. 144-52.
185. Hung, T.H., et al., *Hypoxia-reoxygenation: a potent inducer of apoptotic changes in the human placenta and possible etiological factor in preeclampsia.* Circ Res, 2002. **90**(12): p. 1274-81.
186. Sanchez-Aranguren, L.C., et al., *Endothelial dysfunction and preeclampsia: role of oxidative stress.* Front Physiol, 2014. **5**: p. 372.
187. Vaka, V.R., et al., *Role of Mitochondrial Dysfunction and Reactive Oxygen Species in Mediating Hypertension in the Reduced Uterine Perfusion Pressure Rat Model of Preeclampsia.* Hypertension, 2018. **72**(3): p. 703-711.
188. Covarrubias, A.E., et al., *AP39, a Modulator of Mitochondrial Bioenergetics, Reduces Antiangiogenic Response and Oxidative Stress in Hypoxia-Exposed Trophoblasts: Relevance for Preeclampsia Pathogenesis.* Am J Pathol, 2019. **189**(1): p. 104-114.
189. Nagamatsu, T., et al., *Cytotrophoblasts up-regulate soluble fms-like tyrosine kinase-1 expression under reduced oxygen: an implication for the placental vascular development and the pathophysiology of preeclampsia.* Endocrinology, 2004. **145**(11): p. 4838-45.
190. Jiang, Z., et al., *A Role of sFlt-1 in Oxidative Stress and Apoptosis in Human and Mouse Pre-Eclamptic Trophoblasts.* Biol Reprod, 2015. **93**(3): p. 73.
191. Tong, W., et al., *Chronic Hypoxia in Ovine Pregnancy Recapitulates Physiological and Molecular Markers of Preeclampsia in the Mother, Placenta, and Offspring.* Hypertension, 2022. **79**(7): p. 1525-1535.
192. Peracoli, J.C., M.V. Rudge, and M.T. Peracoli, *Tumor necrosis factor-alpha in gestation and puerperium of women with gestational hypertension and pre-eclampsia.* Am J Reprod Immunol, 2007. **57**(3): p. 177-85.
193. Raghupathy, R., *Cytokines as key players in the pathophysiology of preeclampsia.* Med Princ Pract, 2013. **22 Suppl 1**: p. 8-19.
194. Clayton, S.A., et al., *Mitochondria as Key Players in the Pathogenesis and Treatment of Rheumatoid Arthritis.* Front Immunol, 2021. **12**: p. 673916.
195. Green, D.R., L. Galluzzi, and G. Kroemer, *Mitochondria and the autophagy-inflammation-cell death axis in organismal aging.* Science, 2011. **333**(6046): p. 1109-12.
196. Lopez-Armada, M.J., et al., *Mitochondrial dysfunction and the inflammatory response.* Mitochondrion, 2013. **13**(2): p. 106-18.
197. Blaser, H., et al., *TNF and ROS Crosstalk in Inflammation.* Trends Cell Biol, 2016. **26**(4): p. 249-261.
198. Fiers, W., et al., *More than one way to die: apoptosis, necrosis and reactive oxygen damage.* Oncogene, 1999. **18**(54): p. 7719-30.
199. Kim, J.J., et al., *TNF-alpha-induced ROS production triggering apoptosis is directly linked to Romo1 and Bcl-X(L).* Cell Death Differ, 2010. **17**(9): p. 1420-34.

200. Roca, F.J., et al., *Tumor necrosis factor induces pathogenic mitochondrial ROS in tuberculosis through reverse electron transport.* Science, 2022. **376**(6600): p. eabh2841.
201. Wang, Y. and S.W. Walsh, *TNF alpha concentrations and mRNA expression are increased in preeclamptic placentas.* J Reprod Immunol, 1996. **32**(2): p. 157-69.
202. I, C.W., et al., *Increased expression of NLRP3 inflammasome in placentas from pregnant women with severe preeclampsia.* J Reprod Immunol, 2017. **123**: p. 40-47.
203. Alexander, B.T., et al., *Tumor necrosis factor-alpha-induced hypertension in pregnant rats results in decreased renal neuronal nitric oxide synthase expression.* Am J Hypertens, 2002. **15**(2 Pt 1): p. 170-5.
204. Guo, X., et al., *Upregulation of VEGF by small activating RNA and its implications in preeclampsia.* Placenta, 2016. **46**: p. 38-44.
205. Xu, B., et al., *TNF-alpha inhibits trophoblast integration into endothelial cellular networks.* Placenta, 2011. **32**(3): p. 241-6.
206. Muralimanoharan, S., et al., *Sexual dimorphism in miR-210 expression and mitochondrial dysfunction in the placenta with maternal obesity.* Int J Obes (Lond), 2015. **39**(8): p. 1274-81.
207. Lien, Y.C., et al., *Intrauterine Inflammation Alters the Transcriptome and Metabolome in Placenta.* Front Physiol, 2020. **11**: p. 592689.
208. Batchuluun, B., et al., *Elevated Medium-Chain Acylcarnitines Are Associated With Gestational Diabetes Mellitus and Early Progression to Type 2 Diabetes and Induce Pancreatic beta-Cell Dysfunction.* Diabetes, 2018. **67**(5): p. 885-897.
209. Odegard, R.A., et al., *Risk factors and clinical manifestations of pre-eclampsia.* BJOG, 2000. **107**(11): p. 1410-6.
210. Redman, C., *Pre-eclampsia: A complex and variable disease.* Pregnancy Hypertens, 2014. **4**(3): p. 241-2.
211. Clark, D.E., et al., *A vascular endothelial growth factor antagonist is produced by the human placenta and released into the maternal circulation.* Biol Reprod, 1998. **59**(6): p. 1540-8.
212. Levine, R.J., et al., *Circulating angiogenic factors and the risk of preeclampsia.* N Engl J Med, 2004. **350**(7): p. 672-83.
213. Mor, G., et al., *Inflammation and pregnancy: the role of the immune system at the implantation site.* Ann N Y Acad Sci, 2011. **1221**(1): p. 80-7.
214. Abu-Raya, B., et al., *Maternal Immunological Adaptation During Normal Pregnancy.* Front Immunol, 2020. **11**: p. 575197.
215. Tang, X., et al., *Mitochondria, endothelial cell function, and vascular diseases.* Front Physiol, 2014. **5**: p. 175.
216. Kirkman, D.L., et al., *Mitochondrial contributions to vascular endothelial dysfunction, arterial stiffness, and cardiovascular diseases.* Am J Physiol Heart Circ Physiol, 2021. **320**(5): p. H2080-H2100.
217. Ricci, C.A., et al., *Mitochondria-mediated Maternal-fetal Interactions and Consequences of Mitochondrial Dysregulation Indicate New Roles for Mitochondria in Hypertensive Pregnancies.* medRxiv, 2021: p. 2021.12.18.21268029.

218. Sanchez-Aranguren, L.C., et al., *Soluble Fms-Like Tyrosine Kinase-1 Alters Cellular Metabolism and Mitochondrial Bioenergetics in Preeclampsia.* Front Physiol, 2018. **9**: p. 83.
219. Deer, E., et al., *Vascular endothelial mitochondrial oxidative stress in response to preeclampsia: a role for angiotension II type 1 autoantibodies.* Am J Obstet Gynecol MFM, 2021. **3**(1): p. 100275.
220. Duley, L., *Pre-eclampsia and the hypertensive disorders of pregnancy.* Br Med Bull, 2003. **67**: p. 161-76.
221. Uriho, A., et al., *Effects of resveratrol on mitochondrial biogenesis and physiological diseases.* Advances in Traditional Medicine, 2021. **21**(1): p. 1-14.
222. Bonnefont-Rousselot, D., *Resveratrol and Cardiovascular Diseases.* Nutrients, 2016. **8**(5).
223. Poudel, R., et al., *Effects of resveratrol in pregnancy using murine models with reduced blood supply to the uterus.* PLoS One, 2013. **8**(5): p. e64401.
224. Cudmore, M.J., et al., *Resveratrol inhibits the release of soluble fms-like tyrosine kinase (sFlt-1) from human placenta.* Am J Obstet Gynecol, 2012. **206**(3): p. 253 e10-5.
225. Ding, J., et al., *Efficacy of resveratrol to supplement oral nifedipine treatment in pregnancy-induced preeclampsia.* Endocr Connect, 2017. **6**(8): p. 595-600.
226. Giusti, L., et al., *The Protective Action of Metformin against Pro-Inflammatory Cytokine-Induced Human Islet Cell Damage and the Mechanisms Involved.* Cells, 2022. **11**(15).
227. Sung, J.Y., et al., *Metformin mitigates stress-induced premature senescence by upregulating AMPKalpha at Ser485 phosphorylation induced SIRT3 expression and inactivating mitochondrial oxidants.* Mech Ageing Dev, 2022. **206**: p. 111708.
228. Feng, J., et al., *Mitochondria as an important target of metformin: The mechanism of action, toxic and side effects, and new therapeutic applications.* Pharmacol Res, 2022. **177**: p. 106114.
229. Herzig, S. and R.J. Shaw, *AMPK: guardian of metabolism and mitochondrial homeostasis.* Nat Rev Mol Cell Biol, 2018. **19**(2): p. 121-135.
230. Brownfoot, F.C., et al., *Metformin as a prevention and treatment for preeclampsia: effects on soluble fms-like tyrosine kinase 1 and soluble endoglin secretion and endothelial dysfunction.* Am J Obstet Gynecol, 2016. **214**(3): p. 356 e1-356 e15.
231. Wang, F., et al., *Effect of Metformin on a Preeclampsia-Like Mouse Model Induced by High-Fat Diet.* Biomed Res Int, 2019. **2019**: p. 6547019.
232. Alqudah, A., et al., *Risk of pre-eclampsia in women taking metformin: a systematic review and meta-analysis.* Diabet Med, 2018. **35**(2): p. 160-172.
233. Glueck, C.J., et al., *Height, weight, and motor-social development during the first 18 months of life in 126 infants born to 109 mothers with polycystic ovary syndrome who conceived on and continued metformin through pregnancy.* Hum Reprod, 2004. **19**(6): p. 1323-30.
234. Glueck, C.J., et al., *Effects of metformin-diet intervention before and throughout pregnancy on obstetric and neonatal outcomes in patients with polycystic ovary syndrome.* Curr Med Res Opin, 2013. **29**(1): p. 55-62.

235. De Leo, V., et al., *The administration of metformin during pregnancy reduces polycystic ovary syndrome related gestational complications.* Eur J Obstet Gynecol Reprod Biol, 2011. **157**(1): p. 63-6.
236. Cluver, C.A., et al., *Use of metformin to prolong gestation in preterm pre-eclampsia: randomised, double blind, placebo controlled trial.* BMJ, 2021. **374**: p. n2103.
237. Thompson, J.D. and D. Diers, *Management of nursing intensity.* Nurs Clin North Am, 1988. **23**(3): p. 473-92.
238. Long, J., et al., *Mitochondria Targeted Antioxidant Significantly Alleviates Preeclampsia Caused by 11beta-HSD2 Dysfunction via OPA1 and MtDNA Maintenance.* Antioxidants (Basel), 2022. **11**(8).
239. Gane, E.J., et al., *The mitochondria-targeted anti-oxidant mitoquinone decreases liver damage in a phase II study of hepatitis C patients.* Liver Int, 2010. **30**(7): p. 1019-26.
240. Snow, B.J., et al., *A double-blind, placebo-controlled study to assess the mitochondria-targeted antioxidant MitoQ as a disease-modifying therapy in Parkinson's disease.* Mov Disord, 2010. **25**(11): p. 1670-4.
241. Kirkman, D.L., et al., *Role of mitochondria-derived reactive oxygen species in microvascular dysfunction in chronic kidney disease.* Am J Physiol Renal Physiol, 2018. **314**(3): p. F423-F429.
242. Mason, S.A., et al., *Effect of mitochondrial-targeted antioxidants on glycaemic control, cardiovascular health, and oxidative stress in humans: A systematic review and meta-analysis of randomized controlled trials.* Diabetes Obes Metab, 2022. **24**(6): p. 1047-1060.
243. Yang, Y., et al., *The Potent Antioxidant MitoQ Protects Against Preeclampsia During Late Gestation but Increases the Risk of Preeclampsia When Administered in Early Pregnancy.* Antioxid Redox Signal, 2021. **34**(2): p. 118-136.
244. Ayla, S., et al., *Doxorubicin induced nephrotoxicity: protective effect of nicotinamide.* Int J Cell Biol, 2011. **2011**: p. 390238.
245. Wahlberg, G., et al., *Protective effect of nicotinamide against nephropathy in diabetic rats.* Diabetes Res, 1985. **2**(6): p. 307-12.
246. Rongvaux, A., et al., *Reconstructing eukaryotic NAD metabolism.* Bioessays, 2003. **25**(7): p. 683-90.
247. Li, F., et al., *Nicotinamide benefits both mothers and pups in two contrasting mouse models of preeclampsia.* Proc Natl Acad Sci U S A, 2016. **113**(47): p. 13450-13455.
248. Li, D., et al., *Nicotinamide supplementation induces detrimental metabolic and epigenetic changes in developing rats.* Br J Nutr, 2013. **110**(12): p. 2156-64.
249. Mahmoud, Y.I. and A.A. Mahmoud, *Role of nicotinamide (vitamin B3) in acetaminophen-induced changes in rat liver: Nicotinamide effect in acetaminophen-damged liver.* Exp Toxicol Pathol, 2016. **68**(6): p. 345-54.
250. Tian, Y.J., et al., *Maternal nicotinamide supplementation causes global DNA hypomethylation, uracil hypo-incorporation and gene expression changes in fetal rats.* Br J Nutr, 2014. **111**(9): p. 1594-601.

251. Sun, W.P., et al., *Comparison of the effects of nicotinic acid and nicotinamide degradation on plasma betaine and choline levels.* Clin Nutr, 2017. **36**(4): p. 1136-1142.
252. Kang, H., Y.K. Park, and J.Y. Lee, *Nicotinamide riboside, an NAD(+) precursor, attenuates inflammation and oxidative stress by activating sirtuin 1 in alcohol-stimulated macrophages.* Lab Invest, 2021. **101**(9): p. 1225-1237.
253. Fushima, T., et al., *Nicotinamide ameliorates a preeclampsia-like condition in mice with reduced uterine perfusion pressure.* Am J Physiol Renal Physiol, 2017. **312**(2): p. F366-F372.
254. Zheng, M., M.B. Schultz, and D.A. Sinclair, *NAD(+) in COVID-19 and viral infections.* Trends Immunol, 2022. **43**(4): p. 283-295.
255. Meyer, T., et al., *NAD(+) metabolism drives astrocyte proinflammatory reprogramming in central nervous system autoimmunity.* Proc Natl Acad Sci U S A, 2022. **119**(35): p. e2211310119.
256. Zhao, Y., et al., *NAD(+) improves cognitive function and reduces neuroinflammation by ameliorating mitochondrial damage and decreasing ROS production in chronic cerebral hypoperfusion models through Sirt1/PGC-1alpha pathway.* J Neuroinflammation, 2021. **18**(1): p. 207.
257. Zhou, B., et al., *Boosting NAD level suppresses inflammatory activation of PBMCs in heart failure.* J Clin Invest, 2020. **130**(11): p. 6054-6063.
258. Gariani, K., et al., *Eliciting the mitochondrial unfolded protein response by nicotinamide adenine dinucleotide repletion reverses fatty liver disease in mice.* Hepatology, 2016. **63**(4): p. 1190-204.
259. Pirinen, E., et al., *Pharmacological Inhibition of poly(ADP-ribose) polymerases improves fitness and mitochondrial function in skeletal muscle.* Cell Metab, 2014. **19**(6): p. 1034-41.
260. Zhang, H., et al., *NAD(+) repletion improves mitochondrial and stem cell function and enhances life span in mice.* Science, 2016. **352**(6292): p. 1436-43.
261. Cercillieux, A., E. Ciarlo, and C. Canto, *Balancing NAD(+) deficits with nicotinamide riboside: therapeutic possibilities and limitations.* Cell Mol Life Sci, 2022. **79**(8): p. 463.
262. Freeberg, K.A., et al., *Dietary supplementation with NAD +-boosting compounds in humans: Current knowledge and future directions.* J Gerontol A Biol Sci Med Sci, 2023.
263. Martens, C.R., et al., *Chronic nicotinamide riboside supplementation is well-tolerated and elevates NAD(+) in healthy middle-aged and older adults.* Nat Commun, 2018. **9**(1): p. 1286.
264. Elhassan, Y.S., et al., *Nicotinamide Riboside Augments the Aged Human Skeletal Muscle NAD(+) Metabolome and Induces Transcriptomic and Anti-inflammatory Signatures.* Cell Rep, 2019. **28**(7): p. 1717-1728 e6.
265. Remie, C.M.E., et al., *Nicotinamide riboside supplementation alters body composition and skeletal muscle acetylcarnitine concentrations in healthy obese humans.* Am J Clin Nutr, 2020. **112**(2): p. 413-426.
266. Nascimento, E.B.M., et al., *Nicotinamide Riboside Enhances In Vitro Beta-adrenergic Brown Adipose Tissue Activity in Humans.* J Clin Endocrinol Metab, 2021. **106**(5): p. 1437-1447.

267. Wang, D.D., et al., *Safety and Tolerability of Nicotinamide Riboside in Heart Failure With Reduced Ejection Fraction.* JACC Basic Transl Sci, 2022. **7**(12): p. 1183-1196.
268. Brakedal, B., et al., *The NADPARK study: A randomized phase I trial of nicotinamide riboside supplementation in Parkinson's disease.* Cell Metab, 2022. **34**(3): p. 396-407 e6.
269. Veenhuis, S.J.G., et al., *Nicotinamide Riboside Improves Ataxia Scores and Immunoglobulin Levels in Ataxia Telangiectasia.* Mov Disord, 2021. **36**(12): p. 2951-2957.
270. Lapatto, H.A.K., et al., *Nicotinamide riboside improves muscle mitochondrial biogenesis, satellite cell differentiation, and gut microbiota in a twin study.* Sci Adv, 2023. **9**(2): p. eadd5163.
271. Lee, S.R., et al., *Dietary supplementation with nicotinamide riboside improves fetal growth under hypoglycemia.* J Nutr Biochem, 2023. **116**: p. 109310.
272. Ear, P.H., et al., *Maternal Nicotinamide Riboside Enhances Postpartum Weight Loss, Juvenile Offspring Development, and Neurogenesis of Adult Offspring.* Cell Rep, 2019. **26**(4): p. 969-983 e4.

Appendix:

Supplementary

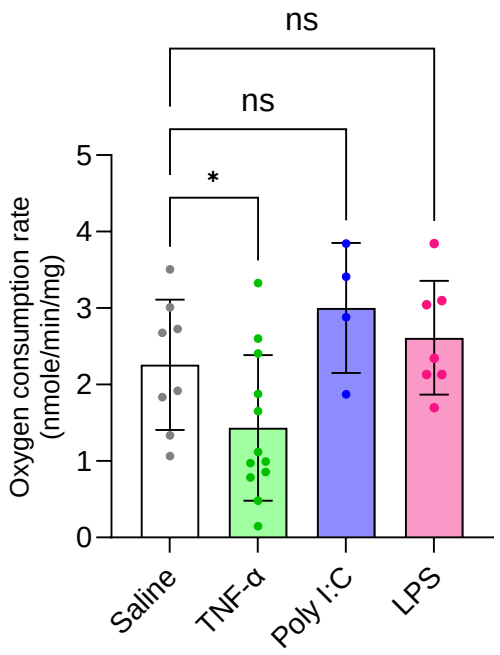


Figure 1: Glutamate/malate driven state-II respiration or oxygen consumption rates. *P＜0.05. Error bar indicates standard deviation (SD). Tumor necrosis factor (TNF-α), Polyinosinic:polycytidylic acid (Poly I:C) and Lipopolysaccharide (LPS).

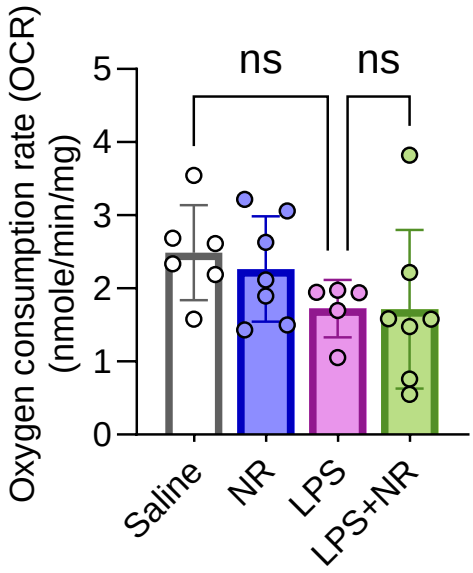


Figure 2: Glutamate/malate driven state-II respiration or oxygen consumption rates. Two-way ANOVA with Holm-Šídák's multiple comparisons test. Error bar indicates standard deviation (SD). LPS= Lipopolysaccharide, NR= nicotinamide riboside.

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