Vascular Adaptations to Pregnancy in a Rat Model of Advanced Maternal Age: Endoplasmic Reticulum Stress as a Potential Target for Therapeutic Intervention

by

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Abstract

Advanced maternal age (≥35 years) increases the risk of pregnancy-specific complications such as reduced fetal growth and preeclampsia. The Davidge laboratory previously showed poor pregnancy outcomes and vascular dysfunction in a rat model of advanced maternal age. However, vascular adaptations, molecular mechanisms, and potential interventions to improve vascular function and adverse pregnancy outcomes in aging were not studied. I **hypothesize** that in a rat model of advanced maternal age; 1) normal pregnancy-induced vascular adaptations are altered, causing adverse pregnancy outcomes, due to increased endoplasmic reticulum (ER) and oxidative stress via NADPH (NOX) oxidases in the vasculature and placenta and 2) that tauroursodeoxycholic acid (TUDCA) treatment (an ER stress inhibitor) by reducing ER and oxidative stress will improve pregnancy outcomes. In the first study, young (3-4 months) and aged (9-9.5 months; ~35 years in humans) non-pregnant and pregnant rats were used, in the second and third studies, young and aged dams with or without TUDCA treatment were studied on gestational day (GD) 20. A calculated dose (150 mg/kg/day) of TUDCA was provided in regular drinking water throughout pregnancy. On GD20 (non-pregnant rats were aged-matched), blood pressure was measured (tail-cuff plethysmography), pregnancy outcomes were recorded, vascular function in mesenteric and main uterine arteries (wire myography), and ER and oxidative stress markers (Western blotting) were assessed in systemic mesenteric arteries and placentas. Endothelium-dependent vasodilation response to methacholine (MCh) was studied in the presence or absence of pan-nitric oxide synthase (NOS) inhibitor (L-NAME), prostaglandin H-synthase (PGHS) inhibitor (meclofenamate), endothelium-dependent hyperpolarization (EDH) inhibitors (apamin and TRAM-34) or NOX inhibitor (apocynin). Vasoconstriction responses to big endothelin-1 (bigET-1) were studied in the presence or absence of matrix metalloproteinases (MMPs) inhibitor

(GM6001), endothelin converting enzyme-1 (ECE-1) inhibitor (CGS35066), chymase inhibitor (chymostatin), or neutral endopeptidase inhibitor (DL-Thiorphan), further, vascular response to ET-1 was also evaluated.

In the first study, I observed adverse pregnancy outcomes in aged dams in comparison to young dams. Enhanced contribution of NO and EDH-mediated relaxation in aged dams compared to young dams. Increased blood pressure and enhanced contribution of NOX enzymes in aged non-pregnant rats compared to all the groups. In addition, aged groups demonstrated greater contractile responses to bigET-1 compared to young groups, independent of pregnancy. There was also increased contribution of ECE-1 in processing bigET-1 to ET-1 in aged groups, while no changes in the presence of other inhibitors (MMPs, chymase, or neutral endopeptidase). There were no differences in the sensitivity to ET-1 between the young and aged groups. In conclusion, reduced blood pressure and enhanced EDH-mediated contribution to vasodilation imply a compensatory beneficial adaptation in aged dams that were successful and able to maintain pregnancy.

In the second study, the results recapitulated adverse pregnancy outcomes in the aged dams vs young dams. NO changes in the MCh responses were observed in mesenteric arteries, while reduced uterine artery relaxation was seen in aged dams vs young dams. Increased levels of phospho-eIF2α, CHOP, and NOX-4 were observed in the aged dams vs young dams. TUDCA treatment decreased blood pressure, improved fetal weight, tended to improve uterine artery vascular function, and reduced the expression of phospho-eIF2α, and CHOP proteins in aged dams vs control dams. In conclusion, this study highlighted the role of ER stress in pregnancy and highlighted the beneficial effect of TUDCA treatment in improving altered vascular function and pregnancy outcomes in advanced maternal age.

In the third study, my data showed increased levels of ER stress proteins GRP78 (in the

male labyrinth zone) and phospho-eIF2α (in male and female junctional zones) in aged dams vs young dams. Whereas TUDCA treatment reduced the levels of ER stress markers only in aged dams (GRP78, phospho-eIF2α, ATF-4, and CHOP) in the male labyrinth zone, and sXBP1 protein in both male and female junctional zones. Thus, TUDCA may have a cytoprotective role in maintaining ER stress proteins to basal levels in advanced maternal age.

In conclusion, my PhD project emphasized the importance of vascular adaptive pathways in pregnancy that could compensate for advanced maternal age and highlighted the complexity of cellular ER stress responses in advanced maternal age that may be modulated in aged dams. Whereas TUDCA intervention, as a promising therapeutic option, may benefit pregnancy outcomes in complicated pregnancies such as advanced maternal age.

Preface

This thesis is an original research work by Mazhar Pasha. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Pregnancy Complications", and AUP # 242 and #3692.

The literature review in Chapter 1, materials and methods in Chapter 2, and data presented in Chapters 3, 4, and 5 are my original work.

The results in Chapter 3 of this thesis have been published as *"Mazhar Pasha, Amy L. Wooldridge, Raven Kirschenman, Floor Spaans, Sandra T. Davidge, and Christy-Lynn M. Cooke. Altered Vascular Adaptations to Pregnancy in a Rat Model of Advanced Maternal Age. Front Physiol. 2021 Jul 28;12:718568."*

Mazhar Pasha was responsible for performing all the experiments, acquisition of data, and analysis as well as preparing the manuscript. Amy L. Wooldridge assisted with the data collection, Raven Kirschenman, assisted with breeding the animals and data collection, Floor Spaans contributed to the manuscript edits, Sandra T. Davidge and Christy-Lynn Cooke were the supervisor and co-supervisor authors and were involved with study conception and design, interpretation of data, and revision of the manuscript.

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Mazhar Pasha was responsible for performing all the experiments, acquisition of data, and analysis as well as preparing the manuscript. Amy L. Wooldridge assisted with the data collection, Raven Kirschenman, assisted with breeding the animals and data collection, Floor Spaans

contributed to the manuscript edits, Sandra T. Davidge and Christy-Lynn Cooke were the supervisor and co-supervisor authors and were involved with study conception and design, interpretation of data, and revision of the manuscript.

The results in Chapter 5 of the thesis have been submitted to PLOSONE-D-22-31856 (under review) as **"***Mazhar Pasha, Raven Kirschenman, Amy L. Wooldridge, Christy-Lynn M. Cooke, and Sandra T. Davidge. The Effect of TUDCA Treatment on Placental Endoplasmic reticulum (ER) Stress in a Rat Model of Advanced Maternal Age"*.

Dedication

This thesis is dedicated to my dear mom and dad.

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I take this opportunity to express my sincere gratitude to all the people who helped me to complete this challenging journey. First and foremost, my supervisor and co-supervisor, Dr. Sandra T. Davidge and Dr. Christy-Lynn Cooke, both are my role models, besides from been great supervisors, both are trusted mentors, always enthusiastic and respectful advisors, who constantly inspired me by providing honest feedback throughout this wonderful journey.

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List of Abbreviations

AA	Arachidonic acid
Ach	Acetylcholine
Ang II	Angiotensin-II
ATF4	Activating factor -4
ATF6	Activating transcription factor 6
ASK1	Apoptosis-signal-regulating kinase
ATP	Adenosine triphosphate
bigET-1	Big endothelin-1
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine-3, 5-monophosphate
CHOP	C/EBP homologous protein
COX	Cyclooxygenase
DCA	Deoxycholic acid
ECE-1 and ECE-2	Endothelin-converting enzymes 1 and 2
EDH	Endothelium-dependent hyperpolarization
eIF2α	Eukaryotic translation-initiation factor-2
eNOS or NOS-III	Endothelial NO synthase
ER	Endoplasmic Reticulum
ET-1	Endothelin-1
ET_A and ET_B	Endothelin A and B receptors
ET-1	Endothelin-1
GD	Gestational day
GRP78	Glucose-regulated protein 78
GTP	Guanosine triphosphate
IKCa	Calcium activated intermediate potassium channels
iNOS or NOS-II	Inducible NO synthase
IP	Prostacyclin receptor
IP3	Inositol trisphosphate
IRE1	Inositol-requiring enzyme-1

JNK	c-Jun N-terminal kinase
L-NAME	N-nitro-L-arginine methyl ester
LCA	lithocholic acid
MCh	Methylcholine
MMPs	Matrix metalloproteases
NEP	Neutral endopeptidase
NO	Nitric oxide
NOS	NO synthase
NOX	NADPH oxidases
nNOS or NOS-I	Neuronal NO synthase
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
(PG)E_2	Prostaglandin
PGE_2	Prostaglandin E_2
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
PIP	Phosphatidylinositol
PKA	protein kinases A
PSS	Physiological saline solution
R	Receptor
ROS	Reactive oxygen species
SKCa	Calcium activated small potassium channels
SNP	Sodium nitroprusside
SpT	Spongiotrophoblast
TGC	Trophoblast giant cells
TP	Thromboxane receptors
TRAF2	TNF-receptor-associated factor 2
TXA_2	Thromboxane A_2
TUDCA	Tauroursodeoxycholic acid
UDCA	Ursodeoxycholic acid
VSMC	Vascular smooth muscle cell relaxation
XBP-1	X-box binding protein-1

CHAPTER 1

General Introduction

1.1 Advanced maternal age

Pregnancy at an advanced age has been increasing gradually in North America, accounting for 20 % of total live births from aged women over 35 years (1-3). In general, advanced maternal age is referred to as maternal age ≥35 years during pregnancy, and with advancing age there is an increased risk of fetal congenital anomalies, chromosomal abnormalities, preterm infant (<37 weeks), and small for gestational age (4-7). Research revealed that advanced maternal age is associated with a higher risk of pregnancy-specific complications, for example, fetal growth restriction, preterm birth, gestational diabetes, preeclampsia (pregnancy-related complication characterized by hypertension, proteinuria, and or end-organ dysfunction), hypertension, placenta previa, and stillbirth (8-13). Further, fertility starts to decline with advancing age, starting at ≥35 years of age (4, 14). However, advancements in assisted reproductive technologies have driven a shift towards childbearing later in life, but this new trend might increase pregnancy complications, as more women of older age become pregnant (15-17).

Clinical studies show that advanced maternal age represents a very important, still understudied population of pregnant women. For example, a cohort study in the United Kingdom demonstrated an 18-20% prevalence of advanced maternal age and was accompanied by higher risks of complications including gestational diabetes, disorders, postpartum hemorrhage, gestational hypertension, and cesarean delivery (12). A study conducted over multiple countries (Asia, the Middle East, Africa, and Latin America) revealed a 12% prevalence of pregnant women with advanced maternal age and were associated with a greater risk of adverse pregnancy outcomes and perinatal outcomes (low fetal body weight, preterm birth, and neonatal mortality (18).

Similarly, a study in South Australia and Finland disclosed that women of advanced age were susceptible to severe maternal and perinatal outcomes (10, 13). Further, several studies also highlight offspring born from a suboptimal uteroplacental environment develop an increased risk of cardiovascular complications later in life (19-22). Thus, advanced maternal age has clinical consequences during pregnancy, at the time of delivery, and also on the long-term health of the mother and the developing baby. The increased susceptibility to pregnancy-related complications with increasing age could be, in part, due to insufficient cardiovascular adaptations during pregnancy (23, 24).

1.2 Cardiovascular adaptations in normal pregnancy

Pregnancy is a dynamic process associated with significant maternal anatomical and physiological adaptations to support the developing fetus. Among the several physiological adaptations, cardiovascular adaptations are one of the most important and it is critical to support a healthy pregnancy (25-27). Cardiovascular adaptations during pregnancy are profound and begin early in pregnancy (~8 weeks of gestation). In brief, the main pregnancy-related hemodynamic changes include increased plasma blood volume (~50%), increased cardiac output (~30–50%, which is the product of stroke volume and heart rate), and reduced systemic peripheral vascular resistance and low blood pressure. The mean arterial pressure decreases (reaching a nadir point) in the second trimester and remains constant during the pregnancy (28, 29). Decreased blood pressure is associated with reduced total peripheral vascular resistance during gestation. The reduced vascular resistance is primarily attributed to the remodeling of uterine vasculature and to a lesser extent, systemic vasculature thus, a major proportion of the cardiac output directs to the uteroplacental unit to nourish the developing fetus. Given that these massive hemodynamic cardiovascular adaptations are required for a healthy pregnancy, pregnancy could be considered a

natural ‘stress test’ on the cardiovascular system that may unmask previously silent (subclinical) pathology (25, 30, 31).

The molecular mechanisms associated with decreased vascular resistance are not well established, but several studies have revealed that during pregnancy there is increased bioavailability of endothelium-released vasodilators such as nitric oxide (NO), prostacyclin (PGI_2), and endothelium-dependent hyperpolarization (EDH) (32-34). Although NO is the major vasodilator, research has also emphasized the importance of PGI_2 in pregnancy-adapted vasodilator responses (33-36). In addition, literature revealed that vasodilation of small resistance arteries such as mesenteric arteries that play a pivotal role in systemic circulation during pregnancy is also mediated by EDH. Though EDH is distinct from NO, studies revealed that if NO-mediated relaxation is reduced, either PGI_2 or EDH may compensate for endothelium-dependent vasodilation (34, 37-41). These vasodilators act both systemically (mesenteric arteries) and locally (uteroplacental unit) to support pregnancy adaptations. In addition, higher circulating levels of estrogens are observed during pregnancy which promotes uterine vascular remodeling and placental angiogenesis (42, 43). Thus, normal maternal cardiovascular adaptations to pregnancy significantly increase the blood supply to the placenta and uterine arteries to nourish and support the developing fetus (44-46), and any changes in the adaptation of these pathways could be linked to pregnancy-related complications such as preeclampsia, and fetal growth restriction (34, 47). The role of various vascular endothelial mediators necessary to maintain healthy pregnancy is discussed in later sections.

1.3 Cardiovascular adaptations in advanced maternal age

In advanced maternal age, normal pregnancy-related vascular adaptations may be impaired, eventually leading to poor pregnancy outcomes. Indeed, using a rat model of advanced

maternal age, previously the Davidge lab has demonstrated reduced fertility rates, adverse pregnancy outcomes (reduced fetal body weight, increased placental weight, reduced litter size, and a higher number of fetal resorptions), and altered vascular function (enhanced active myogenic responses in both uterine and mesenteric arteries) in aged dams in comparison to young dams (23). In addition, studies have also demonstrated similar adverse pregnancy outcomes using other models of advanced maternal age. For instance, Plant *et al.* showed an altered vascular response to pregnancy (immunological, hormonal, and cardiovascular dysfunction) and postnatal fetal growth restriction in the vervet/African green monkey model of advanced maternal age (optimal maternal age: 3–9 years and advanced maternal age: >10 years) (48). Lean *et al.* in C57Bl/6J female mice (control: 8–16 weeks old and aging: 38–42 weeks old) demonstrated impaired uteroplacental vascular function and highlighted the significance of placental dysfunction as the key mechanism for the increased susceptibility to fetal growth restriction and stillbirth in advanced maternal age (49, 50).

Overall, the above studies highlight that pregnancy-related vascular adaptations may be suboptimal or altered in advanced maternal age pregnancies, and thus could increase the risk of developing pregnancy-induced complications. Although pregnancies at an advanced age are considered high-risk clinically, there are limited studies focusing on exploring the mechanistic vascular pathways that could be associated with altered pregnancy adaptations and poor pregnancy outcomes with advanced maternal age (12, 13, 23, 48).

1.4 Vascular Endothelial Mediators During Pregnancy

It is well known that the endothelium plays a key role in order to maintain or regulate the vascular tone during normal pregnancy. The vascular endothelium is in direct contact with the circulating blood, consequently, interacts directly with various circulating endogenous mediators

(acetylcholine, histamine, bradykinin, substance P, and adrenomedullin) and also physical factors (shear stress). These endogenous and physical factors bind to their receptors located on the endothelium and increase the production/release of vasoactive mediators. The importance of various vasoactive mediators produced is studied extensively and the key mediators are 1) vasodilators: NO, PGI_2, and EDH, 2) vasoconstrictors: endothelin-1 (ET-1), thromboxane, reactive oxygen species (ROS), and angiotensin II (34, 51-53).

The sections below emphasize the importance of various endothelium-dependent vasodilators and vasoconstrictors: first, a general overview and then their role in pregnancy and aging (please also note that not all the pathways have been reviewed, but only those relevant to the current thesis have been discussed).

1.4.1 Endothelium-dependent vasodilators

It is clear that during pregnancy sufficient blood flow via uteroplacental is dependent on vasodilation. In general, the overall contribution of vasodilators (NO, PGI_2, and EDH) exists based on nature; young vs aged (reduced NO bioavailability with aging) and vascular bed, for example, it is documented that NO is a major vasodilator in larger conduit arteries (aorta) and endothelium-derived hyperpolarization is dominant in smaller resistance arteries (mesenteric arteries). Moreover, increased levels of PGI_2 are observed when there is decreased bioavailability of NO thus, maintaining overall endothelium-dependent vasodilation response (34, 41).

1.4.1.1 Role of NO in the vasculature

NO is a potent vasodilator synthesized and released by the endothelial cells that plays a significant role in modulating vascular tone and blood pressure; in addition, it also acts as an antioxidant, anti-inflammatory, and antithrombotic agent (52, 54, 55). NO is produced by the

enzyme NO synthase (NOS) from the substrate L-arginine in the presence of oxygen, flavin mononucleotide, reduced nicotinamide-adenine-dinucleotide phosphate, flavin adenine dinucleotide, and BH4 act as a cofactor. NOS exists in three different isoforms; two "constitutive" isoforms such as endothelial (NOS-III or eNOS) produced in the endothelium and neuronal (NOS-I or nNOS) synthesized by neurons, and an "inducible" isoform (NOS-II or iNOS) produced during infection (by bacterial endotoxin), and/or upon stimulation by proinflammatory cytokines (e.g., interleukin-1, interferon-gamma, tumor necrosis factor, etc.) (56, 57). Several factors can stimulate the synthesis of NO, for example, agonists such as acetylcholine, bradykinin, and shear stress activate endothelial receptors that lead to the production of secondary messenger inositol triphosphate which in turn increases intracellular calcium (Ca^{2+}) level. Ca^{2+} binds to calmodulin to form the Ca^{2+}-calmodulin complex, an important regulatory complex for the activity of eNOS and nNOS, whereas iNOS is a Ca^{2+}-independent enzyme. In addition, a Ca^{2+}-independent mechanism via protein kinases (PKA) can also activate eNOS (52, 58).

Once produced, NO diffuses rapidly across the smooth muscle cells and activates the secondary messenger such as the soluble guanylate cyclase enzyme (a heme-dependent enzyme) that catalyzes the formation of cyclic guanosine monophosphate (cGMP; a secondary intracellular messenger). cGMP decreases the intracellular-free Ca^{2+} levels, subsequently causing dephosphorylation of the myosin light chain, thus ultimately resulting in vascular smooth muscle cell (VSMC) relaxation (59, 60). NOS activity can be experimentally antagonized by N-nitro-L-arginine methyl ester (L-NAME), L-N-Methylarginine, L-N, N-Dimethylarginine, etc. Research in pregnant animals revealed that inhibiting eNOS using L-NAME induces vasoconstriction phenotype with reduced uterine artery blood flow, suggesting the pivotal role of NO in vascular adaptation during pregnancy as discussed in the section below (54, 61, 62).

1.4.1.2 Role of NO in vascular adaptations to pregnancy

NO is a potent vasodilator that plays a central role in normal physiological adaptations during pregnancy, reduces systemic peripherical vascular resistance, and regulates fetoplacental blood flow (62-64). Kublickiene *et al*. evaluated flow-mediated responses in myometrial arteries from normal pregnant women and demonstrated that NO, but not prostanoids, play a pivotal role in maintaining sufficient blood flow to the developing fetus during human pregnancy (65). Nelson *et al.* studied the role of NO and expression of NOS protein in a human uterine artery during pregnancy and showed an increased expression of eNOS in the endothelium and nNOS in the adventitia, but no expression of iNOS in both pregnant and non-pregnant uterine arteries. The study also observed a proportional association between the expression of eNOS and NO in the maintenance of human uterine artery vasodilation during pregnancy (66). Owusu *et al.,* in a study, observed higher serum NO metabolites (nitrate/nitrite) levels and lower mean arterial blood pressures in healthy pregnant compared to non-pregnant women, and the study concluded that NO plays a major role in vascular adaptation in pregnancy (67). Hodzic *et al.* observed increased production of NO with gestational age during normal pregnancy compared to preeclampsia, and the concentration of serum NO (after reduction of nitrates to nitrites) in preeclamptic women was positively correlated with blood pressure, uric acid, creatinine clearance, and negatively correlated with platelet count. The study concluded that NO could modulate cardiovascular changes not only during normal pregnancy but also in preeclampsia (68). In addition, Osol *et al.* in a rat model of hypertension (NOS inhibition using L-NAME) in pregnant and non-pregnant rats showed that NO significantly contributes to remodeling the uterine circulation in pregnancy (69). Similarly, studies have demonstrated that the deficiency of NO produces preeclampsia-like syndrome (64, 70, 71). Thus, increased production of NO contributes to the hemodynamic changes that are pivotal for a

healthy pregnancy, conversely, a reduction of NO may be observed in pregnancy-related complications including advanced maternal age.

1.4.1.3 Role of NO in aging

It is known that aging increases the risk of cardiovascular impairments and altered vascular function (72-75). Both humans and animal studies have shown that impaired endothelium-dependent vasodilation with aging is mediated, in part, by a decrease in NO bioavailability (76-79). The molecular mechanisms leading to reduce NO bioavailability could be due to decrease NO production (reduced eNOS activity), increased NO removal (scavenging by superoxide radicles), or both (80-83). Accordingly, we can speculate that with advancing age and associated vascular impairment, the maternal vascular system may not adapt to the increasing cardiovascular demands of pregnancy. In addition to NO, endothelium also produces PGI_2 which acts as a potent vasodilator.

1.4.1.4 Role of PGI_2 in the vasculature

PGI_2 belongs to the endogenous prostanoid family of lipid mediators that acts as a potent vasodilator and also inhibits platelet activation. The increase in endothelial Ca^{2+} levels not only enhance NOS activity but also stimulates the prostaglandin H synthase (PGHS) pathway leading to the formation of prostaglandins, followed by the production of several bioactive metabolites such as prostaglandin (PG)E_2, PGD_2, & $PGF_{2\alpha}$, PGI_2, and thromboxane A_2 (TXA_2) by tissue-specific synthases. In general, PGI_2 counteracts the action of TXA_2; a vasoconstrictor (34). Both PGI_2 and TXA_2 are derived from arachidonic acid (AA) metabolism by the actions of phospholipase A2, following which PGHS (cyclooxygenase) enzymes and specific prostaglandin (PG) synthases process the substrate into the various prostanoids. There are two isoforms: PGHS-

1 and PGHS-2, PGHS-1 is constitutively expressed but can also be induced during shear, whereas PGHS-2 is an inducible isoform (e.g., proinflammatory mediators like cytokines, lipopolysaccharides, etc.) (84, 85).

PGI_2 and TXA_2 act on G-protein-coupled receptors; prostacyclin (IP) and thromboxane (TP) receptors respectively (41, 86). PGI_2 binds to IP receptors and activates adenylyl cyclase to produce secondary intracellular messenger cyclic adenosine monophosphate (cAMP), which eventually activates protein kinase A promoting phosphorylation of the myosin light chain kinase, thus, causing VSMC vasodilation.

1.4.1.5 Role of PGI_2 in pregnancy

The importance of PGI_2 in pregnancy-adapted vasodilator responses has been documented by several studies (33-36). In addition, Goodman *et al.* observed increased PGI_2 biosynthesis in the uterine artery in pregnant compared to non-pregnant ewes (87) and Lewis *et al.* detected increased plasma concentration of PGI_2 metabolite (6-oxo PGF1α) in pregnant compared with the non-pregnant women (88). Moreover, there are reports demonstrating an increased expression of PGHS enzymes (mostly PGHS-1 in endothelial cells) during pregnancy, thus, increasing the cellular capacity for PGI_2 production (89-91). Interestingly, research shows that either PGI_2 deficiency and/or PGI_2/TXA_2 imbalance during pregnancy could be linked to preeclampsia and abnormal fetal growth and development, partly due to increased blood pressure, platelet aggregation, and increased vascular reactivity (92, 93). These studies emphasize the importance of PGI2 in pregnancy-adapted vasodilator responses.

1.4.1.6 Role of PGI_2 in aging

Although several studies highlight how aging affects NO-mediated vasodilation (80-83),

limited research has focused on understanding the effect of aging on PGI_2-mediated vasodilation leading to endothelial dysfunction. Indirect evidence suggests that aging reduces PGI_2-mediated vasodilation, for example, Nakajima *et al.* demonstrated a decreased production of PGI_2 in cultured rat aortic endothelial cells in aged (100-week-old) compared to young (six-week-old) Wistar rats (94). Nicholson *et al.* in a clinical study observed reduced vasodilator effects of PGI_2 in the human forearm in older (61–73 years) compared to younger (19–45 years) subjects, and NOS inhibition (using N^G-monomethyl-L-arginine acetate) reduced PGI_2-mediated vasodilation in older vs younger subjects (95). Further, Qian *et al.* in a literature review emphasized that there is a modified prostaglandins profile associated with age (synthesis and signaling transduction pathways) and the aging-modulated prostaglandin profile could offer an important area to explore and understand the molecular mechanism associated with age-related endothelial dysfunction and cardiovascular disorders (96).

1.4.1.7 Role of EDH in the vasculature

In addition to NO and PGI_2-mediated vasodilation, EDH is also established as an important endothelium-derived vasodilator mainly in small resistance arteries, thus, could be considered an important regulator of blood pressure. The vasodilation effect of EDH is mainly due to enhance VSMC hyperpolarization. Similar to NO and PGI_2, an increase in endothelial Ca^{2+}, is required for EDH pathway activation. Ca^{2+}-activated small and intermediate potassium channels (SKCa and IKCa) causes potassium efflux and hyperpolarization of the endothelium membrane. SKCa and IKCa channels are mainly expressed on endothelium (97, 98). Moreover, hyperpolarization also spreads to the underlying VSMC via myoendothelial gap junctions, preventing the opening of L-type voltage-dependent Ca^{2+} channels, thus, decreasing the Ca^{2+} influx and finally causing VSMC relaxation (99). In general, caution must be applied while concluding the nature of EDH, as most

of the data support a central role of K^+ channels, however, various factors have been proposed to have EDH properties, such as hydrogen peroxide, hydrogen sulfide, epoxyeicosatrienoic acids, and C-type natriuretic peptide (99-101). Though EDH is different from NO, research points out that if NO-mediated relaxation is reduced, EDH may compensate for the overall maintenance of endothelium-mediated vascular relaxation (40).

1.4.1.8 Role of EDH in pregnancy

In addition to NO and PGI_2, studies have demonstrated that EDH acts as the other major endothelium-derived vasodilator (in resistance arteries) during pregnancy. The role of EDH in pregnancy-adapted vasodilation is that the relative contribution of EDH, NO, or PGI_2 is dependent on different vascular bed/vessel sizes (102). Research has demonstrated the upregulation of EDH in normal pregnancy (in small subcutaneous and myometrial arteries), which may be absent in complicated pregnancies (preeclampsia) (103, 104). Several other studies have also demonstrated the importance of EDH as one of the major vasodilators during pregnancy of human omental arteries and myometrial arteries (105-107), and in rat uterine arteries (108).

1.4.1.9 Role of EDH in aging

In general, EDH has been shown to be reduced with aging and is associated with endothelial dysfunction (109). Chennupati *et al.* observed a decreased contribution of EDH to endothelium-dependent vasodilation response but an enhanced effect of NO in saphenous arteries of aged C57BL/6J mice (34 and 64 vs 12-week-old mice) (110).

Figure 1.1 shows a summary of endothelium-dependent vasodilation pathways, recognizing that this is not an exhaustive discussion.

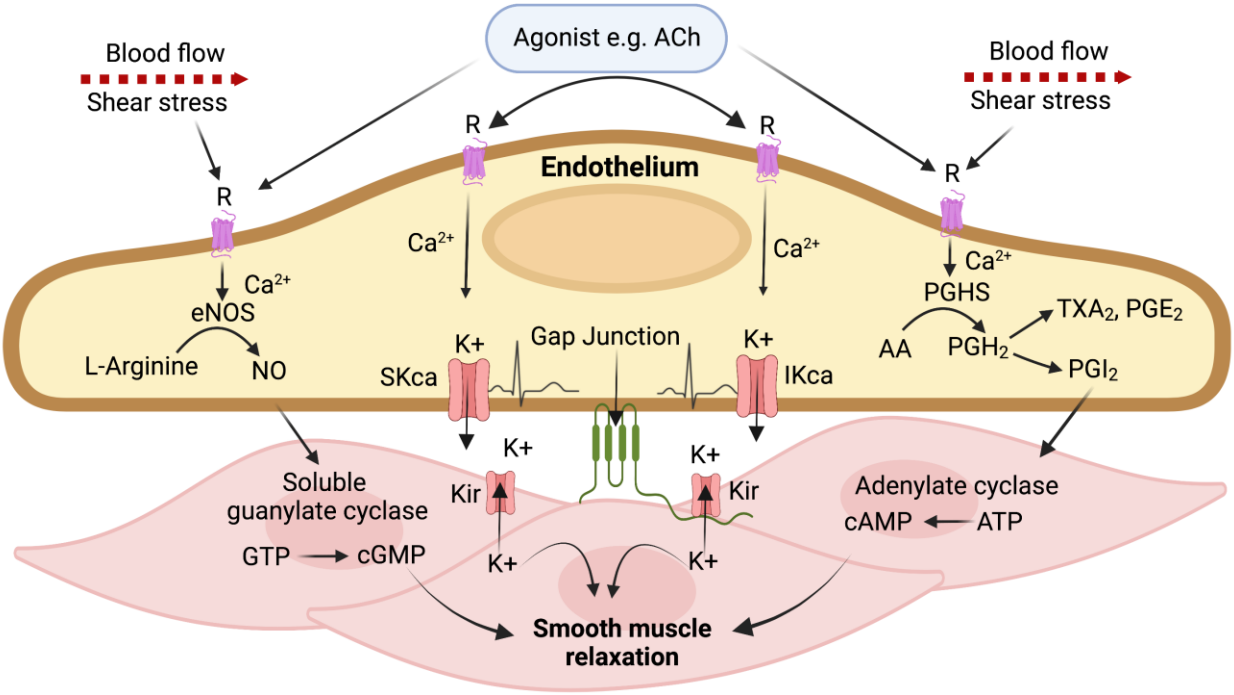

Figure 1.1 Signaling pathways contributing to the endothelium-dependent vasodilation responses

Three endothelium-dependent vasodilation pathways that causes vascular smooth muscle cell relaxation are nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarization (EDH). Ach: acetylcholine, R: receptor, Ca^{2+} calcium ions, eNOS: endothelial nitric oxide synthesis, cGMP: cyclic guanosine-3, 5-monophosphate, GTP: guanosine triphosphate, COX-1: cyclooxygenase-1, AA: arachidonic acid, PGH_2: prostaglandin H_2, TXA_2: thromboxane A_2, PGE_2 : prostaglandin E_2, SKCa: calcium activated small potassium channels, IKCa: calcium activated intermediate potassium channels, Kir: inward rectifying K+ channels, cAMP: cyclic adenosine monophosphate, ATP: adenosine triphosphate. Adapted and modified from Sandoo et al. Open Cardiovasc Med J. 2010 and "Created with BioRender.com."

1.4.2 Endothelium-dependent vasoconstrictor

The vascular endothelium not only produces and releases various vasodilators but also

secretes several vasoconstricting agents such as endothelin-1 (ET-1), angiotensin II, PGHS-derived prostanoids such as TXA_2, endoperoxides, and superoxide anions. ET-1 is known to be one of the most potent endogenous vasoconstrictors that have been linked to reduce endothelial vasodilator capacity during aging and under several pathophysiological conditions (coronary artery disease, Type 2 diabetes, and hypertension) including aging. Several factors enhance the secretion of ET-1 such as shear stress, or stimuli including angiotensin II, thrombin, hypoxia, growth factors, epinephrine, glucose, cytokines, and ROS. In contrast, NO, prostacyclin, cyclic GMP, and atrial natriuretic peptide reduce the release of ET-1 (111, 112). ET-1 is also involved in maintaining/regulating vascular tone via balancing endothelial and VSMC activation, and its placental localization suggests an important role in regulating uteroplacental circulation. Further, overexpression of ET-1 is observed in pregnancy-related complications (52, 113-117). Hence, the main focus will be on ET-1, and discussing other vasoconstrictors were beyond the scope of the current study.

1.4.2.1 Role of ET-1 in the vasculature

There are three isoforms of endothelins with 21 amino acid peptides (ET-1, ET-2, and ET-3), wherein ET-1 is the most predominant isoform (112, 118). The peptide ET-1 is produced from its biologically inactive precursor big endothelin-1 (bigET-1; 1-39 amino acid peptides), and subsequently cleaved by endothelin-converting enzymes (ECE-1/ECE-2). ECE-1 is the major regulatory enzyme present in a variety of cells; indeed, the vascular endothelium cells constitutively produce the ECE-1 enzyme (119-121). In addition, alternative enzymes have been reported: matrix metalloproteases (MMPs; MMP-2 and possibly MMP-9) (122), chymase (123), and neutral endopeptidases (124). Once synthesized, ET-1 interacts with G-protein-coupled receptors; ET_A and ET_B (on the VSMC), and the ET_B receptor (on the endothelium). The ET_A

receptor is the main receptor contributing to the vasoconstrictor effects of ET-1. The binding of ET-1 to its receptors (ET_A and $ET_{B)}$ on the VSMC increases the Ca^{2+} levels via activating phospholipase C to form 1,4,5-triphosphate which initiates the release of intracellular stored Ca^{2+} (sarcoplasmic reticulum), wherein Ca^{2+} binds and form an active complex with calmodulin ultimately activating myosin light chain kinase phosphorylation, thus, causing vasoconstriction (112, 125, 126). In addition, studies have demonstrated that the binding of ET-1 on endothelial cells (ET_B receptor) increases intracellular calcium levels and activity of eNOS, thus causing vasodilation (127, 128).

1.4.2.2 Role of ET-1 in pregnancy

In addition to uteroplacental circulation, ET-1 plays a pivotal role in regulating pulmonary circulation, fetal circulation, and closure of the ductus arteriosus after birth (117). In general, research highlights, an increased ET-1 activity and reduced NO production could be involved in the pathogenesis of hypertensive disorders (preeclampsia) during pregnancy (129), and several studies showed a decreased plasma concentration of ET-1 with the reduced conversion of bigET-1 to ET-1 in normal pregnancy compared to women with preeclampsia or diabetic pregnant women (130-133). Further, ET-1 has also been interrelated with the induction of ER and oxidative stress in preeclampsia, and together have been proposed to contribute to many of the clinical manifestations of preeclampsia (134, 135).

1.4.2.3 Role of ET-1 in aging

It is well documented that aging is associated with changes in the ET-1 system (synthesis, expression, and higher plasma levels) contributing to endothelial dysfunction (73, 136, 137). Further, elevated levels of ET-1 and also increase ET_A receptor activation with aging could modify

vascular tone (favoring vasoconstrictor phenotype). This vasoconstrictor phenotype could be one of the major contributors to the onset and progression of cardiovascular diseases (138, 139). Both human and animal studies have demonstrated a reduction in ET-1 sensitivity with advancing age, suggesting age-related increased ET-1 mediated vasoconstrictor tone (140, 141).

Figure 1.2 shows the summary of the processing of Big-ET-1 to ET-1 by various enzymes and the action of ET-1 on ET_A and ET_B receptors.

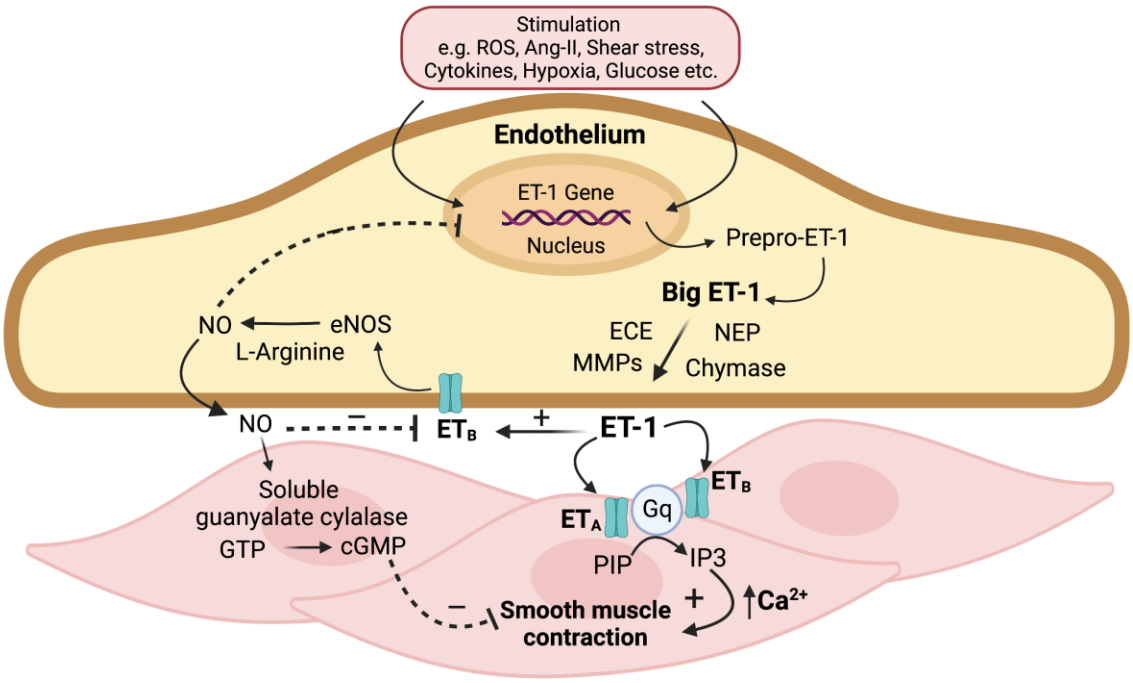

Figure 1.2 Processing of Big-ET-1 to ET-1 by various enzymes and its interaction with NO within the vasculature.

ECE: endothelin converting enzyme, MMPs: matrix metalloproteases, NEP: neutral endopeptidase, and Chymase. ROS: reactive oxygen species, Ang II: angiotensin-II, eNOS: endothelial nitric oxide synthesis, NO: nitric oxide, cGMP: cyclic guanosine-3,5-monophosphate, GTP: guanosine triphosphate, ET-1: endothelin-1, ET_A: endothelin-A receptor, ET_B: endothelin-B receptor, PIP: phosphatidylinositol, IP3: inositol trisphosphate, and Ca^{2+} calcium ions. Adapted

and modified from Cardiovascular pharmacology concepts by Richard E. Klabunde, 2011 and "Created with BioRender.com."

1.5 Summary of various vasoactive mediators produce by the endothelium

The above studies reveal that during pregnancy several hemodynamic changes occur in response to the high metabolic need from both the mother and the developing fetus, and vascular endothelium plays a central role via producing various vasodilators (NO, PGI_2, and EDH) and vasoconstrictor agent (ET-1). To maintain a healthy endothelium and vascular tone, a delicate net balance between vasodilators and vasoconstrictors are essential, in general, during pregnancy vasodilators dominate whereas in aging high circulating levels of vasoconstrictors are observed. Thus, the first research question in my thesis was to understand maternal vascular adaptations that are pivotal to maintaining pregnancy in a rat model of advanced maternal age by comparing it to young and aged non-pregnant groups. Thus, increasing our understanding of how these intricately linked endothelial mediators support pregnancy in advanced maternal age could provide further insights to explore the key molecular mechanisms that could be linked to adverse pregnancy outcomes in advanced maternal age.

Several molecular mechanisms have been identified that contribute to the aging process such as oxidative stress, inflammation, mitochondrial dysfunction, deregulation of autophagy, chronic activation of the renin-angiotensin system, cellular senescence, the loss and shortening of telomeres, epigenetic alterations, and loss of proteostasis (protein homeostasis) (142-148). Multiple factors could lead to loss of proteostasis, often resulting in the accumulation of unfolded/misfolded proteins that trigger ER stress (149). The ER is a multifunctional organelle responsible for protein post-translational modification, and during pregnancy, there is a high requirement for several proteins (peptide hormones, enzymes, growth factors, etc.), and any

disturbance could adversely affect vascular function. Indeed, emerging studies suggest that among the various complex mechanisms, ER stress is thought to mediate many vascular abnormalities in aging vessels and is also documented as one of the important factors contributing to the pathogenesis of adverse pregnancy outcomes in complicated pregnancies including fetal growth restriction (134, 150, 151). Although the importance of ER stress in aging is becoming clear, whether ER stress has a detrimental effect on vascular adaptations and pregnancy outcomes in women of advanced age is not known. Therefore, part of the current project aimed to understand the significance of ER stress on vascular function and its effect on pregnancy outcomes using a rat model of advanced maternal age.

1.6 Oxidative stress and ER stress

Oxidative stress (which occurs due to an imbalance between reactive oxygen species-ROS and the antioxidant system) is thought to mediate many vascular abnormalities in aging vessels (non-pregnant) (152, 153) and is also recognized as one of the key factors contributing to the pathophysiology of adverse pregnancy outcomes (154-156). Although a certain level of oxidative stress is a common feature of normal pregnancy, a persistent increase in oxidative stress is linked with reduced fetal growth (157, 158). Further, emerging research suggests that oxidative stress has been closely linked with ER stress affecting cellular homeostasis (134, 157, 159-161). One of the major enzymes producing ROS in aging dams is NADPH oxidases (NOX), and also the primary source of ROS associated with vascular dysfunction (162, 163). In addition, studies have demonstrated that ER stress increases the levels of ROS via the NOX family (mainly NOX-2/NOX-4) (164, 165), and is recognized as one of the main factors leading to adverse pregnancy outcomes (155, 158, 166).

Overall, these studies delineate the negative effect of ER and oxidative stress during

pregnancy. Further, there is also considerable evidence suggesting the detrimental role of ER and oxidative stress in aging vasculature (in the non-pregnant state) leading to arterial stiffness and endothelial dysfunction (167-171). However, whether increased ER and oxidative stress also contribute to negative effects on vascular adaptations and placental function in pregnant women of advanced age is not known. Therefore, understanding the significance of ER and oxidative stress may provide insight into the development of a novel therapeutic intervention to improve pregnancy outcomes.

1.7 Endoplasmic Reticulum (ER) stress

The ER membrane is responsible for the post-translational processing of proteins, including folding, trafficking, quality control, and maturation of several proteins destined for the plasma membrane, lysosomes, Golgi apparatus, and extracellular space. In general, proteins need to be correctly folded into their native form to function accurately. Nascent proteins are initially bound to calcium-dependent molecular chaperones, such as calnexin, calreticulin, glucose-regulated protein 78 (GRP78), GRP94, etc. In addition, the ER lumen maintains a redox environment, required for the formation of disulfide bonds. Any disturbance of these conditions could increase the accumulation of unfolded proteins, eventually triggering the highly conserved misfolded/unfolded protein response (UPR) pathway. The main aim of the UPR is to restore ER proteostasis for cellular survival via increasing the activity or production of key chaperones that assist in protein folding and/or degradation of unfolded/misfolded proteins. However, prolonged uncontrolled ER stress may lead to apoptosis (programmed cell death) (172-175).

The UPR is activated through the activation of one of the three signaling pathways: inositol-requiring enzyme-1 (IRE1), PKRNA-like endoplasmic reticulum kinase (PERK) or activating transcription factor 6 (ATF6). Under normal or unstress conditions, these three protein

sensors are held in an inactive state via the binding of GRP78 (a key chaperone and a master regulator of ER stress). When the ER is stressed, GRP78 dissociates from the protein sensors finally activating the UPR signaling pathways as discussed below.

1.7.1 PERK pathway

Survival Signaling: Activation of PERK is triggered by the dissociation of GRP78 followed by oligomerization and autophosphorylation. Once activated, PERK causes phosphorylation of eukaryotic translation-initiation factor-2 (eIF2α), eventually represses global protein translation. Further, phospho-eIF2α induces the translation of activating factor -4 (ATF4), which further initiates the transcription of many genes that are intricately linked to redox homeostasis, amino acid metabolism, and ER stress-induced apoptosis. Thus, the PERK-eIF2α-ATF4 pathway not only initiates the pro-survival response but also the pro-apoptosis pathway (176-178).

Apoptosis Signaling: Prolonged or uncontrolled ER stress conditions activate the transcription of C/EBP homologous protein (CHOP) via the PERK pathway. CHOP upregulates a number of proapoptotic factors, such as GADD34, BIM, BAX, PUMA, and DR5. In addition, the CHOP-induced GADD34 causes dephosphorylation of eIF2α, thus, recovers from protein translational repression (178).

1.7.2 IRE1 Pathway

Survival Signaling: IRE1 has both serine-threonine kinase and endoribonuclease (Rnase) activity and is highly conserved across the species. There are two isoforms IRE1α and IRE1β, IRE1α is highly expressed in most of the cells whereas IRE1β is expressed only in intestinal epithelial cells. IRE1 activation via the ribonuclease (RNase) activity causes unconventional splicing of X-box binding protein-1 (XBP-1), an important transcription factor that activates ER-

associated degradation of proteins and also upregulates several ER molecular chaperones that are involved in regulating ER homeostasis (178-180). Further, research also showed that the downregulation of IRE1α and XBP1 had a detrimental effect on the embryo, signifying the importance of these proteins for fetal development (181, 182).

Apoptosis signaling: Like the PERK pathway, chronic ER stress via the IRE1 pathway leads to apoptosis, mainly by recruiting TNF-receptor-associated factor 2 (TRAF2). Eventually, the IRE1-TRAF2 complex activates apoptosis-signal-regulating kinase (ASK1; a mitogen-activated protein kinase), which finally triggers the pro-apoptotic c-Jun N-terminal kinase (JNK) pathway and induces apoptosis (178-180).

1.7.3 ATF6 Pathway

ATF6 exists in two isoforms ATF6α and ATF6β, both are expressed ubiquitously. After dissociation from GRP78, ATF6 activation leads to its translocation to the Golgi apparatus, where site-1 and 2 proteases enzymes cleave it and the active ATF6 translocates to the nucleus to increase the transcription of several UPR target genes, e.g., protein disulfide isomerase, and XBP-1(178-180).

Overall, it is interesting to note that, if the adaptive pathway fails, then prolonged ER stress leads to an increased accumulation of misfolded/unfolded proteins with the ER lumen, eventually activating apoptosis signaling pathways (PERK via CHOP or IRE-1 via JNK pathways). Thus, any cellular stress that disturbs protein folding can affect cell viability (149, 168, 172, 178).

Figure 1.3 shows the summary of ER stress and the UPR pathway.

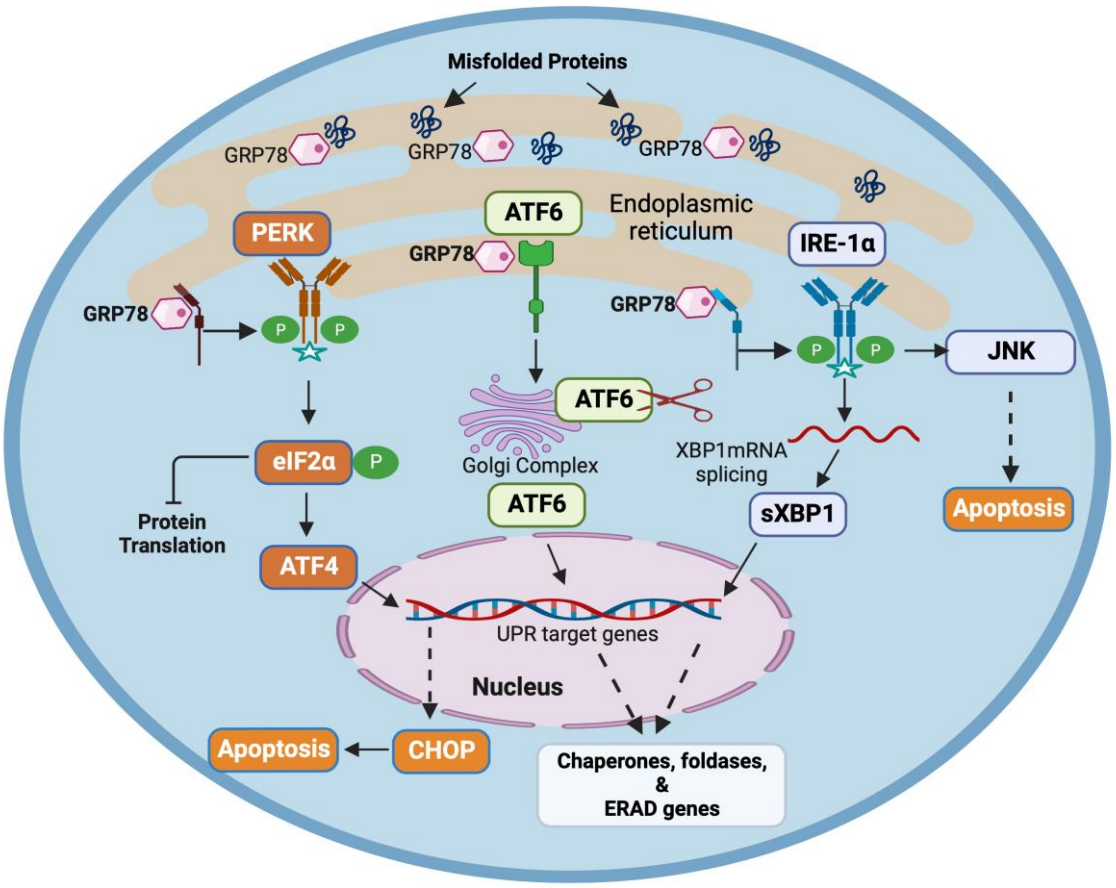

Figure 1.3 ER stress and the unfolded protein response (UPR) signaling pathways.

The UPR gets activated when unfolded/misfolded proteins get accumulated in the ER lumen. GRP78: glucose regulated protein dissociation from IRE1: Inositol-Requiring Enzyme 1, PERK: PKRNA-like endoplasmic reticulum kinase, and ATF6: activating transcription factor 6, causes trans-autophosphorylation of PERK and IRE1 and ATF6 translocation to the Golgi complex and initiates a cascade of UPR activation. eIF2α: eukaryotic translation-initiation factor-2α, ATF4: activating transcription factor 4, CHOP: C/EBP homologous protein, XBP1: X-box binding protein-1, sXBP1: spliced X-box binding protein-1, JNK: c-Jun N-terminal Kinase and ERAD: endoplasmic-reticulum-associated protein degradation. Adapted and modified from Estebanez et al. Front Physiol. 2018 and "Created with BioRender.com."

1.7.4 ER stress in pregnancy

ER stress plays a pivotal role both in normal and complicated pregnancies, and studies have shown ER stress in uteroplacental vasculature in fetal growth restriction, high-altitude pregnancy, and preeclampsia (183-185). Hu *et al.* showed that gestational hypoxia increased ER stress, suppressed Ca^{2+} sparks/STOCs, and increased myogenic tone in uterine arteries of pregnant sheep and was reversed by using ER stress inhibitor (TUDCA/ GSK2606414) (183). Although, not in pregnancy, the contribution of ER stress in vascular dysfunction in systemic hypertension and pulmonary hypertension is well established (186, 187). Further, several studies have emphasized the role of ER stress during complicated pregnancies, for example, abnormal protein profiles lead to increased ER stress and activation of UPR subsequently causing early pregnancy loss (188) and negatively affects blastocyst formation and decreases blastocyst development *in vitro* (189). Increased ER stress reduced oocyte maturation and embryo development (190, 191). Further, placental ER stress has been demonstrated in the pathophysiology of fetal growth restriction and in hypertension disorders like preeclampsia, for example, increased levels of GRP78 and decreased vascular endothelial growth factor in placentae and sera of preeclamptic women (192), increased expression of ATF4 and ATF6β negatively regulated placental growth factor in the syncytiotrophoblast of placentae from preeclampsia patients (193), and increased phosphorylation of eIF2α in placentas suppressed translation initiation in intrauterine growth restriction and preeclampsia patients (194). In addition, Kawakami et al. showed that pregnant mice exposure to tunicamycin altered the formation of the placental labyrinth zone, and increased GLUT3, but decreased GLUT1 mRNA expression, induced fetal growth restriction, and preterm birth (192). Overall, the above studies emphasize the impact of increased ER stress on adverse pregnancy outcomes, however, the consequence of ER stress in advanced maternal age is elusive.

1.7.5 ER stress in aging

It is well established that aging is associated with vascular impairments, and emerging studies also revealed that increased ER stress may further accelerate vascular dysfunction in (non-pregnant) aging vessels (75, 167, 195, 196). Further, studies also highlight that ER stress reduces the expression/activity of folding enzymes and key molecular chaperones with advancing age, thus compromising the protein folding process (196, 197). Although, targeting ER stress has been looked at in other models such as hypertension, diabetes, obesity, and age-related diseases (Alzheimer's and parkinsonism) but its role in pregnancy has not been investigated.

1.8 Maternal Intervention:

Several studies have demonstrated increased ER and oxidative stress with poor pregnancy outcomes in fetal growth restriction (188, 189, 198). Previously, the Davidge lab has observed changes in the vascular function and adverse pregnancy outcomes in aged vs young dams (23), however, the vascular adaptations from non-pregnant to pregnant in young versus aged females have not been investigated. In addition, at the molecular level, advanced maternal age affects both male and female rat placenta with greater induction of apoptosis and oxidative stress in the male placenta compared with the female placenta (50). The literature supports the association between increased ER stress in the placenta and fetal growth restriction (159). Therefore, we focused on selecting a suitable intervention that is effective in reducing ER and oxidative stress in the current research. TUDCA is a water-soluble bile acid, TUDCA is a taurine conjugate of a parent molecule ursodeoxycholic acid (UDCA), used to treat biliary cholestasis of pregnancy and studies have shown the safety of UDCA during pregnancy and lactation (199-203). Compared to UDCA, TUDCA is better absorbed due to higher water solubility (204, 205). Interestingly and relevant to the current research project, in addition to its anti-cholestatic properties, TUDCA can be used as

an inhibitor of ER stress, oxidative stress, and apoptosis, which leads to reduced arterial stiffness and improves vascular dysfunction in diabetic and hypertension mouse models (171, 206, 207).

The beneficial effect of TUDCA in *in vitro* studies are promising; for example, TUDCA by attenuating the expression of active XBP-1 protein improved the rate of two-cell embryo development to blastocysts (208) and TUDCA supplementation of *in vitro* culture medium improved the development of embryos derived from *in vivo* fertilized embryos (209). Similarly, TUDCA treatment in a mouse model increased the rate of implantation and the number of pups compared to the untreated control group (210). Therefore, the use of TUDCA as an intervention drug in the current research project was intriguing, as several studies have highlighted that the rate of early pregnancy loss and infertility is higher in women of advanced maternal age (211-217). Although not previously evaluated in animal models or women of advanced maternal age, an increased ER and oxidative stress in uteroplacental tissues have been linked to fetal growth restriction and preeclampsia (134, 159, 194, 218). Therefore, as part of my thesis, I assessed the effect of TUDCA as an ER stress inhibitor to improve altered vascular function and poor pregnancy outcomes in a rat model of advanced maternal age.

Figures 1.4 and 1.5 shows the structure of UDCA and TUDCA with the proposed molecular mechanism as an ER stress inhibitor, antioxidant, and apoptosis inhibitor.

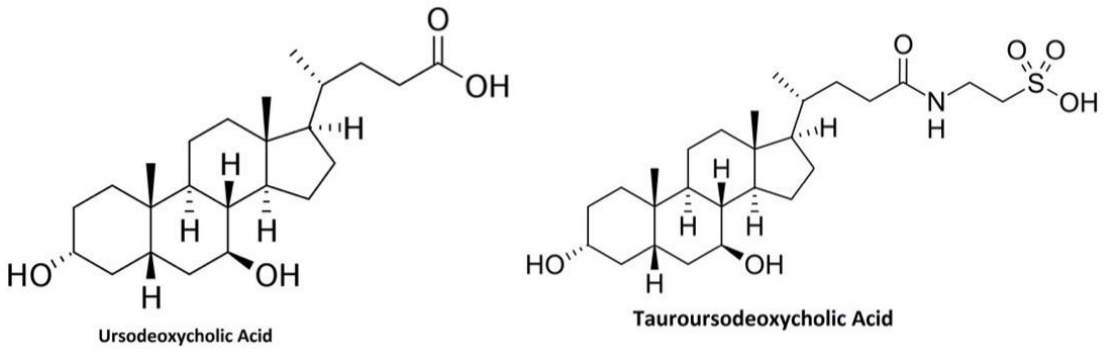

Figure 1.4 Structure of secondary bile acids: UDCA and TUDCA. *UDCA—ursodeoxycholic acid; TUDCA—tauroursodeoxycholic acid.*

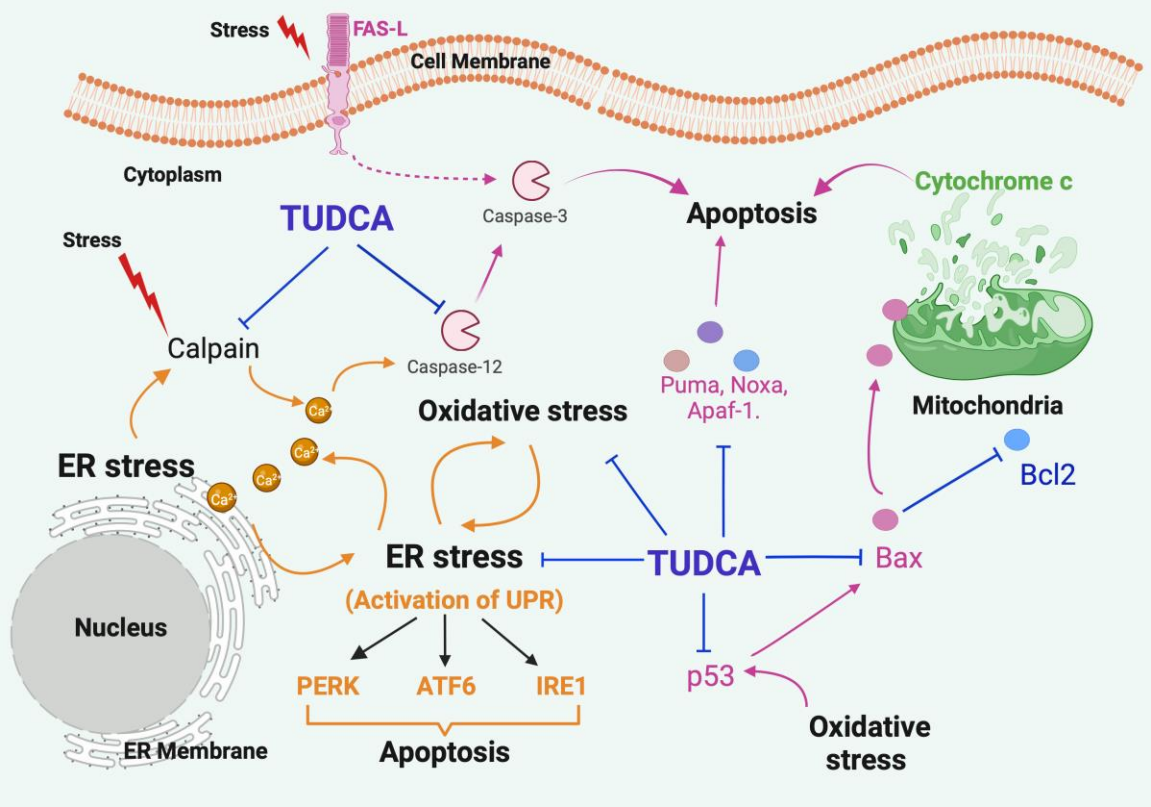

Figure 1.5 Proposed molecular mechanism of TUDCA action as an ER stress inhibitor, antioxidant, and antiapoptotic agent.

TUDCA inhibit ER stress, oxidative stress, and negatively modulates apoptosis (via inhibiting Bax translocation, cytochrome c release, p53, and caspase activation). TUDCA also inhibits apoptosis pathway by modulating intracellular calcium levels and inhibiting calpain. ER: endoplasmic reticulum, IRE1: inositol-requiring enzyme PERK: PKR-like eukaryotic initiation factor 2 kinase, ATF6: activating transcription factor 6, Ca^{2+}: calcium, Apaf-1: apoptotic protease activating factor, pro-apoptotic and anti-apoptotic family of proteins: Bad, Puma, Noxa, and Bcl-2 respectively. Adapted and modified from Amaral et al. JLR, 2009; Choy et al. PLoS One. 2017; and "Created with BioRender.com."

1.9 Background summary:

The goal of my PhD research project was to comprehend the mechanistic vascular

pathways that may be associated with inadequate maternal vascular adaptations and to determine whether increased ER and oxidative stress could be responsible for the poor pregnancy outcomes observed in a rat model. Although pregnancies at advanced age increase the risk of pregnancy-related complications including reduced fetal body weight, limited research has focused on understanding the molecular mechanism(s) leading to altered systemic and uteroplacental adaptations during pregnancy that could subsequently lead to poor pregnancy outcomes. Therefore, understanding the molecular mechanism/s will advance our understanding of why older women are associated with the risk of adverse pregnancy outcomes. Furthermore, I also studied the impact of a maternal intervention (using TUDCA) as proof of principle, which could lead to improved vascular function and pregnancy outcomes using a rat model of advanced maternal age.

2.0 Overarching hypothesis:

I hypothesize that 1) inadequate vascular adaptations in advanced maternal age lead to poor pregnancy outcomes, which may be due to increased ER stress and oxidative stress in the vasculature and placenta and 2) that TUDCA treatment by reducing ER stress will improve pregnancy outcomes.

Aim 1:

To assess the mechanistic pathways of vascular adaptations to pregnancy and its impact on the pregnancy outcomes in a rat model of advanced maternal age.

Aim 2:

To examine the effect of TUDCA on altered vascular function and pregnancy outcomes in a rat model of advanced maternal age.

Aim 3:

To assess the effect of TUDCA on placental ER stress in a rat model of advanced maternal age.

Below figure 1.6 shows the overall summary of the proposed aims.

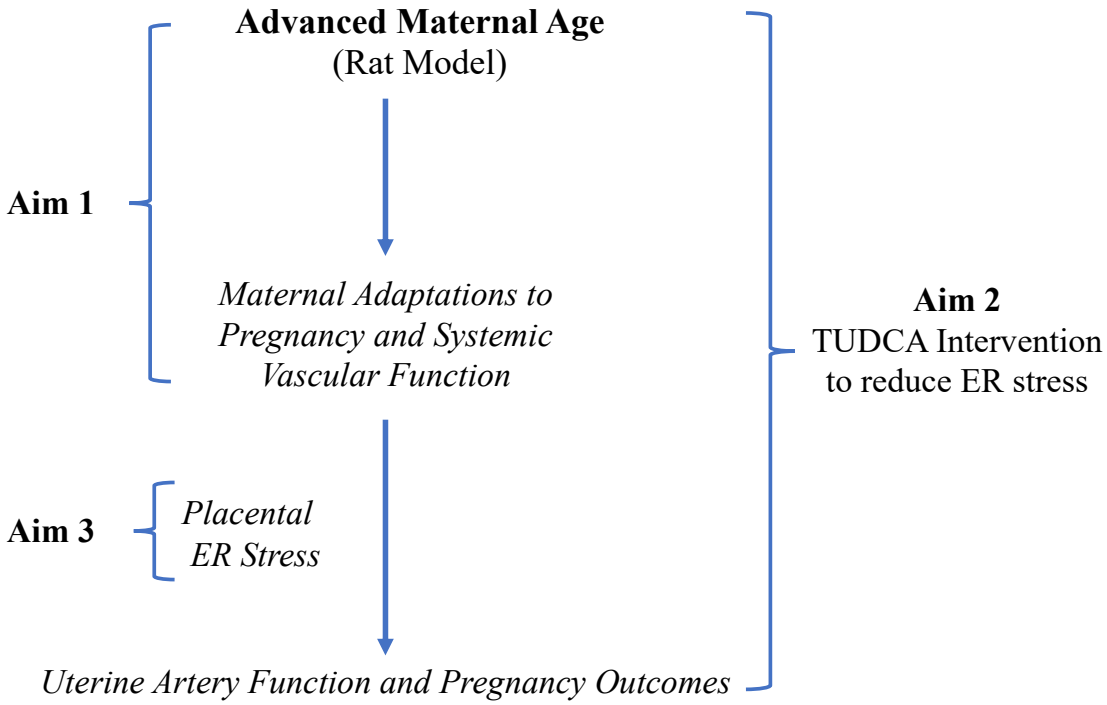

Figure 1.6 Schematic diagram of the research proposal.

TUDCA: tauroursodeoxycholic acid, ER: endoplasmic reticulum.

CHAPTER 2

General Materials and Methods

2.1 Ethical approval

All experimental techniques received prior approval by the University of Alberta Health Sciences Animal Policy and Welfare Committee, following the guidelines of the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (AUP #242 and #3692).

2.2 Animal model

Female and male (for breeding) Sprague Dawley (SD) rats were purchased at 3 months of age from Charles River Canada (St Constant, QC). All rats were housed at an ambient temperature of 22±1°C and a 14:10 h light: dark cycle. A rat model of advanced maternal age was used that was previously established and characterized in our laboratory (23, 219, 220). Briefly, young non-pregnant and pregnant rats were 3 to 4 months of age (corresponding to early reproductive age in humans); aged non-pregnant and pregnant rats were 9.5 to 10 months of age, which corresponds to ≈35 years of age in humans; considering milestones: sexual maturity, skeletal maturity, weaning, and reproductive senescence (219, 221). Young rats were provided *ad libitum* assess to standard rat chow, whereas a restricted diet of six pellets per day was provided to both non-pregnant and pregnant aged rats (*ad libitum* once pregnancy was confirmed) for caloric and nutrient intake based on National Research Council recommendations, to prevent age-related obesity as a confounding factor (222).

Two different experimental designs with a separate cohort of animals were used. The first study (Chapter 3) consisted of young and aged non-pregnant and pregnant rats. For the pregnant

groups, young and aged female rats were mated with young male rats (aged 3–5 months), and once pregnancy was confirmed by the presence of sperm in a vaginal smear (considered as gestational day GD0), following which all rats were provided an *ad libitum* standard chow diet (23, 219). On GD20 (term = 21-22 days), blood pressure was recorded via CODA tail-cuff plethysmography, (Kent Scientific Corporation, CT, USA) rats were anesthetized using isoflurane (4% in oxygen) and euthanized by exsanguination via cardiac puncture. The number of pups and resorption sites were recorded. Fetal biometrics, including body weight, placental weight, crown rump length (CR), and abdominal girth (AG) were recorded. The mesenteric arcade was immediately excised and placed in ice-cold HEPES-buffered physiological saline solution (PSS; composition (in mM): 10 HEPES, 5.5 glucose, 142 NaCl, 1.56 $CaCl_2$, 1.17 $MgSO_4$, 4.7 KCl, 1.18 KH_2PO_4, pH 7.4), after which the mesenteric arteries were isolated for *ex vivo* vascular function via wire myography and for Western blot analysis (described in detail below).

The second and third study (Chapters 4 and 5) consisted of young and aged dams, either control or TUDCA-treated rats. TUDCA treatment was provided via the drinking water throughout pregnancy (starting from GD0 to GD20; to a calculated dose of ~150 mg/kg/day, based on the literature (171, 223, 224). The dose was calculated based on the average weight of the rats and their average daily water consumption. The control groups received regular drinking water. All TUDCA-treated rats were closely monitored for any signs of adverse drug reactions during the study period. On GD20, blood pressure was recorded, after which the rats were anesthetized using isoflurane and euthanized by exsanguination via cardiac puncture. Pregnancy outcomes [litter size, number of resorption sites, fetal body weight, placental weight, crown rump length (CR), and abdominal girth (AG)] were recorded. The mesenteric arcade and main uterine arteries were excised and placed in ice-cold HEPES-buffered PSS for *ex vivo* vascular function and Western

blot analysis (mesenteric arteries). In addition, for Chapter 5, male and female offspring placentas with the labyrinth and junctional zones were separated and frozen for protein quantification via Western blotting.

2.3 Blood pressure measurements

Blood pressure was measured before euthanasia on GD20 by tail-cuff plethysmography (CODA High Throughput System, Kent Scientific, Torrington, CT). All rats were subjected to a 1-week training period (i.e., to get used to the restraint holders/nose cone and occlusion tail cuff) for acclimatization to the system before pregnancy. On the day of the experiments, rats were placed in the restraint holders, and the occlusion tail cuff and volume pressure recording sensor cuff were placed close to the base of the tail to warm up (tail skin surface temperature ~30°C). After a 20 min. acclimatization period, at least 10 consecutive blood pressure measurements (mean arterial pressure, systolic, and diastolic pressure) were recorded and averaged for each rat (219).

2.4 Wire myography

Vascular function was assessed *ex vivo* in isolated systemic small resistance mesenteric and main uterine arteries using a wire myography system. Second-order mesenteric arteries (150–250 μm) were isolated in ice-cold PSS and cut into 2 mm pieces. The 2 mm segments of the mesenteric or uterine artery were mounted onto a wire myograph system (620M DMT, Copenhagen, Denmark) using 40 μm tungsten wires. I studied the uterine and mesenteric vasculature for distinct reasons. Fetal growth is linked to the uteroplacental blood flow via uterine artery, whereas mesenteric arteries play a pivotal role in regulating overall peripheral vascular resistance and control blood pressure. Isomeric tension of the vessels was recorded using LabChart software (AD Instruments; Colorado Springs, USA). All vessels were normalized through a series

of stepwise increase in diameter to their optimal resting tension: 0.8 × IC100; 13.3 kPa for mesenteric arteries and 0.8L = 7.32 kPa for uterine arteries (the internal circumference equivalent to a transmural pressure of 100 mmHg). The rationale for using a tension calculated as 0.8 of the L_{MAX} is based on calculations of length-tension characteristics and the LaPlace equation. In general, for different vascular beds, the calculations can be adjusted by entering the correct *in vivo* pressure (for example, the approximate *in vivo* blood pressure for mesenteric and uterine arteries are 75-100 mmHg and 45-55 mmHg respectively). Therefore, the value for mesenteric arteries was calculated as 0.8 of L100 = 13.3 kPa but for uterine arteries, this should be adjusted to 0.8 of L55 = 7.32 kPa (225).

After normalization, the vessels were exposed to the first wake-up dose of phenylephrine (10 μmol/L; Sigma-Aldrich, St Louis, MO, USA) for 5 minutes. After washing three times with PSS and 10 minutes of rest, the vessels were exposed to a second wake-up dose of phenylephrine followed by a single dose of methylcholine (MCh; 3 μmol/L; Sigma-Aldrich) to assess the functionality of the VSMC and endothelium respectively. After 30 minutes of rest, all the vessels were pre-constriction with the EC_{80} concentration of phenylephrine (3 μmol/L; the mean effective concentration that produces 80% of the maximal response), and vascular responses to MCh were assessed using a cumulative concentration-response curve (CCRCs; 0.003 to 3 μmol/L MCh). Further, to assess relevant pathways involved in endothelium-dependent vasodilation and vasoconstriction, the CCRCs to MCh or BigET-1/ET-1 were performed in the absence or presence of the specific inhibitors (discussed below in Chapter specific methods). Finally, all vessels were washed 4 times with PSS, allowed to rest for 15 minutes, and each bath was exposed to a 124 mmol/L potassium chloride solution (high KCl buffer; containing in mmol/L: 10 HEPES, 124 KCl, 24 NaCl, 2.4 $MgSO_4$, 4.9 $CaCl_2$, 1.18 KH_2PO_4, and 5.5 glucose; pH 7.4) to assess maximum non-

receptor-mediated smooth muscle vasoconstriction responses. All data were recorded using LabChart software (AD Instruments; Colorado Springs, CO, USA) and were summarized as E_{max}: defined as the maximum vasodilation responses to MCh/SNP, sensitivity to MCh/SNP, defined as the negative log of the mean effective concentration that produces 50% of the maximal response (pEC50), and the area under the curve (AUC) and ΔAUC. Data were analyzed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).

2.5 Western blot analysis

Snap frozen mesenteric arteries and placenta samples were isolated and homogenized using lysis buffer (concentration in mmol/L: 20 Tris (pH 7.4), 5 EDTA, 10 sodium pyrophosphates tetrabasic, 100 sodium, and 9 fluoride with 1% Nonidet P-40) containing phosphatase inhibitor (2 mmol/L sodium orthovanadate, Sigma), 1 mmol/L Phenylmethylsulfonyl fluoride (PMSF; Fluka Biochemika), and protease inhibitor cocktail (Thermo Scientific). Bicinchoninic acid assay (Pierce, Rockford, IL) was used to determine the total protein concentration of the samples. 50 µg of proteins (tissue homogenates) were loaded and separated on SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (100V, 2 hours; 0.2µm, Bio-Rad). For normalization, total protein quantification was performed using LI-COR Revert 700 Total Protein Stain and imaged using the LI-COR Odyssey system. Following reversal of the total protein staining, membranes were incubated with BlockOut®-Universal Blocking Buffer (Rockland, PA, USA) for 1 hour at room temperature (to prevent non-specific binding of the antibodies). Membranes were then incubated overnight at 4°C with primary antibodies. Following day, membranes were incubated with the corresponding secondary antibody. Finally, blots were washed several times and were visualized with an LI-COR Odyssey Bioimager (LI-COR Biosciences) and quantified using ImageStudioLite software (LI-COR Biosciences) (primary and secondary antibodies used

are discussed below in Chapter specific methods).

2.6 Chapter 3 Specific Methods

2.6.1 Wire myography: mesenteric arteries

Following normalization and assessing the functionality of the VSMC and endothelium. We assessed the relevant pathways involved in endothelium-dependent vasodilation, the CCRCs to MCh was studied in the presence or absence of specific inhibitors; NOS was inhibited with L-NAME (100 μmol/L; Sigma-Aldrich), PGHS inhibitor meclofenamate (10 μM; Sigma-Aldrich), and EDH-induced vasodilation was inhibited with a combination of apamin (100 nmol/L; Sigma-Aldrich) and 1-(2-chlorophenyl) diphenylmethyl-1H-pyrazole (TRAM-34; 10 μmol/L; Sigma-Aldrich), which blocks small and intermediate-conductance Ca^{2+}-activated potassium channels (SK) respectively. 18 α-glycyrrhetinic acid (3 μmol/L; Sigma-Aldrich) was used to study the contribution of the myoendothelial gap junctions (MEGJs) to EDH response. Contribution of NADPH enzymes were assessed with apocynin (a NOX enzyme inhibitor: 100 μmol/L; Sigma-Aldrich). Simultaneously, to assess endothelium-independent relaxation, a CCRC to the NO donor sodium nitroprusside (0.003 to 2 μmol/L SNP; Sigma-Aldrich) was conducted.

After the MCh/SNP curves, mesenteric arteries were washed three times with PSS and allowed to rest for 30 minutes, vasoconstriction responses to big endothelin-1 (bigET-1) and endothelin-1 (ET-1) were assessed, using CCRCs to ET-1 (1–200 nmol/L; Sigma-Aldrich) or bigET-1 (3–310 nmol/L; AnaSpec, Fremont, USA) to assess the capacity of the vessel to convert bigET-1 to ET-1. Constriction responses to bigET-1 were assessed in the absence or presence of inhibitors of the bigET-1 converting enzymes: CGS35066 (25 μmol/L; Tocris Bioscience, Toronto, Canada), a selective endothelin converting enzyme (ECE-1) inhibitor; GM6001 (30 μmol/L; Calbiochem), a broad spectrum MMPs inhibitor; DL-Thiorphan (25 μmol/L;

Calbiochem), a selective inhibitor of neutral endopeptidase, and chymostatin (100 μmol/L; Sigma-Aldrich), a chymase inhibitor.

2.6.2 Western blotting: mesenteric arteries

The primary antibodies used were phospho-eNOS (Ser1177) (1:500 rabbit polyclonal, Cell signalling technology), total eNOS (1:1000 mouse monoclonal, Thermo Fisher Scientific), or ECE-1 (1:500 mouse monoclonal, Santa Cruz Biotechnology) in phosphate-buffered saline with Tween-20 (Thermo Scientific). The following day, membranes were incubated with the corresponding secondary antibody: IRDye donkey anti-rabbit IgG (for phospho-eNOS) and IRDye donkey anti-mouse IgM (for total-eNOS and ECE-1) at 1:10,000 in PBST buffer (LI-COR Biosciences, Lincoln, NE). Finally, blots were washed several times and were visualized with an LI-COR Odyssey Bioimager (LI-COR Biosciences) and quantified using ImageStudioLite software (LI-COR Biosciences). ECE-1 data were normalized to total protein, whereas phospho-eNOS was normalized to total-eNOS and expressed as percent change compared to the control group (young non-pregnant).

Table 2.1 List of primary antibodies used in chapter 3

Antibody	Species	Company	Dilution in PBST
phospho-eNOS	rabbit polyclonal	Cell Signaling Technology	1:500
total eNOS	mouse monoclonal	Thermo Fisher Scientific	1:1000
ECE-1	mouse monoclonal,	Santa Cruz Biotechnology	1:500

2.6.3 Statistics

Data were plotted using GraphPad Prism 9 and presented as mean ± SEM and were analyzed using a two-way ANOVA with Sidak's *post hoc* test for multiple comparisons. P-values

< 0.05 were considered statistically significant. For the pregnancy outcomes, n represents the number of dams.

2.7 Chapter 4 Specific Methods

2.7.1 Wire myography: mesenteric and uterine arteries

Following normalization and assessing the functionality of the VSMC and endothelium. To assess the relevant pathways involved in endothelium-dependent vasodilation, the CCRCs to MCh was studied in the presence or absence of NOS inhibitor L-NAME (100 μmol/L). Simultaneously, to assess endothelium-independent relaxation, a CCRC to the NO donor sodium nitroprusside (0.003 to 2 μmol/L SNP; Sigma-Aldrich) was conducted. After the MCh/SNP curves, all vessels were exposed to a 124 mmol/L potassium chloride solution to assess maximum non-receptor-mediated smooth muscle vasoconstriction responses.

2.7.2 Western blotting: mesenteric arteries

The primary antibodies used were NOX-2 (1:500 mouse monoclonal, Santa Cruz Biotechnology, Dallas, TX, USA), NOX-4 (1:500 rabbit polyclonal, Proteintech), GRP78 (1:1000 rabbit polyclonal, Cell Signaling Technology, Danvers, MA, USA), phospho-eIF2α (1:500 rabbit polyclonal, Cell Signaling Technology), Total-eIF2α (1:500 mouse monoclonal, Santa Cruz Biotechnology), CHOP (1:500 mouse monoclonal, Cell Signaling Technology), sXBP1 (1:500 rabbit polyclonal, Cell Signaling Technology/Proteintech), or ATF-6 (1:1000 mouse monoclonal, Santa Cruz Biotechnology) in phosphate-buffered saline with Tween-20. The following day, membranes were incubated with the corresponding secondary antibody: IRDye donkey anti-rabbit IgG (for NOX-2, NOX-4, GRP78, phopsho-eIF2α, and sXBP-1) and IRDye donkey anti-mouse IgM (total-eIF2α, CHOP, and ATF-6) at 1:10,000 in PBST buffer (LI-COR Biosciences, Lincoln,

NE). Finally, blots were visualized with an LI-COR Odyssey Bioimager and quantified using ImageStudioLite software. All data were normalized to total protein (except phosph-eIF2α, which were normalized to total-eIF2α) and expressed as percent change compared to the respective control group (either young non-pregnant rats or young pregnant rats).

Table 2.2 List of primary antibodies used in chapter 4

Antibody	Species	Company	Dilution in PBST
GRP78	rabbit polyclonal	Cell Signaling Technology	1:500
NOX-2	mouse monoclonal	Santa Cruz Biotechnology	1:500
NOX-4	rabbit polyclonal	Proteintech	1:500
phospho-eIF2α	rabbit polyclonal	Cell Signaling Technology	1:500
total-eIF2α	mouse monoclonal	Santa Cruz Biotechnology	1:500
CHOP	mouse monoclonal	Cell Signaling Technology	1:500
sXBP1	rabbit polyclonal	Cell Signaling Technology	1:500
ATF-6	mouse monoclonal,	Santa Cruz Biotechnology	1:1000

2.7.3 Statistics

All data were plotted and analyzed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA) or Stata (StataCorp LLC, College Station, TX, USA) and presented as mean ± SEM. Statistical differences were tested using a two-way ANOVA with either planned contrast analysis or Sidak's post-hoc test for multiple comparisons; $p < 0.05$ was considered statistically significant. For the pregnancy outcomes, n represents the number of dams.

2.8 Chapter 5 Specific Methods

2.8.1 Western botting: Male and Female offspring placentas

The primary antibodies used in placentas (male and female labyrinth and junctional zones); GRP78, phospho-eIF2α, Total-eIF2α, CHOP, sXBP1, ATF-6 or ATF-4 (1:500 rabbit polyclonal, Proteintech) in phosphate-buffered saline with Tween-20. The following day, membranes were incubated with the corresponding secondary antibody: IRDye donkey anti-rabbit IgG (for GRP78, phopsho-eIF2α, sXBP-1, and ATF-4) and IRDye donkey anti-mouse IgM (total-eIF2α, CHOP, and ATF-6) at 1:10,000 in PBST. Finally, blots were visualized with an LI-COR Odyssey Bioimager and quantified using ImageStudioLite software. All data were normalized to total protein (except phosph-eIF2α, which were normalized to total-eIF2α) and expressed as percent change compared to the control group (young pregnant rats).

Table 2.2 List of primary antibodies used in chapter 5

Antibody	Species	Company	Dilution in PBST
GRP78	rabbit polyclonal	Cell Signaling Technology	1:500
phospho-eIF2α	rabbit polyclonal	Cell Signaling Technology	1:500
total-eIF2α	mouse monoclonal	Santa Cruz Biotechnology	1:500
ATF-4	rabbit polyclonal,	Proteintech	1:500
CHOP	mouse monoclonal	Cell Signaling Technology	1:500
sXBP1	rabbit polyclonal	Cell Signaling Technology	1:500
ATF-6	mouse monoclonal,	Santa Cruz Biotechnology	1:1000

2.8.2 Statistics

All data were plotted and analyzed using GraphPad Prism 9 and presented as mean ± SEM.

Statistical differences were tested using a two-way ANOVA with Sidak's *post-hoc* test for multiple comparisons; $p < 0.05$ was considered statistically significant.

CHAPTER 3

Altered Vascular Adaptations to Pregnancy in a Rat Model of Advanced Maternal Age

A version of this chapter has been published:

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*Amy L Wooldridge**, Mazhar Pasha,** Palehswan Chitrakar, Raven Kirschenman, Anita Quon, Floor Spaans, Tamara Sáez, Christy-Lynn M Cooke, Sandra T Davidge. Advanced Maternal Age Impairs Uterine Artery Adaptations to Pregnancy in Rats. Int. J. Mol. Sci. 2022, 23(16), 9191.*

Author Contributions: Experimental design and conceptualization, A.L.W., F.S., C.-L.M.C. and S.T.D.; methodology, A.L.W., P.C., R.K., A.Q., F.S., T.S., C.-L.M.C. and S.T.D.; data collection, A.L.W., M.P. and P.C.; manuscript preparation, A.L.W. and P.C.; manuscript review and editing, A.L.W., F.S., C.-L.M.C. and S.T.D. All authors have read and agreed to the published version of the manuscript.

3.1 Introduction

Advanced maternal age increases the risk of pregnancy-related complications such as fetal growth restriction and preeclampsia (8-13). Healthy maternal vascular adaptations to pregnancy play an essential role in normal fetal growth and development. To achieve this, several hemodynamic changes occur, such as increased cardiac output, increased heart rate, and increased blood volume, while there is a decrease in blood pressure due to a reduced systemic vascular resistance during pregnancy (72, 226, 227). Though the mechanism of reduced peripheral resistance is not well established, studies have shown that during pregnancy there is increased bioavailability of a potent vasodilator such as NO (32-34). In addition, vasodilation of small resistance arteries during pregnancy is also mediated by other factors (PGI_2 and EDH) (37-39). Although PGI_2 work as a vasodilator, it is often thought that increased levels of PGI_2 are observed when there is decreased bioavailability of NO thus, maintaining overall endothelium-dependent vasodilation response (34, 41). Similarly, if NO-mediated relaxation is decreased, EDH may compensate for endothelium-dependent relaxation (40). Moreover, studies have shown upregulation of EDH in normal pregnancy (in resistance-sized myometrial arteries and small subcutaneous arteries), that may be absent in pregnancy-related complication such as preeclampsia (103, 104).

In women of advanced maternal age, normal pregnancy-induced vascular adaptations may be impaired, leading to poor pregnancy outcomes and altered vascular function. We have previously demonstrated abnormal pregnancies in aged rats, with reduced capacity to sustain a pregnancy, adverse pregnancy outcomes, and altered vascular function with increased active myogenic responses in both uterine and mesenteric arteries from aged pregnant rats (23).

Aging itself is an independent risk factor for cardiovascular disease (CVD) (228-230). To

maintain vascular homeostasis, there is a fine balance between vasodilator (NO) and vasoconstrictor agents (e.g., ET-1) (231, 232). Although, the pathophysiological mechanisms leading to endothelial dysfunction are likely multifactorial, a hallmark of endothelial dysfunction in the aging vasculature was shown to be reduced bioavailability of NO and enhanced reactivity of ET-1 (233-236). ET-1 is a potent vasoconstrictor polypeptide and has the capacity to induce vascular remodeling, thus ET-1 signaling is believed to be one of the important contributors to the progression of vascular dysfunction in aging and cardiovascular disease (237, 238). In addition, studies have also shown a significant increase in circulating levels of ET-1 in women with pregnancy-related complications compared with normal pregnant women (239, 240). The vasoactive peptide ET-1 is synthesized from its precursor bigET-1, and subsequently cleaved by several enzymes, such as MMPs (122), ECE-1 (119, 120), chymase (123), and neutral endopeptidases (124). Studies have demonstrated that MMPs and ECE-1 play essential roles in the processing of bigET-1 to ET-1 under various pathological conditions such as aging (241), preeclampsia (122, 132), and hypoxia (242). In addition, MMPs also maintain the stability of the extracellular matrix by degrading collagen, elastin, and other extracellular proteins as part of the normal physiological and pathological processes (243). Although MMPs play a significant role during healthy pregnancies (e.g. in trophoblast invasion of the uterus) (244), several studies showed that with advancing age, dysregulation of MMPs activity may contribute to endothelial dysfunction (72, 152, 245). However, the role of MMPs in vascular adaptations to pregnancy with advanced maternal age is not known.

Pregnancy is known to increase the oxidative stress which is considered as normal physiological response due to increase metabolic demands from both mother and the developing fetus. Further, it is known that oxidative stress plays a significant role in the development of age-

related diseases (152, 246, 247). There are several intracellular processes that lead to the generation of ROS, but one of the major enzymes involved in aging is NOX. NOX-induced oxidative stress is also recognized as a key factor in the pathogenesis of adverse pregnancy outcomes such as fetal growth restriction and preeclampsia (158, 248). However, contribution of NOX in advanced maternal age is elusive. Both human and animal studies have shown the importance of systemic vascular adaptations to pregnancy (39, 249-253). However, the impact of age-related vascular impairments during pregnancy, such as altered endothelium-dependent vasodilation (reduced NO bioavailability) and increased endothelium-derived contracting factors (e.g., ET-1) due to their processing enzymes (such as MMPs) remains elusive. Thus, the focus of the current study was to increase our understanding of how these vascular pathways impact the systemic vascular adaptations to pregnancy in an established rat model of advanced maternal age. We used mesenteric arteries as they are a systemic, resistance-sized artery that plays an important role in the control of blood pressure (254). We hypothesize that maternal aging impairs systemic vascular adaptations to pregnancy, due to a more vasoconstrictive phenotype associated with reduced NO contribution and increased activity of MMPs in the aging vasculature.

3.2 Results

3.2.1 Aged dams had worse pregnancy outcomes than young dams

Aged dams had smaller litter sizes ($p < 0.0001$, Figure 3.1A) and more reabsorptions ($p = 0.0014$; Figure 3.1B) compared to the young dams. Fetuses from aged dams had reduced fetal weights (both males and females; $p < 0.0001$, Figure 3.1D) compared with young dams. The crown-rump length abdominal girth ratio was not different between fetuses from aged and young dams (Figure 3.1C). Fetuses from aged dams had a greater placental weight (males: $p = 0.0013$, females: $p = 0.0059$; Figure 3.1E) compared with young dams. The fetal placental weight ratio

was lower in both fetal males and females of aged dams compared to young dams (both $p < 0.0001$, Figure 3.1F).

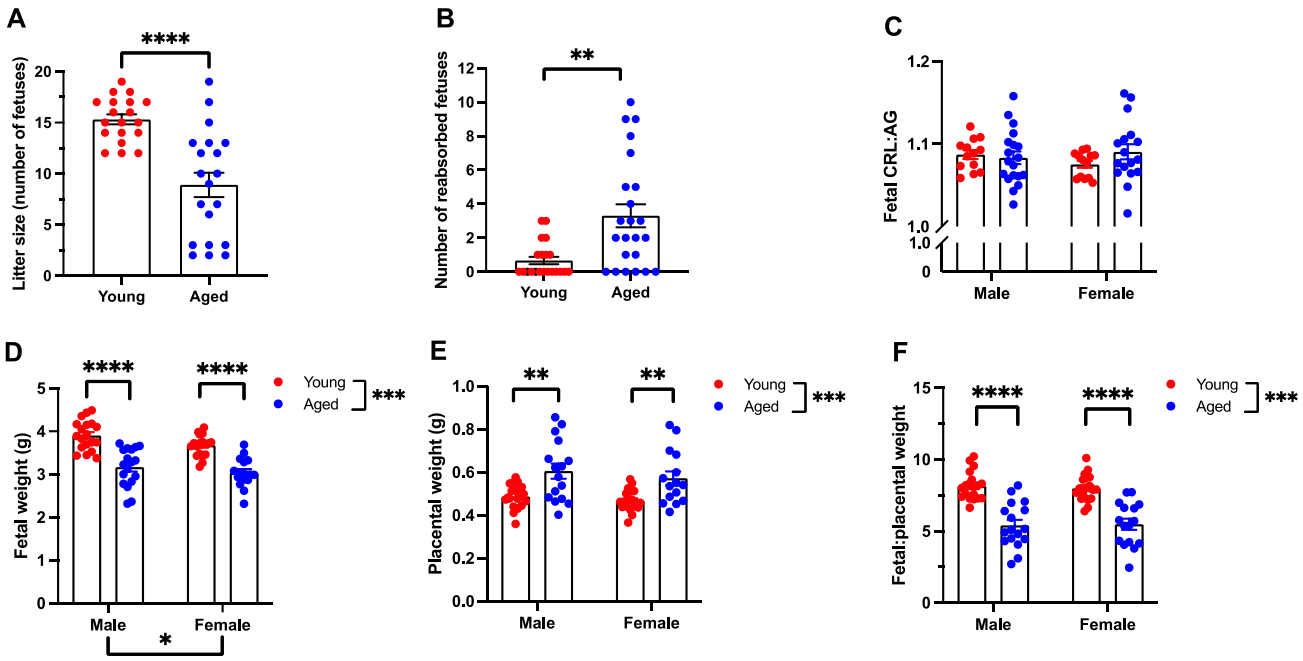


Figure 3.1 Pregnancy outcomes from young and aged dams at GD20.

*(A) Litter size; (B) Reabsorptions; (C) Fetal crown-rump length (CRL)/abdominal girth (AG) ratio; (D) Fetal weight;(E) Placental weight; (F) Fetal/placental weight ratio. Data presented as mean ± SEM; n represents litter average; n = 13–19 dams/group. Data are sex-specific means of litter outcomes, where applicable. Pregnancy outcomes were compared using a t-test (litter size), Mann Whitney U-test (reabsorptions), or a two-way ANOVA (dam age vs. fetal sex) followed by Sidak's post hoc test. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.*

(Pregnancy outcomes data were published as Advanced Maternal Age Impairs Uterine Artery Adaptations to Pregnancy in Rats. Int. J. Mol. Sci. 2022, 23(16), 9191).

3.2.2 Blood pressure and heart rate assessment in young compared to aged non-pregnant and pregnant rats.

Mean arterial pressure (MAP) was increased in aged non-pregnant rats compared to aged

pregnant, young non-pregnant and pregnant rats (Figure 3.2). Pregnancy reduced the MAP in aged pregnant rats compared to aged non-pregnant rats to pressures similar to those of the young pregnant rats. However, no changes in MAP were observed between young non-pregnant and pregnant rats. A similar pattern of changes was observed with the systolic and diastolic blood pressures. Moreover, heart rates tended to be higher in young pregnant rats compare to young non-pregnant rats, while this effect was not observed between the aged non-pregnant and pregnant rats. (Supplementary Table 3.1).

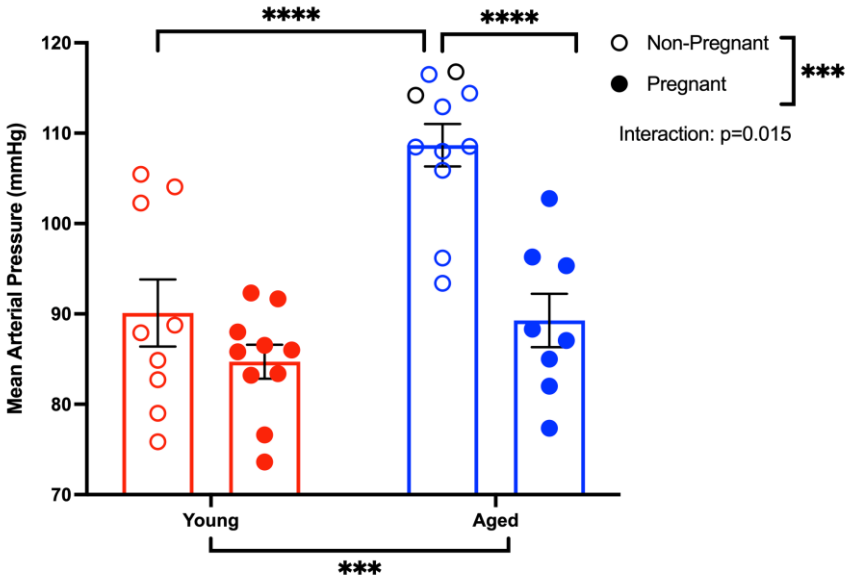


Figure 3.2 Pregnancy reduced the mean arterial pressure in aged dams.

*Mean arterial blood pressure recordings (MAP) of young (3-4 months; in red) and aged (9- 9.5 months; in blue) pregnant (on gestational day 20; closed circles) and non-pregnant (age-matched; open circles) rats. Data presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; ***p<0.001, ****p<0.0001; n= 9-10/group.*

3.2.3 Vasodilation pathways

3.2.3.1 Endothelium-dependent vasodilation and NO contribution to vasodilation

Methacholine (MCh)-induced vasodilation responses were not different in mesenteric

arteries from young or aged, non-pregnant or pregnant rats (Figure 3.3 A, B and Supplementary Table 3.2). However, pre-incubation with L-NAME (to assess NO contribution) decreased the E_{max} to MCh in arteries from both young pregnant and aged pregnant rats, while there was no effect of L-NAME on MCh E_{max} in young and aged non-pregnant rats (Figure 3.4 A, B and D, E). Moreover, L-NAME decreased the sensitivity to MCh (pEC_{50}) only in young non-pregnant (Figure 3.4C) and aged pregnant rats (Figure 3.4F). Similarly, a decrease in the AUC was observed after pre-incubation with L-NAME in young non-pregnant, pregnant, and aged pregnant rats, but not in aged non-pregnant rats (Supplementary Table 3.3). We used Western blot to quantify eNOS protein levels/phosphorylation (at Ser1177) mesenteric arteries. There were no significant differences (p=0.07) in phosphorylation of eNOS between young non-pregnant and pregnant rats, however, phosphorylation of eNOS was significantly increased in vessels from aged pregnant rats compared to aged non-pregnant rats (Figure 3.5). Moreover, no changes in total eNOS protein expression were observed between the groups (Supplementary Figure 3.1).

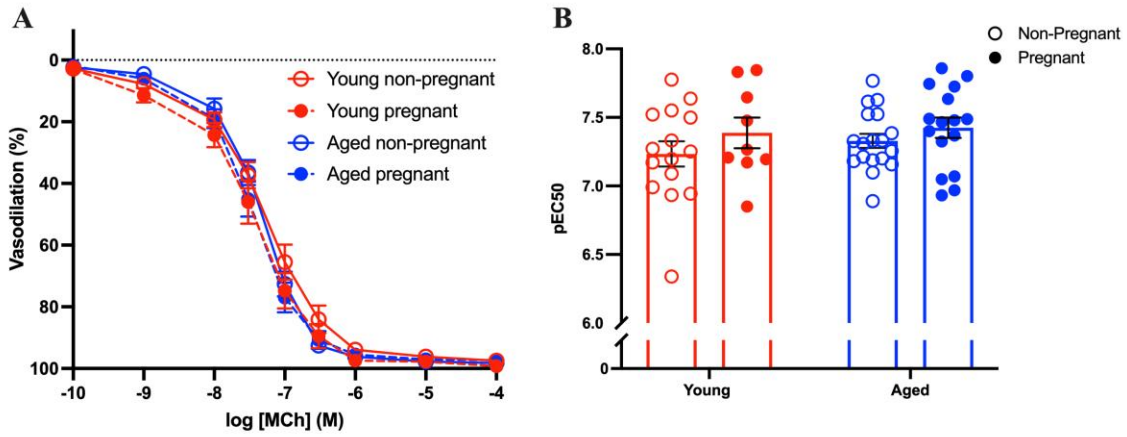


Figure 3.3 No differences in the endothelium-dependent vasodilation in young and aged non-pregnant and pregnant rats.

(A) *Endothelium-dependent vasodilation responses to increasing doses of methylcholine (MCh) in mesenteric arteries of young (3-4 months; in red) and aged (9-9.5 months; in blue) pregnant (on*

gestational day 20; closed circles) and non-pregnant (age-matched; open circles) rats. ***(B)*** *Data summary of sensitivity to MCh (pEC_{50}). Data are presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; n=9-16/group.*

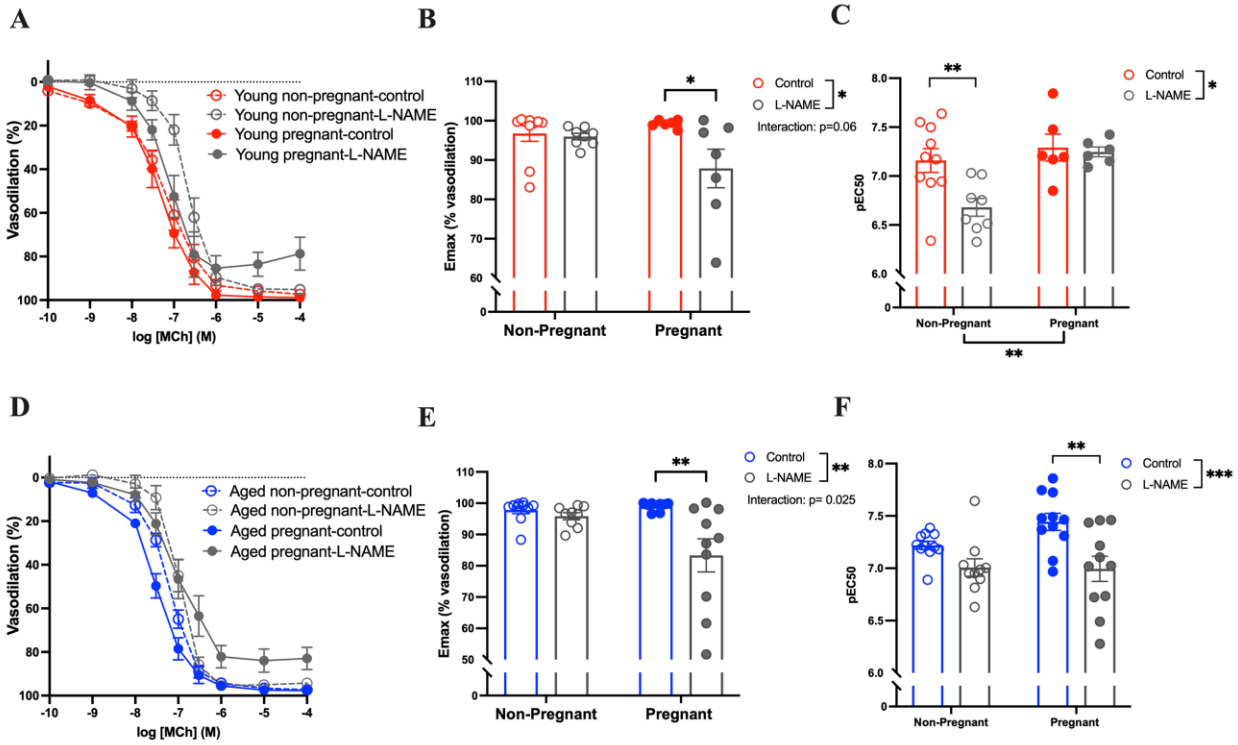


Figure 3.4 Increased nitric oxide-dependent vasodilation during pregnancy in mesenteric arteries from young and aged pregnant rats.

(A, D) *Endothelium-dependent vasodilation responses to increasing doses of methylcholine (MCh) in the presence or absence of L-NAME in mesenteric arteries of young (3-4 months; in red;* ***A, B, & C****) and aged (9-9.5 months; in blue;* ***D, E, & F****) pregnant (on gestational day 20; closed circles) and non-pregnant (age-matched; open circles) rats.* ***(B, E)*** *Data summaries of the maximal vasodilation responses to MCh (E_{max}).* ***(C, F)*** *Data summaries of the sensitivity to MCh (pEC_{50}). Data are presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; *p<0.05, **p<0.01; ***p<0.001; n=6-11/group.*

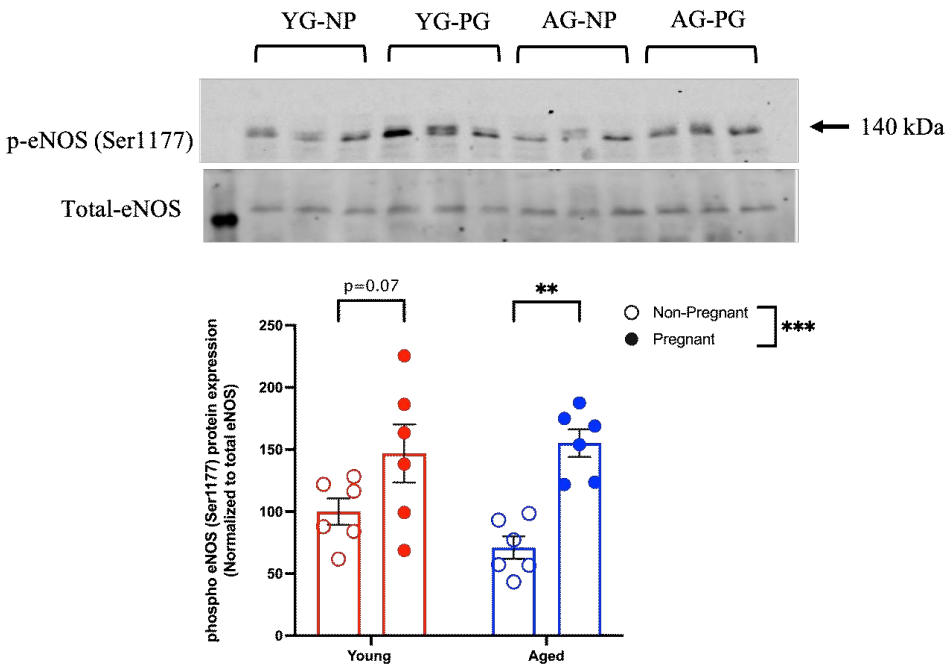


Figure 3.5 Increased phosphorylation of eNOS protein in young and aged pregnant rats. Western *blot analysis of phospho-eNOS (Ser1177) normalized to total eNOS in mesenteric arteries of young (3-4 months; in red) and aged (9-9.5 months; in blue) pregnant (on gestational day 20; closed circles) and non-pregnant (age-matched; open circles) rats. Data are presented as mean±SEM and expressed as percentage of control (i.e., the mean of the non-pregnant young group); analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; **p<0.001; n=6/group. YG-NP=Young non-pregnant; YG-PG=Young pregnant; AG-NP=Aged non-pregnant; AG-PG= Aged pregnant.*

3.2.3.2 PGI_2-mediated vasodilation

An increase in the sensitivity to MCh (pEC50) in the presence of PGHS inhibitor meclofenamate was observed in young rats (non-pregnant and pregnant rats) (Figure 3.6 A, B) while no differences in the sensitivity to MCh (pEC50) was observed in aged rats (aged non-pregnant and pregnant rats (Figure 3.6 C, D).

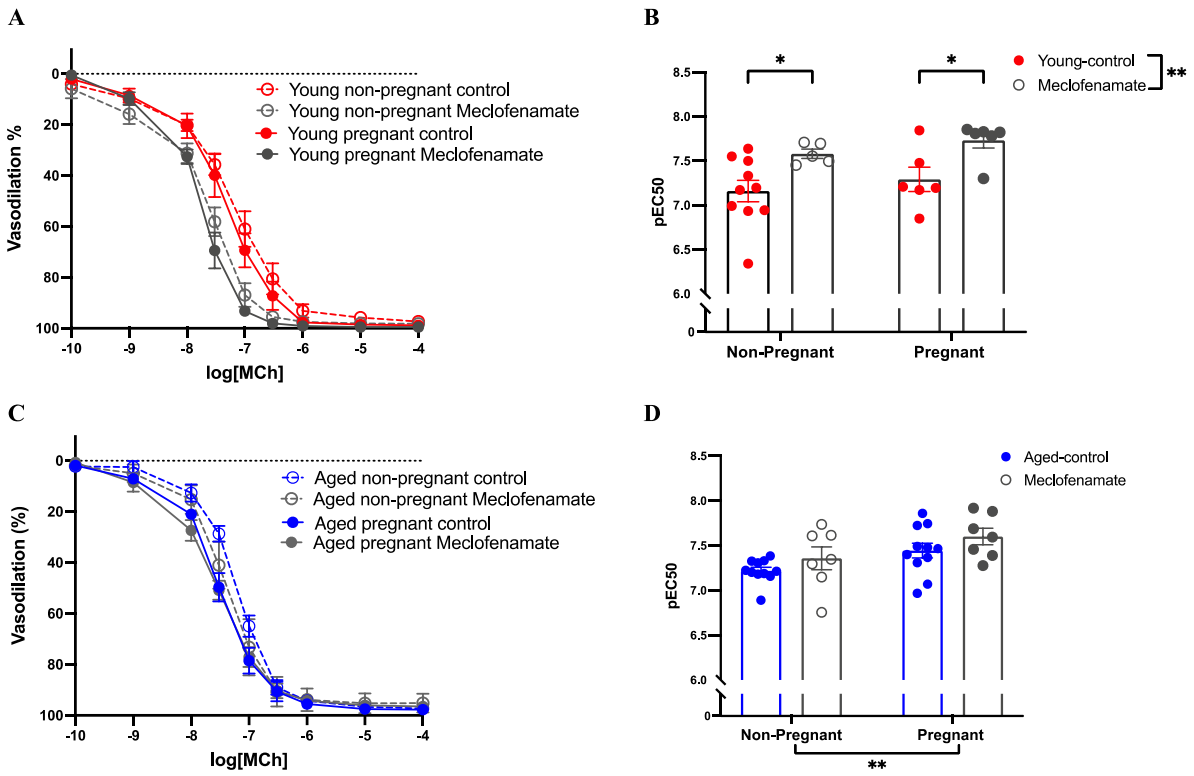


Figure 3.6 Increased sensitivity to prostaglandin-mediated relaxation in mesenteric arteries from young non-pregnant and pregnant rats.

(A, C) *Endothelium-dependent vasodilation responses to increasing doses of methylcholine (MCh) in the presence or absence of meclofenamate in mesenteric arteries of young (3-4 months; in red;* ***A, & B****) and aged (9-9.5 months; in blue;* ***C, & D****) pregnant (on gestational day 20; closed circles) and non-pregnant (age-matched; open circles) rats.* ***(B, D)*** *Data summaries of the sensitivity to MCh (pEC50). Data are presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; *p<0.05, **p<0.01; ***p<0.001; n=5-10/group.*

3.2.3.3 EDH-mediated vasodilation

Assessment of the contribution of EDH (using inhibitors apamin and TRAM-34; which inhibits SK_{Ca} and IK_{Ca} respectively) showed that the E_{max} and sensitivity to MCh (pEC_{50}) was decreased after incubation with apamin and TRAM-34 in young non-pregnant, aged non-pregnant and aged pregnant rats while no effect of apamin and TRAM-34 was observed in young pregnant

rats (Figure 3.7 A-F). Apamin and TRAM-34 also decreased AUC in aged non-pregnant and pregnant rats (Supplementary Table 3.4). Inhibition of myoendothelial gap junctions (MEGJs) by 18α-glycyrrhetinic acid had no effect on MCh-induced vasodilation responses in mesenteric arteries from either young or aged, pregnant, or non-pregnant rats (Supplementary Table 3.5).

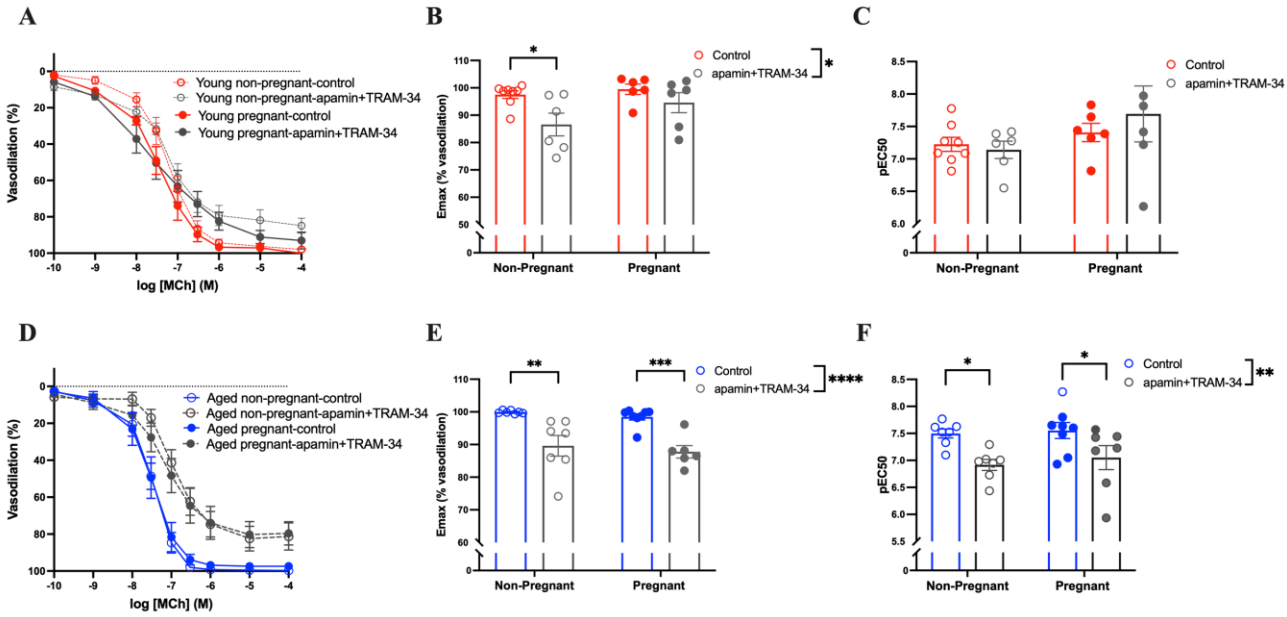


Figure 3.7 Increased endothelium-derived hyperpolarization-mediated relaxation in mesenteric arteries from aged non-pregnant and pregnant rats.

(A, D) *Endothelium-dependent vasodilation responses to increasing doses of methylcholine (MCh) in the presence or absence of apamin/TRAM-34 in mesenteric arteries of young (3-4 months; in red;* ***A, B, C****) and aged (9-9.5 months; in blue;* ***D, E, F****) pregnant (on gestational day 20; closed circles) and non-pregnant (age-matched; open circles) rats.* ***(B, E)*** *Data summaries of the maximal vasodilation responses to MCh (E_{max}).* ***(C, F)*** *Data summaries of the sensitivity to MCh (pEC_{50}). Data are presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; n=6-8/ group.*

3.2.3.4 Contribution of NOX enzymes to vasodilation

No differences in the sensitivity to MCh (pEC50) was observed in the presence of NOX enzymes inhibitor apocynin in young rats (non-pregnant and pregnant rats) (Figure 3.8 A, B).

whereas an increase in the sensitivity to MCh (pEC50) was observed only in aged-non pregnant rats but not in aged pregnant rats (Figure 3.8 C, D).

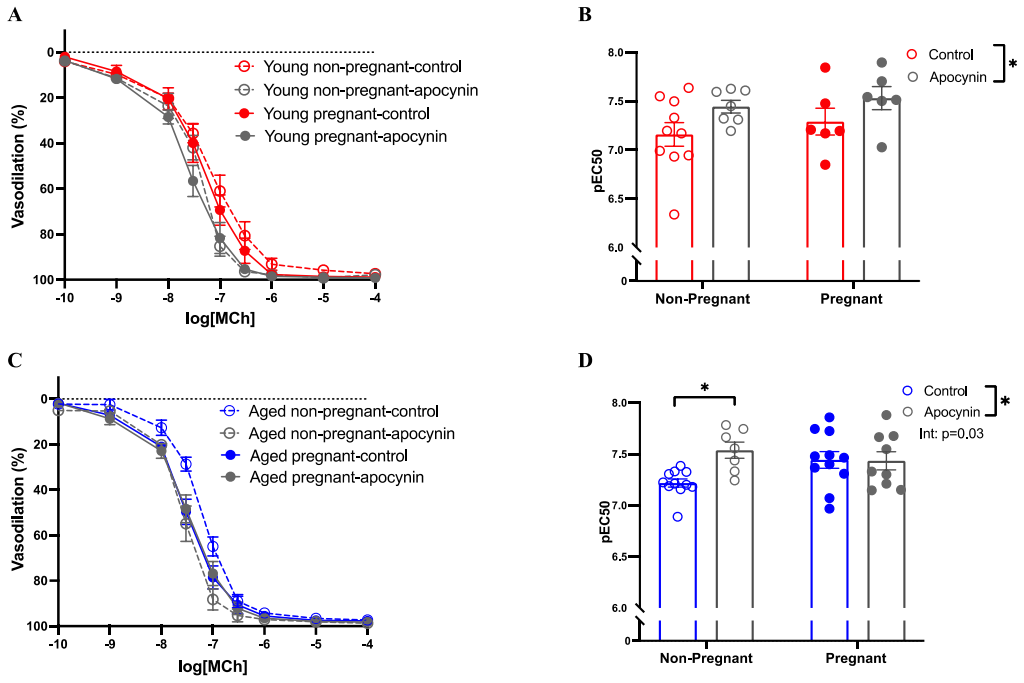


Figure 3.8 Increased sensitivity to apocynin in mesenteric arteries from aged non-pregnant rats.

(A, C) *Endothelium-dependent vasodilation responses to increasing doses of methylcholine (MCh) in the presence or absence of apocynin in mesenteric arteries of young (3-4 months; in red;* ***A, & B****) and aged (9-9.5 months; in blue;* ***C, & D****) pregnant (on gestational day 20; closed circles) and non-pregnant (age-matched; open circles) rats.* ***(B, D)*** *Data summaries of the sensitivity to MCh (pEC_{50}). Data are presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; *p<0.05, **p<0.01; ***p<0.001; n=6-10/group.*

3.2.3.5 Endothelium-independent vasodilation

Sodium nitroprusside (SNP) dose-response curves were conducted to assess potential differences in endothelium-independent relaxation. There were no changes in the vascular responses between the young and aged, non-pregnant and pregnant rats (Supplementary Table 3.6).

3.2.4 Vasoconstrictor pathways:

3.2.4.1 Vascular responses to bigET-1 in mesenteric arteries and contribution of converting enzymes

Vasoconstriction responses to bigET-1 were enhanced in vessels from both aged non-pregnant and pregnant rats compared to the young non-pregnant and pregnant rats, however there was no significant effect of pregnancy (Figure 3.9 A, B and Supplementary Table 3.7). The enhanced constriction responses to bigET-1 in aged rats could be a result of increased conversion of bigET-1 to active ET-1, or due to greater vascular smooth muscle sensitivity to ET-1. Since no differences were evident in ET-1 sensitivity/E_{max} between young and aged non-pregnant and pregnant rats (Figure 3.9 C, D and Supplementary Table 3.7), it appears that there was a greater capacity for aged vessels to convert bigET-1 to ET-1. To determine which enzymes may be contributing to this enhanced conversion, inhibitors of various bigET-1 conversion enzymes were used. Incubating the vessels with the MMP-inhibitor GM6001 did not alter vasoconstriction responses to bigET-1 in either young or aged non-pregnant and pregnant rats (Figure 3.10 A-D and Supplementary Table 3.8). We further evaluated the contribution of other enzymes involved in converting bigET-1 into its vasoactive form. Pre-incubation with the ECE-1 inhibitor CGS35066 did not impact bigET-1 responses in young non-pregnant rats but decreased maximum constriction (E_{max}) was observed in pregnant rats (Figure 3.11 A, B and Supplementary Table 3.8), while constriction to bigET-1 was significantly reduced in the aged non-pregnant and pregnant rats (Figure 3.11 C, D). However, Western blot analysis showed that there were no changes in ECE-1 protein levels between the groups (Figure 3.11 E). Chymase inhibitor chymostatin and neutral endopeptidase inhibitor thiorphan did not alter bigET-1 vasoconstriction in either young or aged rats (Supplementary Table 3.9). In addition, no changes in the vasoconstriction capacity to

KPSS were observed between the young (non-pregnant: 5.17±0.41; n=16 vs pregnant: 5.51±0.48 mN/mm; n=12) and aged rats (non-pregnant; 5.85±0.32; n=15 vs pregnant: 5.68±0.38 mN/mm; n=15), suggesting that overall constrictor capacity of the vascular smooth muscle cells was not different between groups.

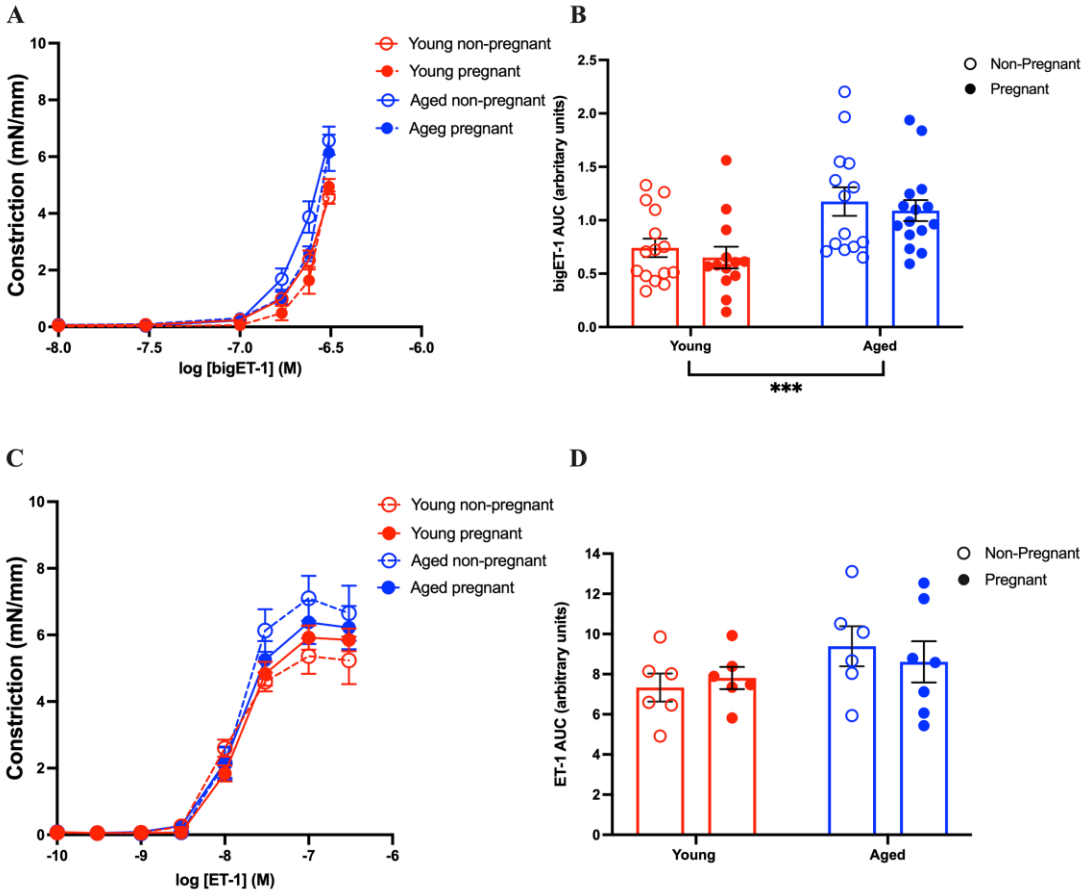


Figure 3.9 Increased vascular responses to bigET-1 in aged non-pregnant and pregnant rats, and no changes in vascular responses to ET-1.

(A, C) *Vascular contraction responses to bigET-1 and ET-1 in mesenteric arteries of young (3-4 months; in red) and aged (9-9.5 months; in blue) pregnant (on gestational day 20; closed circles) and non-pregnant (age-matched; open circles) rats.* ***(B, D)*** *Data summaries of area under the curve (AUC) of the bigET-1 (**B**) and ET-1 (**D**) responses. Data are presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; ***p<0.001; n=6-16/ group.*

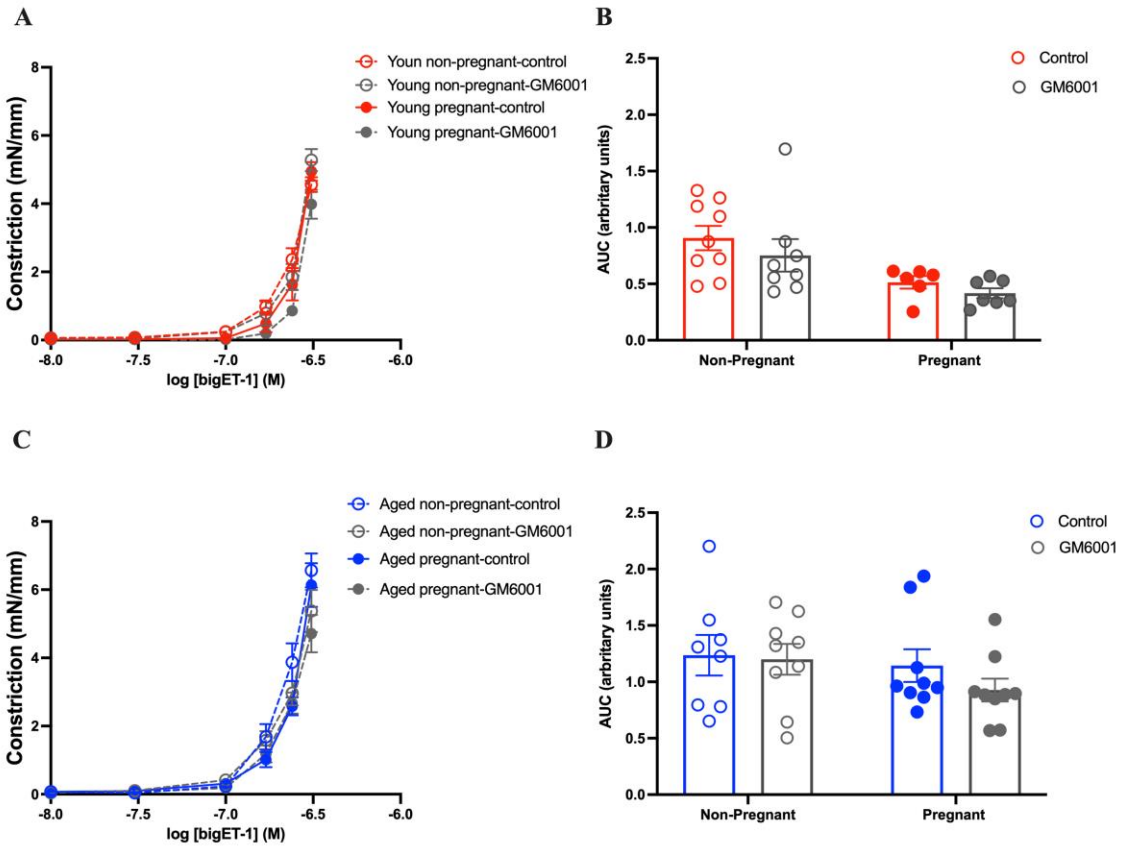


Figure 3.10 No significant contribution of matrix metalloproteases in converting to bigET-1 in young and aged non-pregnant and pregnant rats.

(A, C) *Vascular contraction response to bigET-1 in in the presence or absence of MMPs inhibitor GM6001 in mesenteric arteries of young (3-4 months; in red****; A, B****) and aged (9-9.5 months; in blue;* ***C, D****) pregnant (on gestational day 20; closed circles) and non-pregnant (age-matched; open circles) rats.* ***(B, D)*** *Data summaries of area under the curve (AUC) of the bigET-1 responses. Data are presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; n=6-8/ group.*

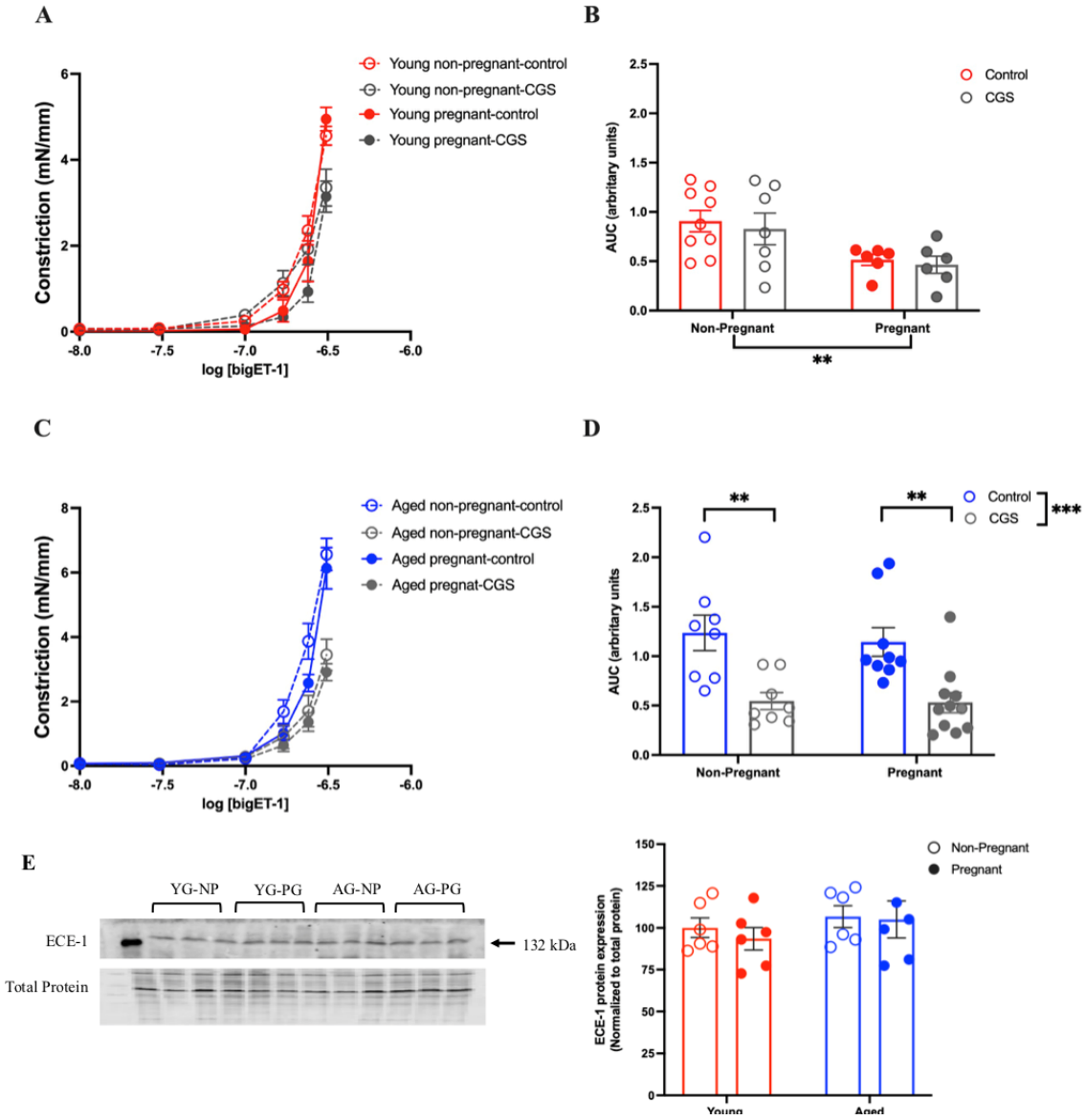


Figure 3.11 Increased contribution of endothelin converting enzyme (ECE) in converting bigET-1 in aged non-pregnant and pregnant rats.

(A, C) *Vascular contraction response to bigET-1 in in the presence or absence of ECE-1 inhibitor CGS35066 (CGS) in mesenteric arteries of young (3-4 months; in red;* ***A, B****) and aged (9-9.5 months; in blue;* ***C, D****) pregnant (on gestational day 20; closed circles) and non-pregnant (age-matched; open circles) rats.* ***(B, D)*** *Data summaries of area under the curve (AUC) of the bigET-1 responses.* ***I*** *Western blot analysis of ECE-1 protein normalized to total protein in mesenteric arteries; the ECE-1 data is expressed as percentage of control (the mean of the non-pregnant young group). Data are presented as mean±SEM; analyzed by two-way ANOVA with Sidak's*

*multiple comparisons post-hoc test; **p<0.001; ***p<0.001; n=6-8/ group. YG-NP=Young non-pregnant; YG-PG=Young pregnant; AG-NP=Aged non-pregnant; AG-PG= Aged pregnant.*

Supplementary Data

	Young		Aged	
	Non-pregnant	Pregnant	Non-pregnant	Pregnant
Diastolic pressure (mmHg)	90.1 ± 3.71	84.7 ± 1.87	108.7 ± 2.35***	89.3 ± 2.95
Systolic pressure (mmHg)	120.3 ± 3.21	113.1 ± 3.38	147.0 ± 4.19***	120.6 ± 3.06
Heart Rate (BPM)	375.2 ± 12.6	419.4 ± 15.0#	390.3 ± 13.49	404.3 ± 13.35

Supplementary Table 3.1 Systolic, diastolic blood pressure (mmHg) and heart rate (BPM) readings from young and aged non-pregnant and pregnant rats on gestational day 20.
*Data presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; #p=0.058; ****p<0.0001 compared to aged pregnant, young non-pregnant and pregnant rats; n= 9-10/group.*

	Young		Aged	
	Non-pregnant	Pregnant	Non-pregnant	Pregnant
E_{max} (% vasodilation)	98.83 ± 4.81	99.21± 1.00	97.82 ± 3.42	99.29 ± 1.54
AUC (arbitrary units)	330.64 ± 11.66	345.15 ± 13.68	323.49 ± 9.16	351.95 ± 9.66

Supplementary Table 3.2 Endothelium-dependent vasodilation responses to methacholine (MCh) in mesenteric arteries from young and aged non-pregnant and pregnant rats. *Summarized as the maximal vasodilation responses (E_{max}; % vasodilation) and area under the curve (AUC; arbitrary units) to MCh. Data presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; n=6-11/group.*

	Young		Aged	
	Non-pregnant	Pregnant	Non-pregnant	Pregnant
Control (AUC)	330.64 ± 11.66	345.15 ± 13.68	323.49 ± 9.16	351.95 ± 9.66
L-NAME (AUC)	263.40 ± 13.96**	272. 94 ± 24.92*	291.76 ± 8.56	265.11 ± 21.35**

Supplementary Table 3.3 Contribution of the nitric oxide (NO) to endothelium-dependent vasodilation responses to methylcholine (MCh) in mesenteric arteries from young and aged non-pregnant and pregnant rats.

*Summarized as the area under the curve (AUC; arbitrary units) to MCh after pre-incubation with the NO-inhibitor L- NAME or without inhibitor (control). Data presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; *p<0.05, **p<0.01 – L-NAME versus Control; n=6-11/group.*

	Young		Aged	
	Non-pregnant	Pregnant	Non-pregnant	Pregnant
Control (AUC)	327.91 ± 11.81	349.37 ± 25.09	366.27 ± 13.56	359.38 ± 19.46
Apamin/TRAM-34 (AUC)	266.68 ± 26.84	351.28 ± 28.86	255.10 ± 24.63*	274.63 ± 34.65*

Supplementary Table 3.4 Contribution of the EDH to endothelium-dependent vasodilation responses to methylcholine (MCh) in mesenteric arteries from young and aged non-pregnant and pregnant rats.

*Summarized as the area under the curve (AUC; arbitrary units) to MCh after pre-incubation with EDH inhibitor - apamin/TRAM-34 or without inhibitor (control). Data presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; *p<0.05 – Apamin/TRAM-34 versus Control; n=7-8/group.*

	Young		Aged	
	Non-pregnant	Pregnant	Non-pregnant	Pregnant
Control (pEC_{50})	7.26 ± 0.31	7.41 ± 0.35	7.50 ± 0.22	7.55 ± 0.42
18α-glycyrrhetinic acid (pEC_{50})	7.36 ± 0.42	7.73 ±0.15	7.43 ± 0.49	7.76 ± 0.42
Control (E_{max})	97.50 ± 1.39	101.15 ± 2.97	99.94 ± 0.18	97.91 ± 1.19
18α-glycyrrhetinic acid (E_{max})	96.42 ± 1.12	98.75 ± 0.45	98.44 ± 0.45	98.30 ± 0.51
Control (AUC)	315.41 ± 19.65	349.37 ± 25.09	366.27 ± 13.56	359.38 ± 19.46
18α-glycyrrhetinic acid (AUC)	330.19 ± 23.46	371. 21 ± 24.04	363.41 ± 18.08	377.95 ± 16.20

Supplementary Table 3.5 Contribution of the myoendothelial gap junctions (MEGJs) to endothelium-dependent vasodilation responses to methylcholine (MCh) in mesenteric arteries from young and aged non-pregnant and pregnant rats.

Summary of the sensitivity to MCh (pEC_{50}), maximal vasodilation responses (E_{max}; % vasodilation) and area under the curve (AUC; arbitrary units) to MCh after pre-incubation with the MEGJ-inhibitor 18a-glycyrrhetinic acid or without inhibitor (control). Data presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; n=5-8/group.

	Young		Aged	
	Non-pregnant	Pregnant	Non-pregnant	Pregnant
SNP E_{max} (% vasodilation)	96.68 ± 1.59	93.32 ± 8.80	93.93 ± 7.59	95.59 ± 3.00
SNP pEC50	7.55 ± 0.08	7.85 ± 0.16	7.43 ± 0.10	7.65 ± 0.10
SNP (AUC)	315.41 ± 19.65	300.68 ± 10.74	242.75 ± 7.32	271.86 ± 11.68

Supplementary Table 3.6 Endothelium-independent vasodilation responses to sodium nitroprusside (SNP) in mesenteric arteries from young and aged non-pregnant and pregnant rats.

Summarized as the maximal vasodilation responses (E_{max}; % vasodilation), sensitivity to SNP (pEC_{50}), and area under the curve (AUC; arbitrary units) to SNP. Data presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; n=5-8/group.

	Young		Aged	
	Non-pregnant	Pregnant	Non-pregnant	Pregnant
Big-ET-1 (E_{max}; mN/mm)	4.56 ± 0.22	4.95 ± 0.27	6.56 ± 0.50	6.14 ± 0.64
ET-1 (E_{max}; mN/mm)	5.37 ± 0.53	5.95 ± 0.37	7.10 ± 0.68	6.42 ± 0.65

Supplementary Table 3.7 Vascular responses to bigET-1 and ET-1 in mesenteric arteries from young and aged non-pregnant and pregnant rats.

Summarized as the maximal vasocontraction responses (Emax; mN/mm) to bigET-1 and ET-1. Data presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; n=6-16/group.

	Young		Aged	
	Non-pregnant	Pregnant	Non-pregnant	Pregnant
Control (E_{max})	4.56 ± 0.22	4.95 ± 0.27	6.56 ± 0.50	6.14 ± 0.64
GM6001 (E_{max})	5.28 ± 0.32	3.99 ± 0.42	5.38 ± 0.62	4.71 ± 0.55
CGS35066 (E_{max})	3.96 ± 0.12	3.49 ± 0.15*	3.46 ± 0.49**	2.92 ± 0.27**

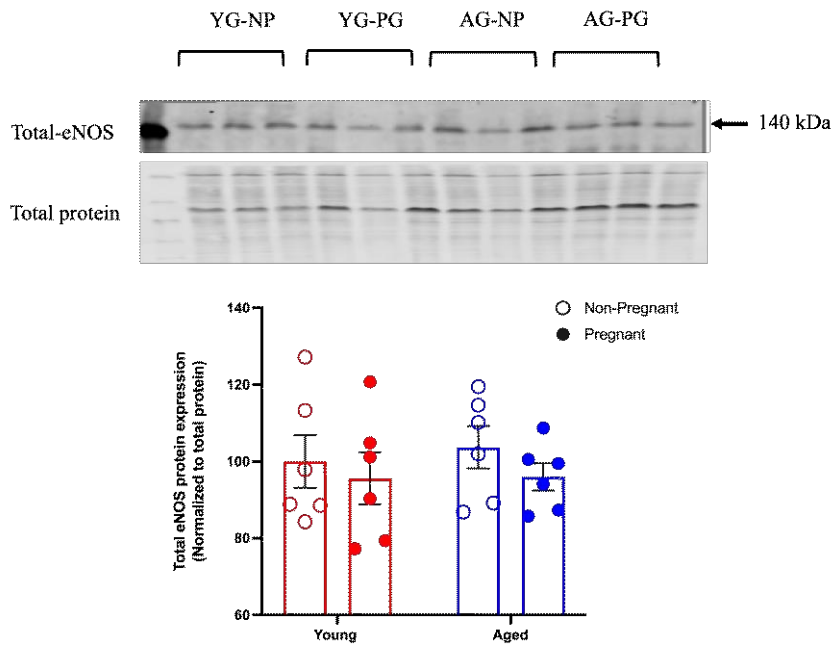
Supplementary Table 3.8 Contribution of matrix metalloproteases (MMPs) and endothelin converting enzymes (ECE-1) to the conversion of bigET-1 to ET-1 in mesenteric arteries from young and aged non-pregnant and pregnant rats.

*Summarized as the maximal vasocontraction responses (Emax; mN/mm) to bigET-1 responses after pre-incubation with GM6001 (MMPs inhibitor) or CGS35066 (ECE-1 inhibitor), or without inhibitor (control). Data presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; *p<0.05, **p<0.01– CGS35066 versus Control; n=6-16/ group.*

	Young		Aged	
	Non-pregnant	Pregnant	Non-pregnant	Pregnant
Control (AUC)	0.49 ± 0.06	0.77 ± 0.18	1.09 ± 0.22	0.92 ± 0.13
Chymostatin (AUC)	0.45 ± 0.10	0.48 ± 0.15	0.74 ± 0.18	0.73 ± 0.16
Thiorphan (AUC)	0.78 ± 0.14	0.82 ± 0.20	1.29 ± 0.23	0.96 ± 0.20
Control (E_{max})	3.13 ± 0.27	4.23 ± 0.47	6.78 ± 0.76	7.79 ± 1.13
Chymostatin (E_{max})	3.21 ± 0.53	2.98 ± 0.54	6.11 ± 1.23	6.12 ± 1.03
Thiorphan (E_{max})	3.87 ± 0.24	3.41 ± 0.33	6.42 ± 0.73	6.32 ± 0.83

Supplementary Table 3.9 Contribution of chymases and neutral endopeptidases to the conversion of bigET-1 to ET-1 in mesenteric arteries from young and aged non-pregnant and pregnant rats.

Summarized as the area under the curve (AUC; arbitrary units) and maximal vasocontraction responses (Emax; mN/mm) to bigET-1 responses after pre-incubation with chymostatin (chymase inhibitor) or thiorphan (neutral endopeptidase inhibitor), or without inhibitor (control). Data presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; n=6-8/ group.



Supplementary Figure 3.1 Total eNOS protein expression in mesenteric arteries of young and aged pregnant rats.

Western blot analysis of total eNOS protein expression, normalized to total protein, in mesenteric arteries of young (3-4 months; in red) and aged (9-9.5 months; in blue) pregnant (on gestational day 20; closed circles) and non-pregnant (age-matched; open circles) rats. Data are presented as mean±SEM and expressed as percentage of control (i.e., the mean of the non-pregnant young group); analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; n=6/group. YG-NP=Young non-pregnant; YG-PG=Young pregnant; AG-NP=Aged non-pregnant; AG-PG= Aged pregnant.

3.3 Discussion

The goal of the current study was to understand the vascular pathways that may be contributing to impaired vascular adaptations to pregnancy in a rat model of advanced maternal age. Overall, our data demonstrated altered vascular function in the aged non-pregnant rats, while these changes appear to be compensated for during pregnancy with a decrease in blood pressure compared to young dams accompanied by an increased contribution of NO and EDH-mediated

relaxation in aged pregnant rats. Furthermore, we demonstrated that contractile responses to bigET-1 were greater in mesenteric arteries of the aged groups compared with the young groups, independent of pregnancy. In contrast to our hypothesis, no contribution of MMPs in converting bigET-1 to active ET-1 was observed, while an increased contribution with ECE-1 was found in aged rats (both non-pregnant and pregnant). However, a similar contractile response to ET-1 suggests that the vascular smooth muscle sensitivity to ET-1 was not different between the groups. We speculate that, although aging contributes to changes in various pathways of endothelium-dependent vasodilation, pregnancy in aged dams may confer vascular protection.

3.3.1 Advanced maternal age and pregnancy outcomes

Aged dams had poor pregnancy outcomes compared to young dams, including smaller litter sizes, higher number of reabsorption rates, and a lower fetal/placental weight ratio. These results align with our previous observations using same rat model of advanced maternal age (23). We now extend those findings to report that maternal age affected fetal and placental outcomes similarly in both male and female fetuses and placentas. We speculate that the observed fetal growth restriction could be due to insufficient maternal hemodynamic adaptation during pregnancy. Nevertheless, this might not be the only possible mechanism involved, thus, designing future experiments to evaluate placental nutrient exchange might explain other mechanisms contributing to the fetal and placental changes observed with advanced age. We then assessed blood pressure and systemic vascular pathways that may be contributing to impaired vascular adaptations to pregnancy.

3.3.2 Advanced maternal age and blood pressure

It is well established that aging alters the structural and functional properties of the vascular wall, leading to arterial stiffness with increased blood pressure, resulting in an overall constrictive phenotype (39, 40, 68, 69). In the current study, the higher blood pressure (systolic, diastolic, and

mean arterial pressure) in non-pregnant aged rats compared to the other groups may have been expected or anticipated, considering the prevalence of age-related arterial stiffness and hypertension (233, 255). Generally, a mild decrease in blood pressure is observed over the course of a normal pregnancy, reaching a nadir around mid-gestation, likely reflecting a reduced systemic vascular resistance (256). We did not observe a difference in blood pressure between young non-pregnant and pregnant rats, which may, in part, be due to the fact that blood pressure was assessed in late gestation; thus, missing the gestational window in which maximal differences in blood pressure are evident. Indeed, our lab previously performed a telemetric assessment of blood pressure (GD5 to GD21) in a selective reduced uteroplacental perfusion rat model (sRUPP and RUPP model of preeclampsia) and observed diurnal changes in blood pressure in sRUPP and RUPP compared to sham animals (257). Thus, we speculate that the Tail cuff method used in the current study might be considered a limitation, and telemetry could have been more sensitive and thus have shown differences between groups.

Interestingly, a significant reduction in blood pressure was observed in aged pregnant rats compared to the aged non-pregnant rats, such that young and aged pregnant rats had similar blood pressures. In aged pregnancies, this reduction in blood pressure may be due to an increased contribution of endothelium-dependent vasodilator pathways that we observed in aged pregnant rats (i.e., NO and EDH, discussed below). In line with our observations, Gaillard *et al.,* in a population-based prospective cohort study, showed that blood pressure differences appear to be small and within the normal physiological range between young (20–24.9; 25–29.9, and 30–34.9 years) and older women (35–39.9 and >40 years) during pregnancy (258). In contrast, Plant *et al.* showed an increased in diastolic blood pressure, but no changes in the systolic blood pressure in pregnant vervet/African green monkeys of advanced maternal age (3–9 years were considered

optimal maternal age, while those 10 years and older were considered to be advanced maternal age) (48). In addition, a trend in increase heart rate was observed in young pregnant rats compared to non-pregnant rats, consistent with the well-characterized cardiovascular adaptation to pregnancy seen in women (259). Interestingly, this trend, not observed between the aged non-pregnant and pregnant rats. This difference may be due to an impaired cardiac sympathetic activity in aging or may be related to cardiac remodeling that is known to occur in aging (260-262). Overall, our study is unique in that we analyzed blood pressure changes compared to the non-pregnant state thus allowing us to assess the cardiovascular adaptations to pregnancy and with aging. As blood pressure was elevated in the aged non-pregnant rats, these animals may be entering pregnancy with a compromised cardiovascular capacity; and unless significant vascular adaptations to pregnancy occur, the aged cardiovascular system would likely be unable to support normal fetal growth and development.

3.3.3 Advanced maternal age and endothelium-dependent vasodilation

Normal pregnancy is associated with an increased endothelium-dependent vascular relaxation, with increased NO contribution during pregnancy (263, 264). In the current study, overall endothelium-dependent vasodilation to MCh in mesenteric arteries was similar between the young and aged non-pregnant and pregnant rats. Furthermore, pre-treatment with the NOS inhibitor L-NAME revealed a significant decrease in E_{max} in both young and aged pregnant rats. These data suggest that in our model, NO-mediated vasodilation is enhanced during pregnancy, however age does not appear to impact this adaptation. Interestingly, this greater NO contribution in pregnancy may be due to increased eNOS phosphorylation at Ser1177 /activity, which was increased in aged pregnant rats (and tended to be higher in young pregnant rats) compared to both non-pregnant rat groups. Several studies clearly demonstrated increased contribution of NO in

young pregnant animals in different vascular beds including mesenteric arteries (39, 62, 64, 67, 68, 265-267), but there is paucity of research investigating systemic vascular contribution of NO in advanced maternal age. In non-pregnant animals, the shift in MCh-induced vasodilation by L-NAME in young but not aged rats suggests that aging is associated with reduced NO-mediated vasodilation, while the NO pathway may be upregulated in pregnancy in order to support the required cardiovascular adaptions. These data support evidence in the literature that the NO pathway is important in vascular adaptations to pregnancy (34, 57), including in our rat model of advanced maternal age.

One of the vasoactive factors produced via the PGHS pathway is PGI_2 which acts as a potent vasodilator. We observed enhanced sensitivity to MCh in the presence of meclofenamate in young non-pregnant and pregnant rats, while no changes in the aged non-pregnant and pregnant rats, suggesting modulation of PGI_2 only in the young group. Previously our lab revealed an increased contribution of NOS- and PGHS-dependent vasodilation in both mesenteric and uterine arteries from pregnant mice (12 weeks) (39). Goodman *et al.* observed upregulation of PGI_2 in the uterine artery in pregnant compared to non-pregnant ewes (87). In contrast, Conrad and Colpoys demonstrated that infusion of PGHS inhibitor (indomethacin) into pregnant rats did not induce hypertension (268). We speculate that the difference in response of PGI_2 may depend on the species and vascular bed or could be due to inhibition of a vasoconstrictor as opposed to PGI_2 (a vasodilator). In general, it is documented that NO is a major vasodilator in larger conduit arteries (aorta) and endothelium-derived hyperpolarization is dominant in smaller resistance arteries (mesenteric arteries). Although PGI_2 acts as a vasodilator, it is often thought that reduced bioavailability of NO increases levels of PGI_2 to maintain overall endothelium-dependent vasodilation response (34, 41).

In addition to NO, we also observed an EDH contribution to vasodilation (as assessed by inhibiting SK and IK channels with apamin and TRAM-34) in mesenteric arteries of young non-pregnant, aged non-pregnant and aged pregnant rats, however, this contribution of EDH was absent in young pregnant rats. Thus, EDH contribution to endothelium-dependent vasodilation (specifically via SK and IK channels) appears to be minimal in young pregnant rats, while this pathway may be enhanced in aged pregnant rats. Thus, the EDH pathway may serve as an additional endothelium-dependent mechanism to compensate for the reduced NO or enhanced vasoconstrictor state to support pregnancy. In some cases, epoxyeicosatrienoic acids, hydrogen peroxide, and electrical coupling via MEGJs acts as hyperpolarizing factors (269-272). We did not observe any contribution of MEGJs to MCh-induced vasodilation. Although assessing all other EDH pathways was beyond the scope of the current study, and it will be interesting to evaluate these specific pathways in future experiments. In contrast to our findings, others have shown a loss of EDH-mediated vasodilation in rat mesenteric arteries with aging and hypertension, in part due to decrease synthesis/release of EDH or a defective electrical coupling between endothelial and smooth muscle cells (273, 274). Furthermore, in normal pregnancy, EDH-mediated vasodilation was elevated due to increased synthesis/reduced degradation in addition to NO/prostanoid synthesis, contributing to the vascular adaptations (37). Thus, EDH contribution to vasodilation exists based on the species, nature (young vs aged), vascular bed, and state (pregnant vs non-pregnant), and the enhanced EDH-dependent relaxation in conditions such as vascular aging may be a compensatory mechanism to maintain a balance of vasoactive factors. Overall, our data indicate that NO is involved in endothelium-dependent vasodilation in both young and aged pregnant rats, while enhanced EDH-mediated vasodilation in mesenteric arteries from aged pregnant rats (and a decrease in blood pressure) suggests that beneficial vascular adaptations occur

in the aged rats that were able to maintain pregnancy.

3.3.4 Advanced maternal age and NOX dependent vasodilation

We also investigated the contribution of NOX enzymes to oxidative stress in the vasculature (mesenteric arteries) via pretreatment with apocynin. The results showed an increased sensitivity to apocynin only in aged non-pregnant rats, while no changes were observed in the young group (non-pregnant and pregnant rats) and aged pregnant rats. The increased sensitivity in aged non-pregnant rats could be linked to higher blood pressure observed in aged non-pregnant rats, as mesenteric arteries contribute to the total peripheral resistance, and therefore regulate blood pressure. The increased contribution of NOX in aged non-pregnant is not surprising and in line with the aging literature, as one aspect of aging is enhanced production of ROS (162, 275, 276). Interestingly, no contribution of NOX enzymes in the age pregnant rats could be related to enhanced NO and EDH-mediated vasodilation in mesenteric arteries.

3.3.5 Advanced maternal age and vasoconstriction

The ET-1 pathway plays an essential role in the maintenance of vascular tone, however, a greater ET-1 activity is associated with pathological conditions, such as aging, and may impair vascular function (141, 233, 277, 278). ET-1 production involves enzymatic cleavage of bigET-1. In the current study, constriction responses to bigET-1 were increased in the aged groups, and since the vascular smooth muscle response to ET-1, as well as to KPSS (suggesting no change in non-receptor mediated vasoconstriction responses) was not different among the groups, this enhanced bigET-1 responsiveness appeared to be due to increased bigET-1 processing in aged arteries. We postulated this may be due to upregulation of MMPs in the aged animals. However, we did not observe a significant contribution of MMPs-mediated constriction to bigET-1 in

mesenteric arteries from any of our group. It is possible that our aged rat model (9.5 months ~35 years of human age) is in fact too "young" and as such, these animals do not yet demonstrate an aged-associated increase in MMP expression/activity, which has been described by others at older ages (non-pregnant; 23-month-old; CB6F1 mice and 30 month-old F344XBN rats) (226, 241, 278-280). Moreover, MMP-contribution may be vascular bed-dependent, as suggested by other studies (277, 281, 282). Thus, in the mesenteric arteries of our aged rats, MMPs may not play a significant role, while other enzymes may contribute to bigET-1 conversion, such as ECE-1.

ECE-1 has been shown to play a dominant role in the processing of bigET-1 in aging vasculature (283, 284). Indeed, we showed a significant ECE-1 contribution to bigET-1 cleavage in aged mesenteric arteries, independent of pregnancy state, while in young rats there was no role for ECE-1 conversion of bigET-1. Interestingly, we did not observe any changes in the ECE-1 expression between the groups, thus the increased ECE-1 contribution may be due to an enhanced ECE-1 activity rather than expression. Although the cleavage of bigET-1 to active ET-1 is primarily through MMPs and ECE-1, alternative pathways including chymase and neutral endopeptidases are also involved (285-287). However, our data did not support a major role for these enzymes. Nevertheless, a constitutive conversion of bigET-1 to the potent vasoconstrictor ET-1 occurs by several enzymes to maintain normal vascular tone. To the best of our knowledge, we are the first to demonstrate the enhanced contribution of ECE-1 in converting bigET-1 to ET-1 in a rat model of advanced maternal age. Our findings of increased NO and EDH contribution to vasodilation, together with enhanced ECE-1-mediated conversion of bigET-1 to ET-1 in mesenteric arteries, suggests an important link between endothelium-derived NO and EDH signaling and the bigET-1/ ET-1 pathways. Indeed, future studies are warranted to determine how these mechanisms are interacting and contributing to systemic vascular adaptations to pregnancy

a rat model of advanced maternal age.

3.4 Conclusion

The population of women becoming pregnant at an advanced age is increasing globally and poses many health challenges due to their increased risk of pregnancy complications. As such, women of advanced age represent a very important and yet understudied demographic of pregnant women. Our study explores various vascular pathways involved in the adaptations to pregnancy, which were distinct in advanced maternal age compared to young rats. Although NO is involved in the vasodilation in both young and aged pregnant rats, enhanced EDH mediated endothelium-dependent vasodilation in aged mesenteric vasculature and lower MAP suggests a beneficial adaptation in these rats that were able to maintain pregnancy. In addition, increased contribution of ECE-1 may provide a more dominant conversion pathway for bigET-1 in aging vasculature. This study increases our understanding of the vascular pathways involved in the systemic vascular adaptations to pregnancy. Given the increasing trend toward delaying pregnancy, understanding the vascular adaptations that may be compromised, thus contributing to an increased risk of adverse pregnancy outcomes in women with advanced maternal age, may help to develop effective treatment and prevention strategies.

CHAPTER 4

The Effect of Tauroursodeoxycholic Acid (TUDCA) Treatment on Pregnancy Outcomes and Vascular Function in a Rat Model of Advanced Maternal Age

A version of this chapter has been published:

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4.1 Introduction

As previously discussed in Chapters 1 and 3, in a rat model of advanced maternal age we observed poor pregnancy outcomes, altered vascular function in systemic arteries, and increased active myogenic responses in both mesenteric and uterine arteries from aged dams compared to young dams (23, 288). Although pregnancies at advanced maternal age are considered high-risk clinically, the underlying pathophysiological molecular mechanisms associated with the adverse pregnancy outcomes, particularly related to vascular dysfunction in advanced maternal age are not well established.

Among the various complex mechanisms, emerging studies show that an increase in ER stress and increased levels of ROS are associated with subsequent endothelial dysfunction in (non-pregnant) aging vessels (75, 289). The ER is a major site for the folding and trafficking of both intracellular and secretory proteins, and any disturbance in the protein-folding environment can lead to the accumulation of misfolded or unfolded proteins, triggering the evolutionarily conserved unfolded protein response (UPR). Please refer to the Figure 1.3 (ER stress and the unfolded protein response (UPR) signaling pathways) page # 42.

Several studies have reported ER stress to play a role in adverse outcomes during pregnancy (189, 190). Further, increased placental ER stress has been associated with fetal growth restriction and early-onset preeclampsia (159, 218). In addition, ER stress is tightly linked to oxidative stress. Protein folding in the ER requires a tightly controlled redox environment and excess ROS generation can severely impact cellular homeostasis (160, 163). Prolonged ER stress in aging is linked to oxidative stress via production of pro-oxidants by NOX family (especially the NOX-2 and NOX-4 isoforms), thereby contributing to vascular dysfunction (164, 165, 171, 290-293). NOX-induced oxidative stress is also recognized as a key factor in the pathogenesis of

adverse pregnancy outcomes such as fetal growth restriction and preeclampsia (158, 248). *In vivo* and *in vitro* studies also demonstrated that chemically induced ER stress (using tunicamycin) increases not only ER stress proteins but also oxidative stress leading to endothelial dysfunction (168, 170, 294). Literature also shows the contribution of ER stress in adverse pregnancy outcomes in complicated pregnancies such as fetal growth restriction and preeclampsia (151, 184, 295). Previously, we have observed poor pregnancy outcomes with fetal growth restriction phenotype in advanced maternal age rat model, and we speculate that increased ER and oxidative stress could contributes to impaired pregnancy outcomes and altered vascular adaptations in pregnancies at advanced maternal age.

In the current study, we first assessed if maternal aging was associated with increased vascular ER stress, followed by a second set of experiments to evaluate the potential benefits of TUDCA (an ER stress inhibitor) on vascular function and pregnancy outcomes in a rat model of advanced maternal age. TUDCA is a bile acid derivative that occurs naturally in the body and is used to treat cholelithiasis and cholestatic liver disease (204, 296). Both *in vitro* and *in vivo* studies have shown a beneficial effect of TUDCA on embryo survival (208, 210). However, whether treatment with TUDCA can improve pregnancy outcomes in advanced maternal age has not been investigated. Therefore, we hypothesized that (1) impaired vascular function is due to increased ER stress in advanced maternal age and (2) TUDCA treatment will improve vascular function and pregnancy outcomes in a rat model of advanced maternal age by reducing ER stress.

4.2 Results

4.2.1 Increased expression of ER stress markers and NOX-4 in aged non-pregnant and pregnant rats

We previously showed that advanced maternal age in a rat model was associated with

impaired pregnancy outcomes and altered vascular function (23, 288). Therefore, in the first study, we assessed if the expression of ER stress markers and NOX in systemic (mesenteric) arteries was increased in advanced maternal age pregnancies. GRP78 and phosph-eIF2α expression was increased in the aged groups with no effect of pregnancy (Figure 4.1A and 4.1B). CHOP expression was increased in the aged groups, while the expression of CHOP was reduced in young pregnant rats compared to young non-pregnant rats, without differences between aged pregnant and non-pregnant rats (significant interaction; Figure 4.1C). sXBP-1 protein levels were higher only in the aged non-pregnant rats compared to the young non-pregnant group (Figure 4.1D). There were no changes in the expression of ATF-6 among the groups (data not shown). As ER stress increases ROS production via NOX, we measured the expression of two key isozyme NOX-2 and NOX-4 isoforms. There were no differences in NOX-2 protein expression between the groups (Figure 4.1E). However, NOX-4 protein expression was higher in aged non-pregnant rats compared to young non-pregnant rats and tended to be higher in aged dams compared to young dams (Figure 4.1F).

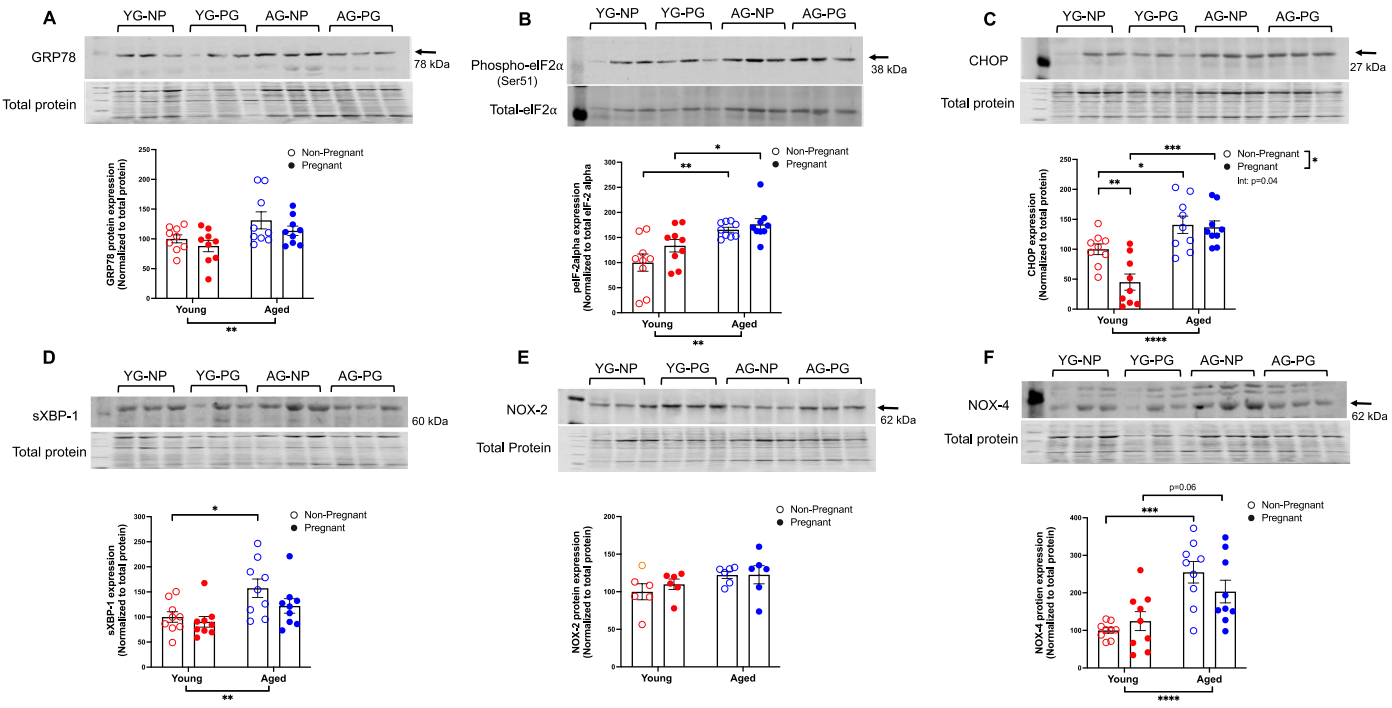

Figure 4.1 Increased expression of ER stress markers and NOX-4 in aged non-pregnant and pregnant rats.

*Expression levels of (A) GRP78, (B) phosph-eIF2α (Ser51), (C) CHOP, (D) sXBP-1, (E) NOX-2, and (F) NOX-4 proteins normalized to total protein in mesenteric arteries of young (3-4 months; in red) and aged (9-10 months; in blue) pregnant (gestational day 20; closed circles) and non-pregnant (age-matched; open circles) rats. Representative blots are shown above the graphs, data are presented as mean±SEM and expressed as percentage of control (i.e., the mean of the young non-pregnant group); analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; n=6-9/group. YG-NP=Young non-pregnant; YG-PG=Young pregnant; AG-NP=Aged non-pregnant; AG-PG= Aged pregnant.*

4.2.2 Reduced ER stress protein expression in TUDCA-treated aged dams

The second study was designed to assess if treatment with the ER stress inhibitor TUDCA could reduce the ER stress that had been shown to be increased in the systemic vasculature of the aged dams, as well as improve pregnancy outcomes and vascular function. No changes were seen

in the expression of GRP78 protein between young and aged control and TUDCA-treated groups (Figure 4.2A). However, phospho-eIF2α and CHOP expression was increased in aged control dams compared to young control dams, and this was reduced by TUDCA treatment in the aged dams, without the effect of TUDCA treatment in the young dams (Figure 4.2 B, C). NOX-4 expression was increased in the aged dams compared to young saline-treated dams, and this aging effect was no longer significant in the TUDCA-treated control compared to TUDCA-treated aged dams (significant interaction; Figure 4.2D).

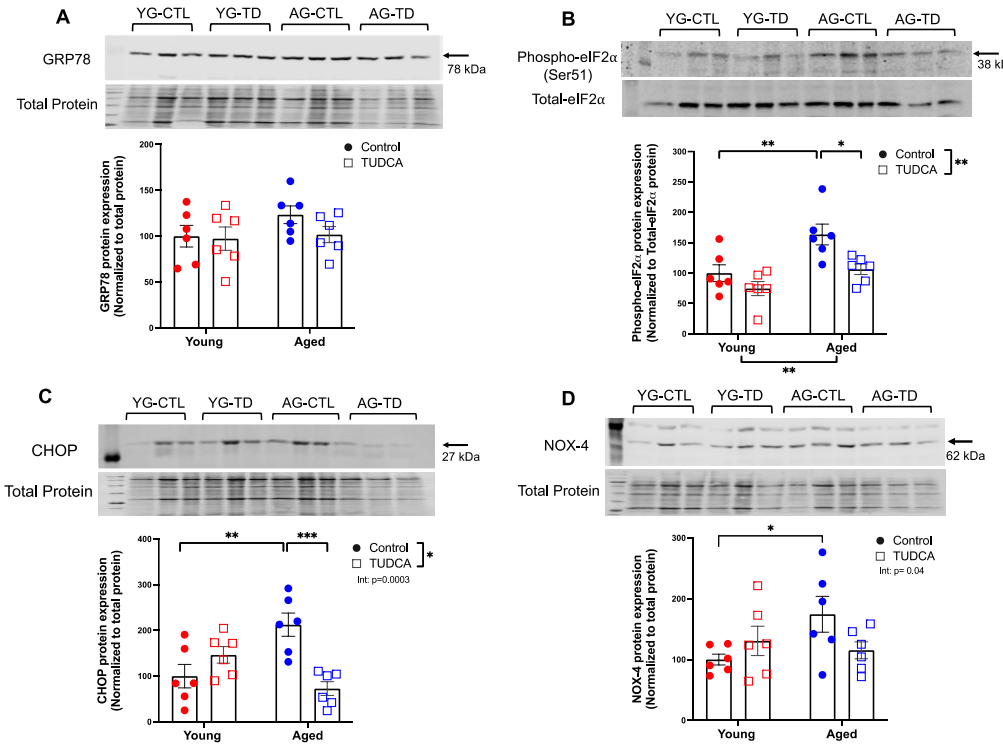

Figure 4.2 Reduced expression of ER stress markers in TUDCA-treated aged dams.

Expression levels of (A) GRP78, (B) phosph-eIF2α (Ser51), (C) CHOP, and (D) NOX-4 proteins normalized to total protein in mesenteric arteries of young (3-4 months; in red) and aged dams (9-10 months; in blue) with (open squares) or without (closed circles) TUDCA-treatment on gestational day 20. Representative blots are shown above the graphs, and data are presented as mean±SEM and expressed as percentage of control (i.e., the mean of the young control dams);

*analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; n=6/group. The number of asterisks defines the level of statistical significance observed among the data in the graphs: *p<0.05, **p<0.01, and ***p<0.001; main ANOVA effects are depicted below the x-axis (aging) or besides the legend (TUDCA); post-hoc test results are shown within the graphs. YG-CTL=Young control dams; YG-TD=Young TUDCA-treated dams; AG-CTL= Aged control dams; AG-TD=Aged TUDCA-treated dams.*

4.2.3 Reduced blood pressure in aged TUDCA-treated dams

TUDCA treatment reduced blood pressure (systolic, diastolic, and mean arterial pressure) in only the aged dams (Figure 4.3 A-C).

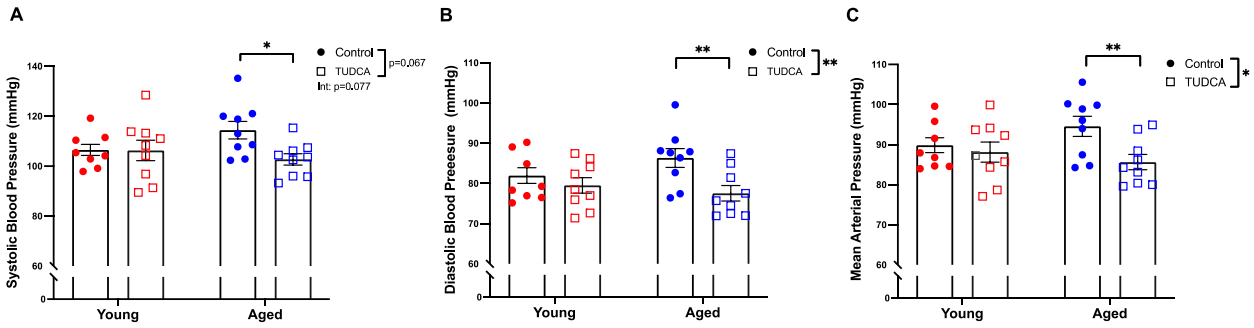

Figure 4.3 Blood pressure was reduced in TUDCA-treated aged dams.

*(A) Systolic, (B) diastolic, and (C) mean arterial blood pressure of young control (3-4 months; in red) and aged dams (9-10 months; in blue) with (open squares) or without (closed circles) TUDCA-treatment on gestational day 20. Data presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; n=8-9/group. The number of asterisks defines the level of statistical significance observed among the data in the graphs: *p<0.05 and **p<0.01; main ANOVA effects are depicted below the x-axis (aging) or besides the legend (TUDCA); post-hoc test results are shown within the graphs.*

4.2.4 Mesenteric artery vascular function was not impacted by age or TUDCA treatment

In mesenteric arteries, no differences in MCh-induced endothelium-dependent vasodilation responses were observed between the groups (Figure 4.4 A, B). In addition, there were no

differences in endothelium-independent relaxation responses to SNP (Figure 4.4 C, D). As NO is a potent vasodilator and plays an important role in regulating blood flow during pregnancy, we evaluated NO contribution using L-NAME, and showed that NO contribution was similar in all the groups (Figure 4.5A-C).

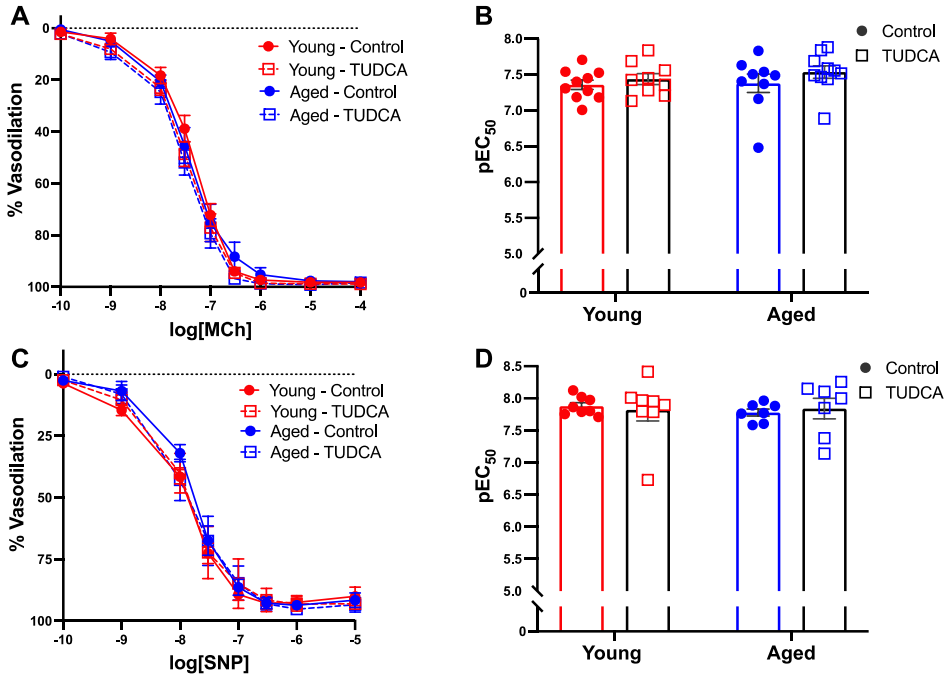

Figure 4.4 No differences in the endothelium-dependent or endothelium-independent vasodilation responses in mesenteric arteries between the groups.

Endothelium-dependent vasodilation responses to increasing doses of (A) MCh or (C) SNP in mesenteric arteries of young control (3-4 months; in red) and aged dams (9-10 months; in blue) with (open squares) or without (closed circles) TUDCA-treatment on gestational day 20. (B, D) Data summaries of the sensitivity to MCh and SNP (pEC50). Data are presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; n=8-9/group.

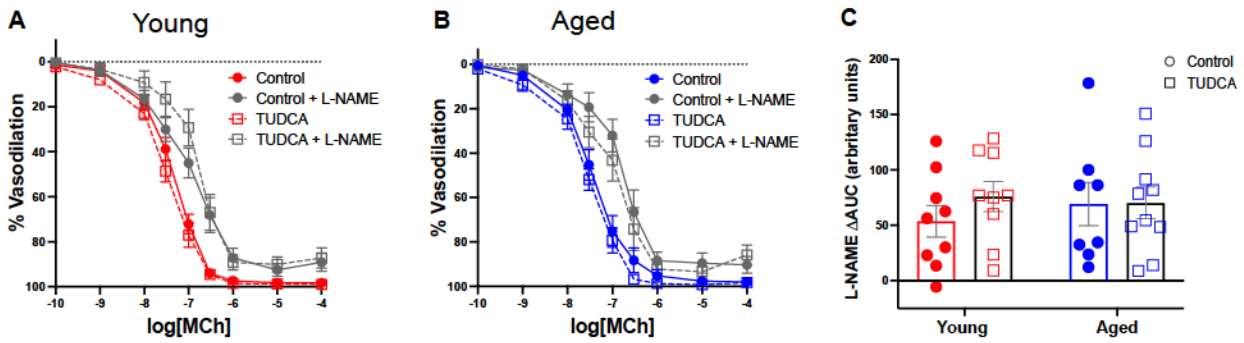

Figure 4.5 TUDCA did not changed the contribution of nitric oxide to vasodilation in mesenteric arteries.

(A, B) Endothelium-dependent vasodilation responses to increasing doses of MCh in the presence or absence of L-NAME in mesenteric arteries of young control (3-4 months; in red) and aged dams (9-10 months; in blue) with (open squares) or without (closed circles) TUD-CA-treatment on gestational day 20. (C) Data summary of A+B, as differences in area under the curve (ΔAUC) in the presence or absence of L-NAME. Data are presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; n=8-9/group.

4.2.5 Increased fetal body weight in aged TUDCA-treated dams

Fetal body weight was lower in control aged dams, while TUDCA treatment increased fetal body weight in the aged dams without any changes in the young control dams (Figure 4.6A). Placental weights were not different among the groups (Figure 4.6B). The fetal/placental weight ratio was decreased in control aged dams compared to young dams (Figure 4.6C). No differences in the crown-rump length/abdominal grith (CR/AG) ratio were observed between the groups (Figure 4.6D). Litter sizes were significantly reduced in aged control, but not TUDCA-treated aged compared to young dams (Figure 4.6E). The number of fetal resorptions were higher in the aged dams, which tended to be reduced with TUDCA treatment (Figure 4.6F).

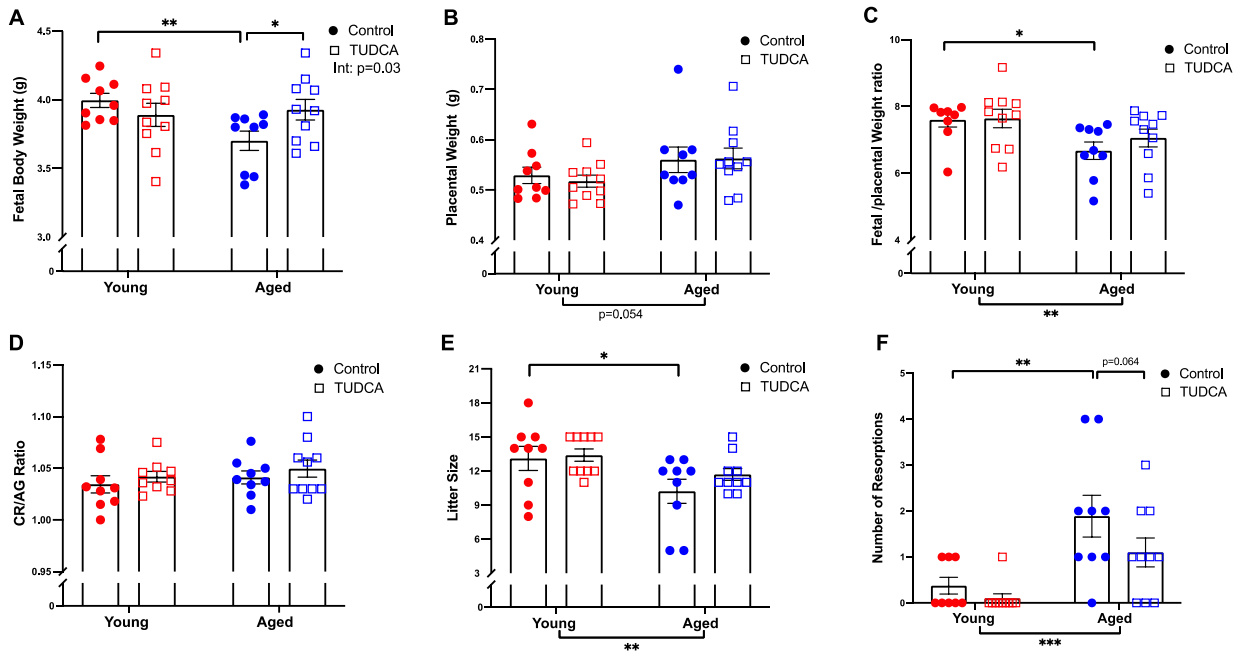

Figure 4.6 TUDCA-treatment increased fetal body weight in aged dams.

*Pregnancy outcomes such as (A) fetal body weight, (B) placental weight, (C) fetal/placental weight ratio, (D) fetal crown-rump (CR)/abdominal girth (AG) ratio, (E) litter size, and (F) number of resorptions in young control (3-4 months; in red) and aged dams (9-10 months; in blue) with (open squares) or without (closed circles) TUDCA-treatment on gestational day 20. Data are presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; n=9-10/group. The number of asterisks defines the level of statistical significance observed among the data in the graphs: *p<0.05, **p<0.01, and ***p<0.001; main ANOVA effects are depicted below the x-axis (aging) or besides the legend (TUDCA); post-hoc test results are shown within the graphs.*

4.2.6 TUDCA treatment tended to improve uterine artery function in aged dams

MCh-induced maximum vasodilation responses were reduced in uterine arteries of aged control dams compared to young control dams, whereas TUDCA treatment tended to increase maximum vasodilation responses in aged dams, without effect in uterine arteries of the young dams (Figure 4.7A, B). There were no changes in uterine artery endothelium-independent

vasodilation responses to SNP between the groups (Figure 4.7 C, D). Moreover, pre-incubation with L-NAME revealed a similar NO contribution among the groups (Figure 4.8 A, B and C).

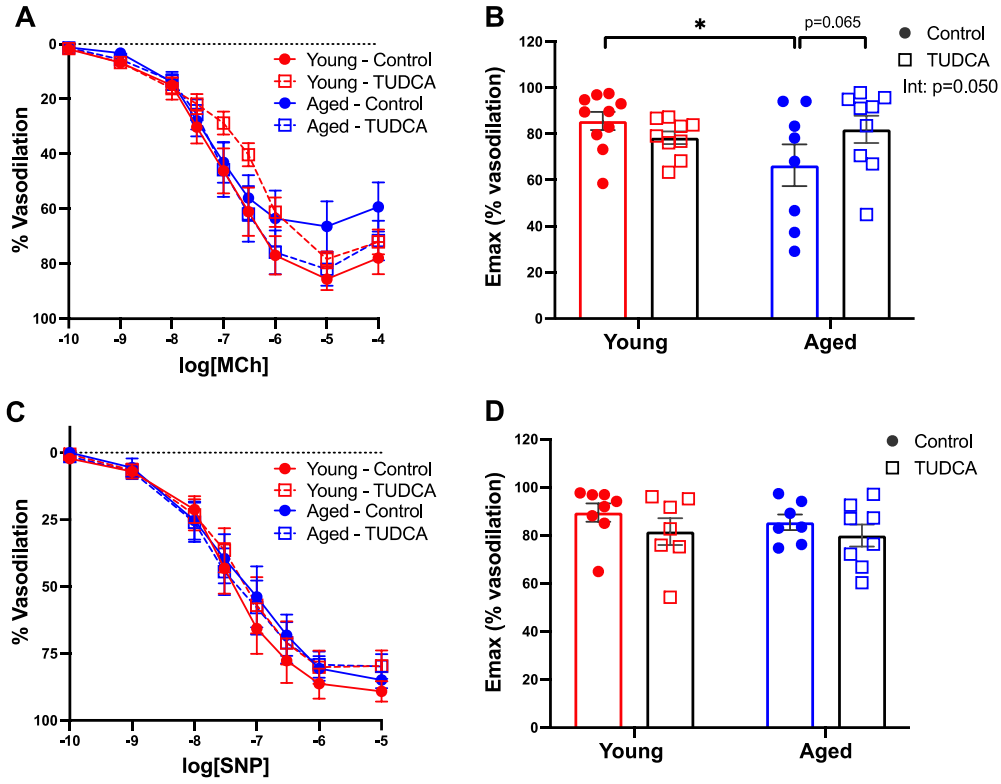

Figure 4.7 TUDCA tended to improve uterine artery endothelium-dependent vasodilation responses in aged dams.

*Endothelium-dependent and endothelium-independent vasodilation responses to increasing doses of (A) MCh or (C) SNP in uterine arteries of young control (3-4 months; in red) and aged dams (9-10 months; in blue) with (open squares) or without (closed circles) TUDCA-treatment on gestational day 20. (B, D) Data summaries of maximal vasodilation responses to MCh and SNP (Emax). Data are presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; n=8-9/group. The number of asterisks defines the level of statistical significance observed among the data in the graphs: *p<0.05; main ANOVA effects are depicted below the x-axis (aging) or besides the legend (TUDCA); post-hoc test results are shown within the graphs.*

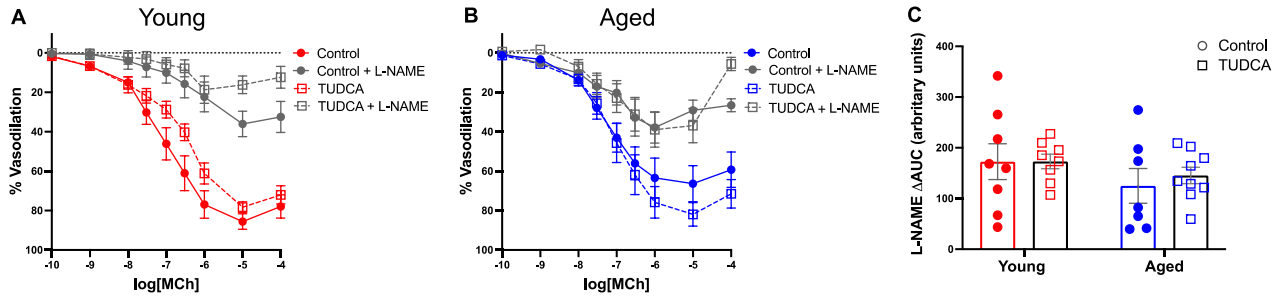

Figure 4.8 No effect of TUDCA treatment on nitric oxide contribution to vasodilation in uterine arteries between the groups.

(A, B) Endothelium-dependent vasodilation responses to increasing doses of MCh in the presence or absence of the pan nitric oxide synthase inhibitor L-NAME in uterine arteries of young control (3-4 months; in red) and aged dams (9-10 months; in blue) with (open squares) or without (closed circles) TUDCA-treatment on gestational day 20. (C) Data summary of A+B, as differences in area under the curve (ΔAUC) in the presence or absence of L-NAME. Data are presented as mean±SEM; analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; n=8-9/group.

4.3 Discussion

The main objective of the current study was to evaluate whether ER stress contributes to impaired vascular adaptations and pregnancy outcomes in advanced maternal age, and to assess whether targeting ER stress using TUDCA could reduce ER stress and improve vascular function and pregnancy outcomes. Our data showed that TUDCA treatment in a rat model of advanced maternal age reduced ER stress in mesenteric arteries, decreased blood pressure, increased fetal body weight, and tended to improve vasodilation responses in uterine arteries, suggesting a beneficial effect.

In a previous study, we observed altered vascular adaptations to pregnancy in mesenteric arteries of aged dams compared to young dams (288). Given the association of ER stress with adverse pregnancy outcomes (134, 208), we speculated that ER stress may be involved in

mediating this vascular dysfunction. In the current study, we assessed the expression of various ER stress markers and observed a higher expression of GRP78 in the aged rats compared to the young rats, but no changes in aged non-pregnant rats compared to young non-pregnant rats. Further, we observed an increased expression of phospho-eIF2α, CHOP, and sXBP-1 from aged non-pregnant rats compared to young non-pregnant rats. In general, under conditions of ER stress, GRP78 is released from the UPR sensors activating the downstream signaling pathways to regain ER homeostasis. One way of achieving homeostasis is by increasing the expression of phospho-eIF2α (to reduce the global protein synthesis) via the PERK pathway, however, prolonged ER stress leads to activation of pro-apoptotic factors such as CHOP. In the current study, a possible explanation for the differential expression of ER stress proteins (i.e., no changes in GRP78, but increased phospho-eIF2α and CHOP) could be that there was an increased expression of GRP78 at an earlier stage of the pregnancy, which eventually returned to basal conditions once it activated the downstream targets (the UPR response, phospho-eIF2α, and CHOP). This has been previously reported, for example, Kumar *et al.* observed differential regulation of ER stress markers (no changes in the expression of GRP78 together with increased CHOP expression) in anterior ischemic optic neuropathy in adult mice (297) . Similarly, Karaskov *et al.* observed no changes in the levels of GRP78 but increased expression of phospho-eIF2α, CHOP, ATF4, and XBP1protein in INS-1 pancreatic ß-cells exposed to palmitate (a known ER stress inducer) (298). An increased expression of ER stress proteins in aged non-pregnant rats is in line with aging literature (i.e., an age-related increase in vascular ER stress) (167, 170, 299). In addition, elevated levels of phospho-eIF2α and CHOP in aged dams compared to young dams suggest that the age-related increase in ER stress persists in pregnancy and could contribute to vascular dysfunction and impair pregnancy outcomes. Interestingly, CHOP expression was reduced in young pregnant dams compared to their

non-pregnant controls, which was not seen in the aged rats, suggesting this may be a pregnancy adaptation that did not occur with advanced maternal age.

Evidence in the literature supports a strong interplay between ER and ROS, their association with vascular changes in aging vasculature, and NOX as the primary source of ROS (153, 159, 160, 300). Galan *et al*. showed an increased expression of ER stress proteins (phospho-eIF2α, CHOP, and ATF6), and NOX-2 and NOX-4 were associated with vascular dysfunction in mesenteric arteries and aortas using C57BL/6J (control) and p47phox-/-mice (NOX lacking) injected with tunicamycin (an ER stress inducer) (170). In addition, Lee *et al.* using C57BL/6J mice, and the NOX-4 KO mice model showed an increased expression of IRE1 and NOX-4 could be linked to vascular dysfunction in aging (167). Thus, the increased expression of NOX-4 in the aged groups suggests upregulation of NOX-4 under ER stress conditions that could contribute to altered vascular function.

After finding signs of systemic vascular ER stress, together with previously reported impaired pregnancy outcomes and altered vascular function (288); we wanted to assess if pregnancy outcomes and vascular function could be improved by ameliorating ER stress. To the best of our knowledge, we are the first to assess the effect of an ER stress inhibitor, such as TUDCA, on pregnancy outcomes and vascular function in a rat model of complicated pregnancy. TUDCA is a naturally occurring bile acid, and chemically, TUDCA is a taurine conjugate of ursodeoxycholic acid (UDCA), which is approved by the Food and Drug Administration for the treatment of primary biliary cholangitis and is also safely used in pregnancy to treat intrahepatic cholestasis compared to UDCA, TUDCA is better absorbed by the intestine and liver because of its higher water solubility at various pH (201, 205). In addition to its anti-cholestatic properties, it has been shown that TUDCA is an effective inhibitor of ER stress. For instance, TUDCA improved

endothelial dysfunction in both animal models (hypertension and diabetic mouse models) and in clinical studies (Type 2 diabetes mellitus) (206, 207, 301). Here, we showed that TUDCA treatment in aged dams reduced the expression of phospho-eIF2α, CHOP, and NOX-4 in systemic arteries. This is in line with other studies in both rats and mice which showed that endothelial dysfunction is associated with upregulation of pelF2α/ATF4/CHOP in mesenteric resistance arteries and aortas, albeit these studies were not performed during pregnancy (166, 171). Moreover, others have previously shown that TUDCA treatment improved vascular function by reducing ER stress and NOX-2 and NOX-4 expression in mesenteric arteries and aortas in a hypertension and diabetic mouse model (non-pregnant mice) (164, 170, 171). Collectively, our data suggest that ER stress may contribute to impaired vascular adaptations in aged dams and that TUDCA treatment may have beneficial effects on ER/oxidative stress in the systemic vasculature.

Mesenteric resistance arteries play a significant role in the systemic circulation, regulating overall peripheral vascular resistance during normal pregnancy (39, 171, 254). However, our data showed no differences in mesenteric artery endothelium-dependent vasodilation responses or NO contribution between the young and aged groups. These findings contrast with other studies, which showed that TUDCA increased NO bioavailability by reducing ER stress and improved vascular function in mesenteric arteries and aorta from tunicamycin-treated mice and in db/db mice (171) (207). A potential explanation for these differences in vascular outcomes after TUDCA treatment could be that the other studies were conducted in non-pregnant rodents, and chemically induced ER stress causes profound vascular endothelial dysfunction with reduced NO, however the phenotype of the vascular dysfunction in our aged dams was more subtle. (288). Further, it is possible that the rat age of our model may not be sufficiently ‘old’ enough to significantly affect endothelium-dependent relaxation in mesenteric arteries; age-related vascular changes are likely

ongoing. The latter was more evident in uterine arteries, with reduced uterine artery maximum vasodilation response and fetal growth restriction in the offspring (discussed below), implying that the vascular changes could be at a subthreshold level.

TUDCA reduced blood pressure in aged dams compared to untreated aged dams, such that blood pressure was similar to that of young dams. Although the effects of TUDCA on blood pressure in pregnancy are not well established, research in non-pregnant animal models has demonstrated that TUDCA, by alleviating ER stress (i.e., reduced expression of markers such as GRP78, phospho-eIF2 , CHOP, IRE1α, XBP1, and ATF6) decreases blood pressure, reduces arterial stiffness, and improves endothelial dysfunction in spontaneously hypertensive rats (207, 302, 303). It may be speculated that the reduction in ER stress that we observed contributed to the reduction in blood pressure by TUDCA in aged dams, although the specific mechanisms leading to this effect require further investigation.

We hypothesized that TUDCA may be able to improve pregnancy outcomes in aged dams. Indeed, TUDCA treatment increased fetal body weight and tended to reduce the number of fetal resorptions in aged dams, suggesting that TUDCA can have a beneficial effect on pregnancy outcomes with advanced maternal age. Moreover, there was no (negative) impact of TUDCA on pregnancy outcomes in the young dams. Of note, TUDCA improved fetal body weight in aged dams without changes in the placental weight. In general, the major determinant of fetal growth is the ability of the placenta to supply nutrients and oxygen via simple diffusion and/or using various transporter-mediated systems, such as glucose and amino acid transport system (304) (305-307). Indeed, there are numerous studies demonstrating placental insufficiency as the primary cause of fetal growth restriction (306, 308, 309). Studies showed that ER stress and ROS can directly impact mammalian targets of rapamycin and O-GlcNAc transferase pathways, two key nutrient-sensing

proteins involved in amino-acid and glucose transport, causing fetal growth restriction (310-312). By reducing placental ER and oxidative stress, TUDCA could thus indirectly control these key nutrient sensors in aged dams to improve fetal weight, however, this remains to be studied in future experiments.

The higher number of fetal resorptions in aged control dams could be attributed to various reasons; impaired decidualization or reduced uterine prostaglandins synthesis that may affect trophoblast cells invasion and eventually reduce the ability of the blastocyst implantation (313, 314). Further, *in vitro* studies showed that ER stress negatively affects blastocyst formation, decreases blastocyst development, and reduces oocyte maturation and embryo development (188-190). Lin *et al*. demonstrated that the TUDCA supplementation of the embryo culture medium improved the rate of implantation/number of livebirth rates of transferred mouse embryos in surrogate mice (210). Thus, increased ER stress could be linked to the higher number of resorptions observed in aged dams, and the reduced resorption rate in aged dams after TUDCA treatment suggests that reducing ER stress may (partially) prevent fetal resorptions.

One of the most important physiological changes for normal pregnancy outcomes is the remodeling of the uterine arteries to supply well-oxygenated blood to the developing fetus (315-318). Previously, using our advanced maternal age rat model, we demonstrated changes in uterine artery vascular function in advanced maternal age compared to young dams (23). In the current study, TUDCA tended to improve endothelium-dependent vasodilation responses in aged dams (without effect in young dams), suggesting increased uterine artery ER stress may have impaired uterine artery vascular function. Further, TUDCA may have improved the uterine artery function by reducing the ER stress, similar to what was observed in the mesenteric arteries, but due to the limited tissue amount of the uterine arteries, we could not measure the levels of ER stress markers.

ER stress has been shown to impair uterine artery function. For instance, Hu *et al*. showed that ER stress suppresses Ca2+ sparks/STOCs and increases myogenic tone in uterine arteries in an animal model of pregnant sheep acclimatized to high altitude, which was reversed using TUDCA/PERK inhibitor (GSK2606414) (183). In addition, no changes in vascular responsiveness to the NO-donor SNP (endothelium-independent response) were found between the groups, suggesting the effect of TUDCA treatment was endothelium-dependent. However, there were no differences in NO contribution which suggests that the improved uterine artery relaxation in aged dams after TUDCA treatment is not NO dependent and may be due to adaptations in other endothelial vasodilatory pathways (an area that remains to be investigated). Overall, our data indicates that TUDCA improved uterine artery vascular function and adverse pregnancy outcomes in complicated pregnancies.

4.4 Conclusion

Advanced maternal age is associated with an increased risk of pregnancy complications such as fetal growth restriction, preeclampsia, preterm birth, and stillbirth. Our study demonstrated the presence of ER stress in mesenteric arteries from rats of advanced maternal age, and that inhibition of ER stress by TUDCA reduced expressions of ER stress proteins in the mesenteric vasculature. TUDCA treatment also reduced blood pressure, improved fetal body weight, and uterine artery function in aged dams, signifying its beneficial role in advanced maternal age pregnancies. Clinically, maternal aging is frequently associated with co-morbidities, such as hypertension, diabetes, obesity, or cardiovascular disease-associated endothelial dysfunction, therefore, designing future studies that include a second hit (such as high salt or high-fat diet or chemically induced ER stress) are warranted to confirm the potential benefit of TUDCA in these complex pregnancies. In summary, our studies are the first to illustrate the role of ER stress in

pregnancies at an advanced maternal age, and for TUDCA as a potential therapeutic that may benefit pregnancy outcomes in this high-risk population.

CHAPTER 5

The Effect of TUDCA Treatment on Placental Endoplasmic reticulum (ER) Stress in a Rat Model of Advanced Maternal Age

***Mazhar Pasha**, Raven Kirschenman, Amy L. Wooldridge, Christy-Lynn M. Cooke, and Sandra T. Davidge. The Effect of TUDCA Treatment on Placental Endoplasmic reticulum (ER) Stress in a Rat Model of Advanced Maternal Age (Manuscript under review: PLoS ONE- 22-31856).*

5.1 Introduction

Using a rat model of advanced maternal age, we have demonstrated poor pregnancy outcomes (fetal growth restriction, reduced number of pups, and increased resorptions rate) and altered vascular function (enhanced NO and EDH vasodilation) in mesenteric arteries in aged dams compared to young dams (Chapter 3) (23, 288). Further, in Chapter 4, we observed increased expression of ER stress markers (phospho-eIF2α and CHOP) in mesenteric arteries of aged dams compared to young dams, and through an intervention study using TUDCA, we demonstrated reduced expression of ER stress marker in mesenteric arteries (phospho-eIF2α and CHOP proteins), improved fetal body weight, and tendency to improve uterine artery function compared to control aged dams (319). However, the effect of TUDCA on placental ER stress in advanced maternal age has not been investigated.

Aside from adequate maternal cardiovascular adaptations, the placenta is arguably the most critical, vital organ during pregnancy, but paradoxically also the most complex and least understood. The placenta is a multifunctional organ that plays a significant role in fetal growth and development during pregnancy. It acts as an interface between the mother and fetus and serves as the lung, liver, kidney, gut, and exocrine/endocrine glands. The major functions include hormone synthesis, gas exchange, nutrient uptake, waste elimination, secretion of hormones, growth factors, etc. Thus, placenta insufficiency/dysfunction could seriously limit the supply of adequate oxygen and nutrients to the developing fetus, eventually could lead to pregnancy complications and poor pregnancy outcomes (320-322).

Because my research project focuses on a rat model, the background will primarily describe the structure and function of the rat placenta, which differs from the human placenta; however, a comparative physiological review of species was beyond the scope of this thesis. The placenta of

rat is histologically divided into the labyrinth zone, junctional zone, and metrial gland or the decidualized mesometrial triangle (323-326). The labyrinth zone is comprised of both fetal and maternal vasculature and the specialized syncytiotrophoblasts. Syncytiotrophoblast is a multinucleated cell formed by the fusion of underlying cytotrophoblasts and is in direct contact with the maternal blood circulation, thus, it's the major site having high metabolic activity, and is involved in the exchange of nutrients, gases, and waste products between the fetus and the mother. (25, 29, 30). Whereas, the junctional zone constitutes the main endocrine compartment of the placenta, producing several key hormones, growth factors, and cytokines. The junctional zone comprises three cell types: spongiotrophoblast, trophoblast giant cell, and glycogen cells. Further, invasive trophoblast cells arise from the junctional zone and participate in uterine vascular remodeling (31). Thus, both zones play an important role in regulating maternal and fetal physiology for the maintenance of healthy pregnancy, and normal fetal growth and development (25, 29, 30). The figure 5.1 below shows the different placental zones and cell types.

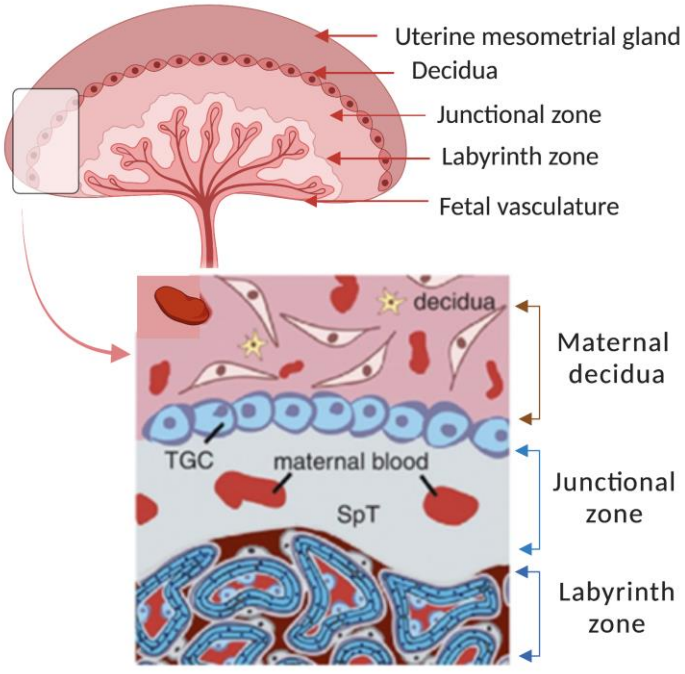

Figure 5.1 Placenta and various cell types of labyrinth and Junctional zones

The labyrinth zone is composed of cytotrophoblast cells in contact with the maternal blood, and fetal vascular endothelium. The junctional zone comprised of spongiotrophoblast (SpT), trophoblast giant cells (TGC), and the glycogen cells. Adapted from Maltepe et. al. J Clin Invest. 2010.

Placental ER stress has been highlighted in the pathophysiology of fetal growth restriction and preeclampsia, for example, Mochan *et al.* demonstrated an increased expression of GRP78 and decreased vascular endothelial growth factor levels in placentae and sera of preeclamptic pregnant women (192). Mizuuchi *et al.* showed that increased ER stress proteins (ATF4 and ATF6β) negatively regulate placental growth factor in syncytiotrophoblast of placentae from preeclampsia and in trophoblast-like cells (JEG-3 and BeWo cells) exposed to tunicamycin (chemical inducers of ER stress) or hypoxia-reoxygenation (193). In addition, Kawakami *et al.* revealed that pregnant mice exposure to prolonged ER stress (tunicamycin) altered the formation of the placental labyrinth zone, and decreased GLUT1 mRNA expression, but increased GLUT3, induced fetal growth restriction and preterm birth (327). Overall, the above studies highlight the detrimental role of increase ER stress and poor pregnancy outcomes, however, the role of placental ER stress in advanced maternal age is unknown. Our group has previously demonstrated increased levels of placental oxidative stress in male and female placentas and elevated levels of apoptosis only in the male placenta of aged dams compared to young dams, indicating that there may be sex differences in the mechanisms behind placental dysfunction in aged dams (328). In the current study, I assessed the effect of TUDCA on placental ER stress in the male and female offspring labyrinth and junctional zones. We hypothesize that increased placental ER stress could be linked to poor pregnancy outcomes and TUDCA treatment, by reducing the placental ER stress, improves pregnancy outcomes in a rat model of advanced maternal age.

5.2 Results

5.2.1 Male-Labyrinth Zone: TUDCA-treatment reduced the expression of ER stress markers in aged dams.

For the labyrinth zone of the placenta, quantification of various ER stress markers via Western blotting showed an increased expression of GRP78 in male offspring placentas from aged control dams compared to young control dams. TUDCA intervention reduced the placental expression of GRP78 in aged TUDCA-treated dams compared to aged control, in contrast, increased levels of GRP78 were observed in young TUDCA-treated dams vs young control (Figure 5.2A). In addition, a reduced expression of phospho-eIF2α, ATF-4, and CHOP was seen only in TUDCA-treated aged dams vs aged control, while no changes were observed in the young group (young control and young-TUDCA-treated dams; Figure 5.2 B, C, and D). No changes in the expression of ATF-6α and sXBP-1 proteins were seen in male placentas of both young and aged control and TUDCA treated dams (Figure 5.2 E and F).

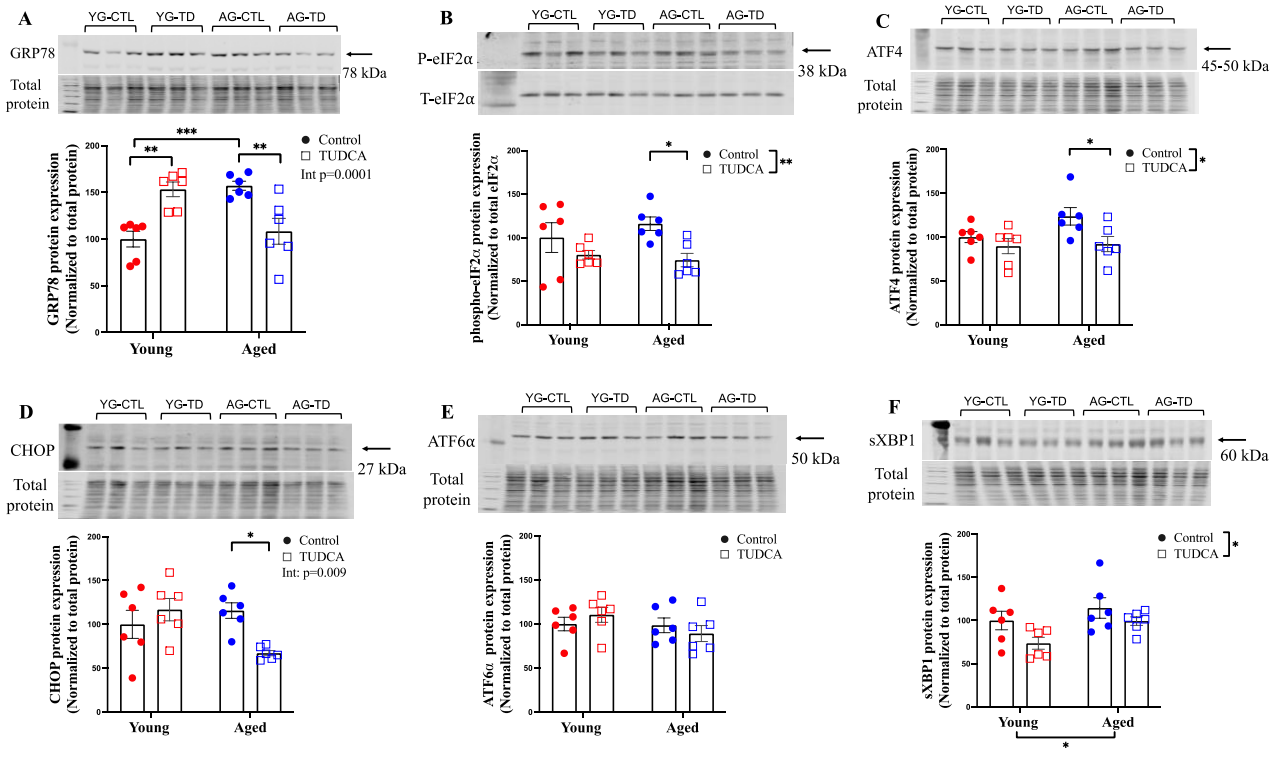

Figure 5.2 TUDCA reduced the expression of ER markers in the labyrinth zone of placentas of male offspring aged dams.

*Western blot analysis of male offspring labyrinth zone (A) GRP78, (B) phospho-eIF2 α, (C) ATF-4, (D) CHOP, (E) ATF-6α, and (F) sXBP-1 normalized to total protein (except phopsho-eIF2α normalized to Total eIF2α) in male placenta labyrinth zone of young (3-4 months; in red) and aged control dams (9-9.5 months; in blue) with (open squares) or without (closed circles) TUDCA-treatment on gestational day 20. Data are presented as mean±SEM and expressed as percentage of control (i.e., the mean of the young control dams); analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; *p<0.05, **p<0.01, ***p<0.001; n=6/group; n=6/group. YG-CTL=Young control dams; YG-TD=Young TUDCA-treated dams; AG-CTL=Aged control dams; AG-TD=Aged TUDCA-treated dams.*

5.2.2 Female-Labyrinth Zone: No changes in the expression of ER markers with TUDCA-treatment.

For the labyrinth zone of the placenta, no changes in the expression of GRP78 in female offspring placentas were observed in either the young and aged groups (with or without TUDCA treatment) (Figure 5.3A). phospho-eIF2α expression was higher in aged control dams vs young control dams, while no changes were observed with TUDCA-treated young and aged dams (significant interaction p=0.023; Figure 5.3B). No changes were seen in the expression of ATF-4, CHOP, ATF-6α, and sXBP-1 in female placentas from both young and aged control and TUDCA-treated dams (Figure 5.3 C, D, E, and F).

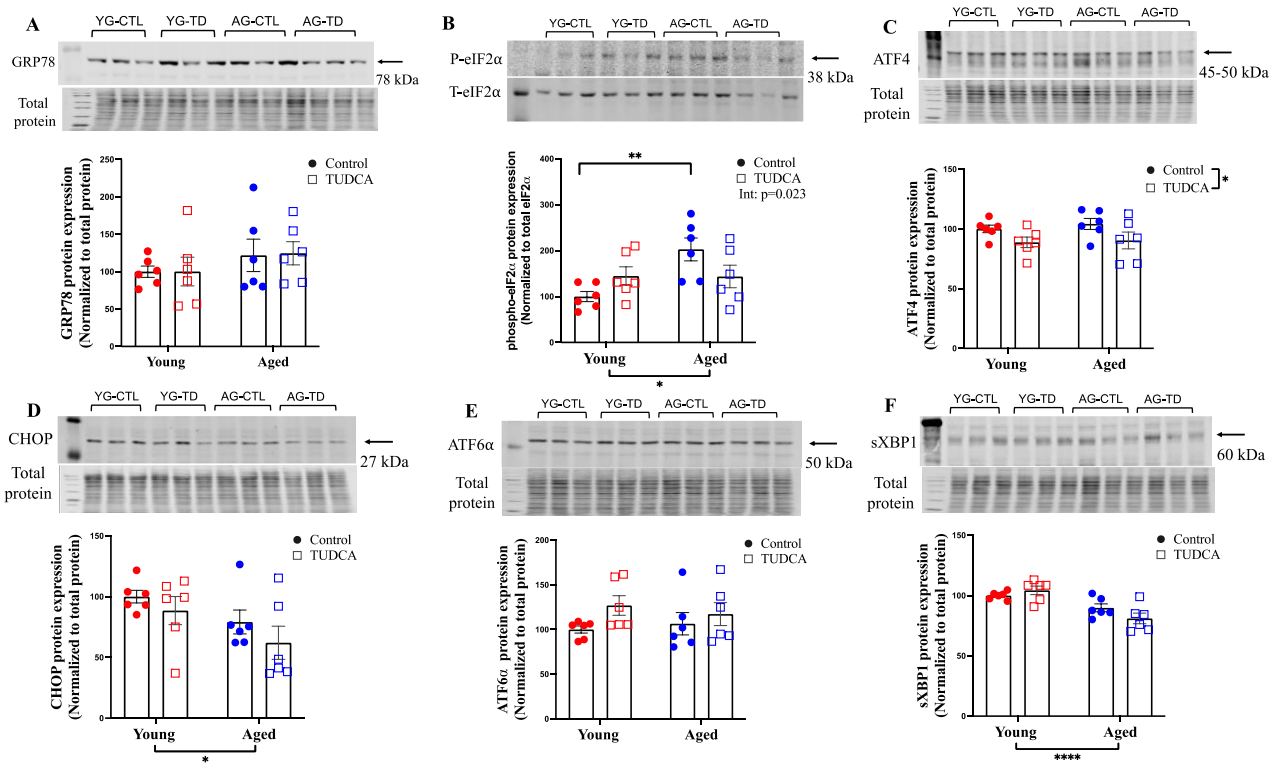

Figure 5.3 TUDCA-treatment did not alter the expression of ER stress markers in the labyrinth zone of placentas of female offspring from young and aged groups.

*Western blot analysis of female labyrinth zone (A) GRP78, (B) phospho-eIF2α, (C) ATF-4, (D) CHOP, (E) ATF-6α, and (F) sXBP-1 normalized to total protein (except phopsho-eIF2α normalized to Total eIF2α) in female placenta labyrinth zone of young (3-4 months; in red) and aged control dams (9-9.5 months; in blue) with (open squares) or without (closed circles) TUDCA-treatment on gestational day 20. Data are presented as mean±SEM and expressed as percentage of control (i.e., the mean of the young control dams); analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; *p<0.05, **p<0.01, ****p<0.0001; n=6/group; n=6/group. YG-CTL=Young control dams; YG-TD=Young TUDCA-treated dams; AG-CTL=Aged control dams; AG-TD=Aged TUDCA-treated dams.*

5.2.3 Male-Junctional Zone: TUDCA-treatment reduced the expression of sXBP-1 protein in aged dams.

For the junctional zone of the placenta, no changes in the expression of GRP78, ATF-4,

CHOP, and ATF-6α in male offspring placentas were observed between young and aged groups (with or without TUDCA treatment; Figure 5.4 A, C, D, and E). A trend in increased expression of phopsho-eIF2α was noticed in aged control dams compared to young control dams, while TUDCA treatment did alter the expression in both young and aged groups (significant interaction p=0.067; Figure 5.4B). TUDCA treatment reduced the protein expression of sXBP-1 only in male offspring placentas from aged dams, but no changes were observed in young control and TUDCA treated dams (Figure 5.4F).

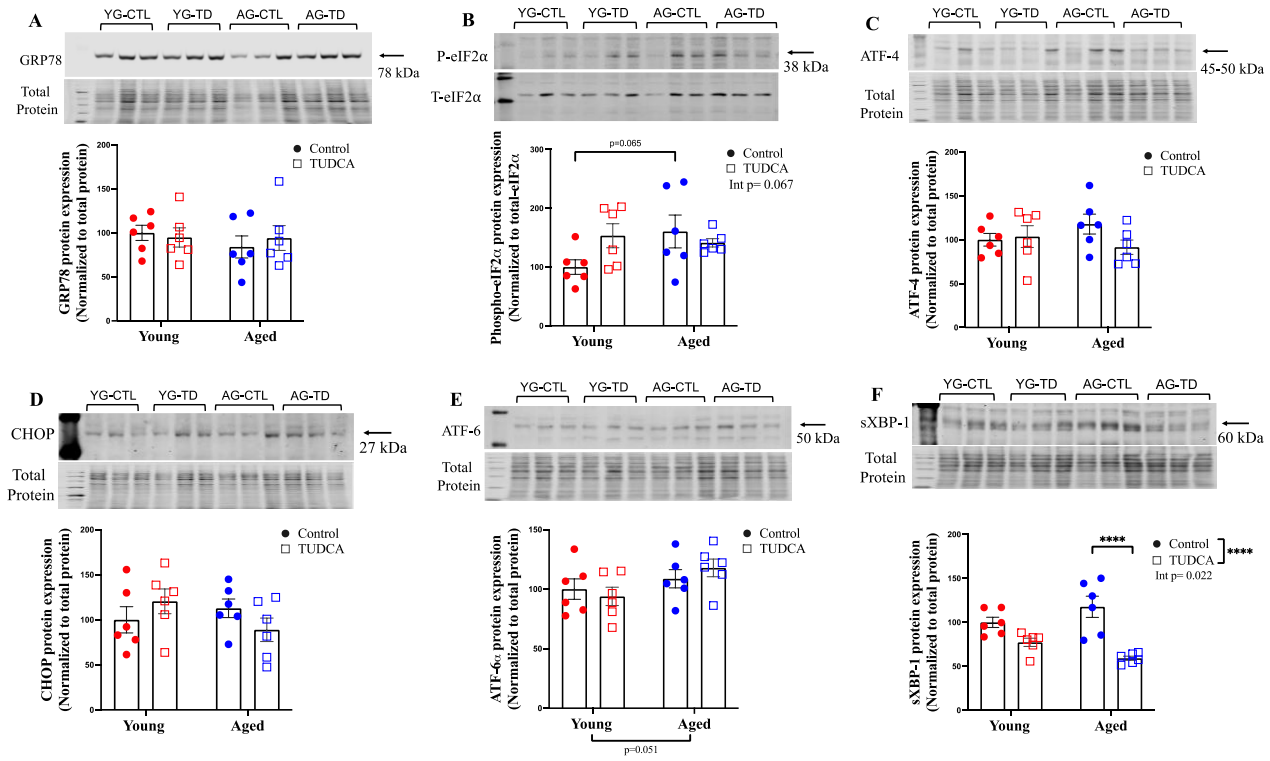

Figure 5.4 TUDCA-treatment reduced the expression of sXBP-1 protein in the junctional zone of placentas of male offspring from aged dams.

Western blot analysis of male junctional zone (A) GRP78, (B) phospho-eIF2α, (C) ATF-4, (D) CHOP, (E) ATF-6α, and (F) sXBP-1 normalized to total protein (except phopsho-eIF2α normalized to Total eIF2α) in male placenta junctional zone of young (3-4 months; in red) and aged control dams (9-9.5 months; in blue) with (open squares) or without (closed circles) TUDCA-treatment on gestational day 20. Data are presented as mean±SEM and expressed as percentage

*of control (i.e., the mean of the young control dams); analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; ****p<0.0001; n=6/group; n=6/group. YG-CTL=Young control dams; YG-TD=Young TUDCA-treated dams; AG-CTL=Aged control dams; AG-TD=Aged TUDCA-treated dams.*

5.2.4 Female-Junctional Zone: TUDCA-treatment reduced the expression of sXBP-1 protein in aged dams.

For the junctional zone of the placenta, no changes in the expression of GRP78, phospho-eIF2α, ATF-4, CHOP, and ATF-6α in female offspring placentas were seen between young and aged groups (with or without TUDCA treatment; Figure 5.5 A, B, C, D, and E). TUDCA treatment reduced the expression of sXBP-1 only in female offspring placentas from aged dams but had no effect in the young control and TUDCA treated dams (Figure 5.5F).

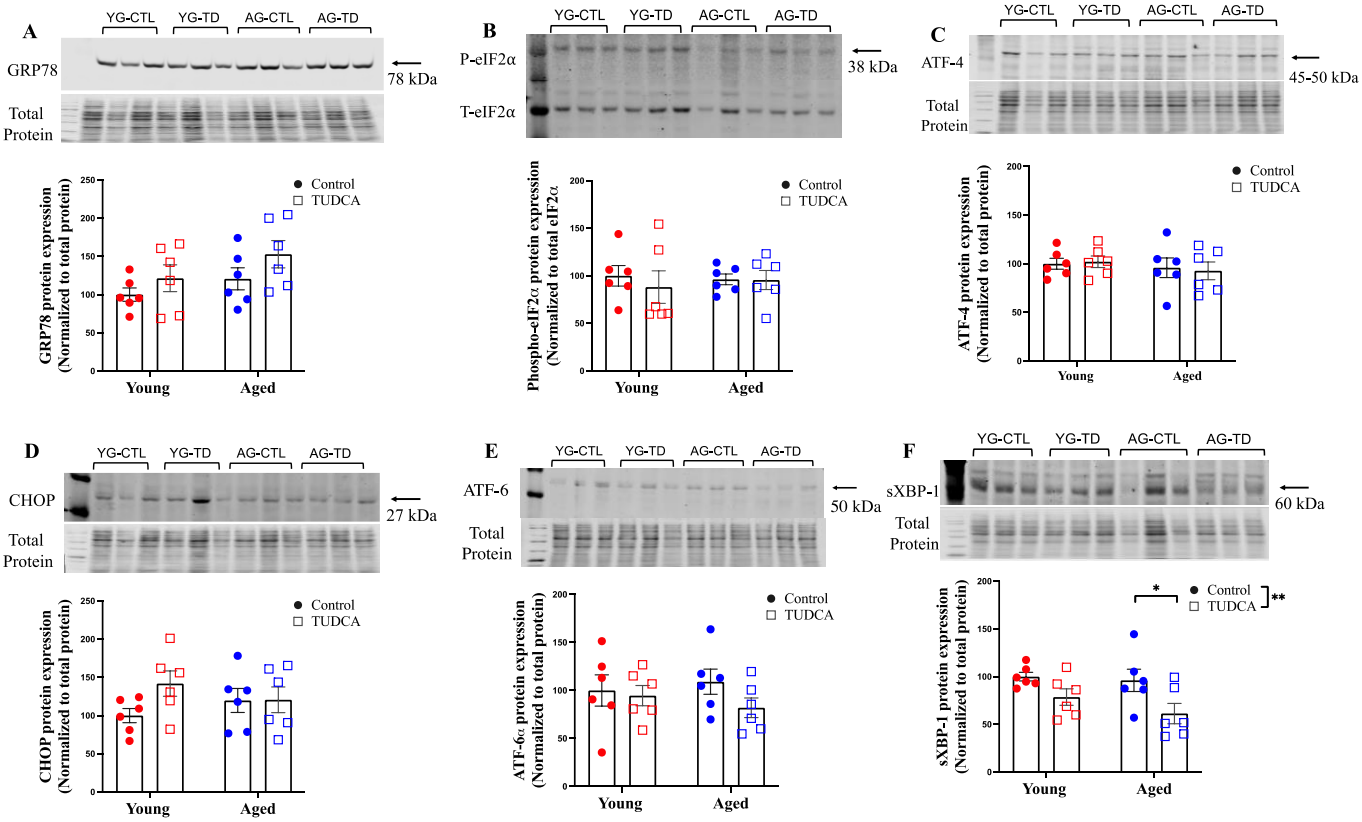

Figure 5.5 TUDCA-treatment reduced the expression of sXBP-1 protein in the junctional zone of placentas of female from aged dams.

Western blot *analysis of female junctional zone (A) GRP78, (B) phospho-eIF2α, (C) ATF-4, (D) CHOP, (E) ATF-6α, and (F) sXBP-1 normalized to total protein (except phopsho-eIF2α normalized to Total eIF2α) in female placenta junctional zone of young (3-4 months; in red) and aged control dams (9-9.5 months; in blue) with (open squares) or without (closed circles) TUDCA-treatment on gestational day 20. Data are presented as mean±SEM and expressed as percentage of control (i.e., the mean of the young control dams); analyzed by two-way ANOVA with Sidak's multiple comparisons post-hoc test; *p<0.05; n=6/group; n=6/group. YG-CTL=Young control dams; YG-TD=Young TUDCA-treated dams; AG-CTL= Aged control dams; AG-TD=Aged TUDCA-treated dams.*

5.3 Discussion

In the current study, we assessed the effect of TUDCA intervention on placental ER stress in both male and female offspring labyrinth and junctional zones in a rat model of advanced maternal age. Our data showed that TUDCA treatment reduced ER stress markers (GRP78, phospho-eIF2α, ATF-4, and CHOP) in the male labyrinth zone and sXBP-1 in the male and female junctional zone without any effect in the female labyrinth zone, suggesting a sexually dimorphic response.

In Chapter 3, we observed poor pregnancy outcomes, altered vascular function, and increased ER stress in mesenteric arteries, and as highlighted in the Chapter 4, TUDCA treatment reduced blood pressure, improved fetal body weight, uterine artery function, and reduced ER markers in mesenteric arteries (phospho-eIF2α and CHOP) in aged dams compared to aged control. These findings suggest a potential beneficial role of TUDCA in advanced maternal age pregnancies (319) However, the effect of TUDCA on placental ER stress had not been investigated. Several studies have demonstrated that increased placental ER stress is associated

with adverse pregnancy outcomes in complicated pregnancies (fetal growth restriction and preeclampsia) (159, 193, 194, 327). Therefore, we speculated an increase in placental ER stress could be a link to poor pregnancy outcomes (fetal growth restriction) that was observed in our rat model of advanced maternal age.

Generally, under conditions of ER stress, the master regulator GRP78 is released from the three key UPR sensor proteins (PERK, ATF-6α, and IRE1α) leading to the activation of downstream pathways to restore protein homeostasis. One way of achieving homeostasis is by phosphorylation of eIF2α (phospho-eIF2α via the PERK pathway) which reduces the global protein synthesis of protein, thus, reducing the ER workload. However, if ER fails to regain proteostasis or under the conditions of prolonged ER stress, there is paradoxical activation of ATF-4 and CHOP (a pro-apoptotic protein) eventually leading to apoptosis. In addition, upregulation of sXBP-1 via IRE1α and /or ATF6α promotes cell survival (149, 168, 172, 178).

In the male offspring labyrinth zone, we observed a higher expression of GRP78 protein in aged dams compared to young dams and TUDCA treatment reduced the expression of GRP78 in aged dams vs aged control. The observed up-regulation of GRP78 in aged dams is in line with aging literature (161, 170, 196), and in sera (using GRP78 ELISA Kit) and placentae (using immunohistochemistry and Western blotting) of intrauterine growth restriction and early onset preeclampsia (192, 193). It is known that pregnancy is a stress factor culminating in acute/mild ER stress and one way to respond to this stress is via upregulation of GRP78. In contrast, under excessive or chronic ER stress, initially, upregulation of GRP78 activates the UPR pro-survival pathway, but eventually activates pro-apoptotic protein such as CHOP via the PERK pathway or JNK via IRE1α. This could have been the case with aged dams (increased GRP78), and TUDCA treatment significantly reduced the expression of GRP78. In addition, TUDCA also reduced the

expression of phospho-eIF2α, ATF-4, and CHOP only in aged dams compared to aged control, but no changes were seen in young-TUDCA treated dams vs young control. Thus, we could speculate that TUDCA is acting as a protective agent to prevent ER stress by keeping these proteins (GRP78, phospho-eIF2α, ATF-4, and CHOP) at basal levels in aged dams. Although, not in pregnancy, several studies have demonstrated the cytoprotective effect of TUDCA not only by alleviating ER stress but also by preventing apoptosis (171, 329-333).

TUDCA treatment increased the levels of GRP78 in young-TUDCA treated dams compared to young control. As highlighted previously, acute ER stress is common in normal pregnancy, and one way to respond to this stress is via upregulation of GRP78, which was evident in young-TUDCA treated dams. Thus, induction of GRP78 could acts as a pro-survival arm of the UPR and also as a molecular chaperone regulating protein homeostasis. Supporting this notion, Luo *et al.* demonstrated that mouse embryos with the knockout of GRP78 showed reduced proliferation, apoptosis, and embryonic lethality (334). In the current study, the exact mechanism is not clear, we speculate that TUDCA might work differentially under the conditions of acute vs chronic ER stress and likely increases the levels of molecular chaperone GRP78 under acute ER stress conditions in young dams. On the contrary, reduces the levels of GRP78 under chronic ER stress, which could be beneficial to avoid cells entering into the apoptosis pathway that was evident in aged-TUDCA treated dams.

Further, in aged untreated animals, there were no differences in other ER stress markers aside from GRP78 and we speculate the reason could be due to the fact that we assessed ER stress at late gestation (GD20). It is possible that changes in ER stress markers occurred earlier in pregnancy in aged animals and thus our study missed the gestational window to observe any effects. This could be considered for future experiments.

In the female labyrinth zone, an increase in the expression of phospho-eIF2α was observed in aged dams compared to young control, although TUDCA treatment did not significantly reduce the expression of phosph-eIF2α in aged dams, there was a significant interaction (p=0.023), whereby the aging effect was no longer significant after TUDCA treatment. The higher expression of phosho-eIF2α in female placentas in aged dams suggests that these dams were in the adaptive phase (protective phase) and could have the capacity to restore ER homeostasis via negative feedback dephosphorylation of phospho-eIF2α to terminate the global translation of proteins, consequently, no effect of TUDCA treatment was observed. Novoa *et al.* demonstrated in PERK−/− and GCN2−/− cells that growth arrest and DNA damage protein 34-GADD34 causes dephosphorylation of eIF2α, via negative feedback mechanism and inhibits stress-induced gene expression, thus may promote recovery from translational inhibition of UPR. In addition to ER stress via PERK-mediated eIF2α activation, other conditions such as viral infection (PKR), amino acid starvation (general control non-derepressible-2), and heme deficiency (heme-regulated inhibitor kinase) also phosphorylates eIF2α (335-338), thus, examining all the conditions in the male and female labyrinth and junctional zones were beyond the scope of the current study. We speculate the reason for no changes in the expression of other ER stress markers (GRP78, ATF-4, CHOP, AFT-6α, and sXBP-1) between young and aged groups could be that in the female labyrinth zone there is no activation of the UPR pathway. Previously, we have demonstrated increased oxidative stress in the placenta of female (+57%) and male fetuses (+90%), and increased apoptosis (cleaved caspase 3 via immunohistochemistry) only in the placenta of males from aged dams but not in female placentas. The data, also demonstrated that female fetuses of aged dams had beneficial changes in placental transport and endocrine function (up-regulation of insulin-like growth factor 2), compared to young dams, while with male fetuses, there were no beneficial

changes in placental transport/hormone expression (reduced insulin-like growth factor 2) in aged dams compared to young dams (328). Thus, advanced maternal age could alter placental phenotype in a sex-specific manner. As ER and oxidative stress are closely linked with cellular homeostasis and apoptosis, therefore, in the current study, we speculate that possibly these female placentas have a beneficial effect or may be less sensitive to ER stress compared to male placentas.

In the male (but not female) junctional zone, we observed a trend toward increased expression of phospho-eIF2α in aged control dams vs young control dams ($p=0.065$). There were no changes in the levels of other ER stress proteins (GRP78, ATF-4, CHOP, AFT-6α, and sXBP-1) observed between the young and aged control placental junctional zone. Closely related to our findings, Yung *et al.* in homozygous $Eif2s1^{tm1RjK}$ mutant mice (mice lacking translational and transcriptional regulation in the ER) observed an approximately two-fold increase in phospho-PERK (an upstream sensor of phospho-eIF2α) in the junctional zone, but not in the labyrinth zone. In addition, no changes in the expression of other ER stress markers (GRP78, GRP94, and sXBP-1 mRNA), nor an increase in apoptosis was observed in either zone (218). While this study did not study male and female labyrinth and junctional zone separately, this observation could still suggest the existence of low-grade ER stress in the junctional zone and perhaps these pathways could have been highly conserved at least in the junctional zone across the species (rat vs mice). TUDCA treatment reduced the expression of sXBP-1 in both male and female junctional zones compared to aged control, suggesting its effect only on the IRE1α arm of ER stress. Though not in pregnancy, Groenendyk *et al.* in HeartCRT+ (a mouse model of cardiac fibrosis) have demonstrated that TUDCA treatment reduced XBP1 splicing and prevented the activation of IRE1α (339). Overall, we speculate that the differential expression of ER stress proteins in the labyrinth and junctional zone could be one of the possible pathophysiology features that could be linked to observed poor

pregnancy outcomes in aged dams, and TUDCA by repressing ER stress, improved pregnancy outcomes (i.e., the fetal body weight) in aged rats.

5.4 Conclusion

Advanced maternal age is associated with an increased risk of fetal growth restriction, preeclampsia, and preterm birth. The current study demonstrated acute ER stress with upregulation of GRP78 (in the male labyrinth zone) and phospho-eIF2α (in male and female junctional zones) in aged dams compared to young dams. Whereas TUDCA intervention reduced the expression of ER stress markers only in aged dams (GRP78, phospho-eIF2α, ATF-4, and CHOP) in the male labyrinth zone, and sXBP1 protein in both male and female junctional zones. These findings highlight the complexity of cellular stress responses in advanced maternal age, and in particular that ER stress markers may be modulated in aged dams in a sexually dimorphic manner. Furthermore, the results from this study suggest that TUDCA treatment may have a cytoprotective role in the placenta by maintaining ER stress proteins to basal levels.

CHAPTER-6

6.1 Summary and Key Findings

The main focus of my PhD thesis was to assess the mechanistic vascular pathways that may be contributing to altered vascular adaptations and adverse pregnancy outcomes in a rat model of advanced maternal age. I also evaluated whether increased ER and oxidative stress contribute to impaired vascular adaptations and pregnancy outcomes and assessed whether reducing ER stress using TUDCA could improve altered vascular function and pregnancy outcomes in a rat model of advanced maternal age.

I used an established rat model of advanced maternal age, wherein, aged dams had shown adverse pregnancy outcomes including reduced fetal growth, lower fetal/placental weight ratio smaller litter sizes, and higher fetal reabsorptions compared to young dams (23). In the same rat model, my first part of the thesis was to evaluate vascular adaptations to pregnancy. In order to achieve this, it was important to include aged-matched non-pregnant groups to which findings could be compared, thus allowing for the evaluation of the vascular adaptations both with pregnancy and aging. My results recapitulated the salient features of poor pregnancy outcomes (fetal growth restriction, increased placental weight, smaller litter sizes, and higher number of fetal resorption rate), in addition, the results demonstrated an increased blood pressure and altered endothelial function in the aged non-pregnant rats, while these alterations appear to be compensated with reduced blood pressure and increased involvement of NO and EDH-mediated relaxation in systemic arteries in aged dams vs young dams. Independent of pregnancy, the aged group also demonstrated increased contractile responses to bigET-1 compared to the young group. No changes in bigET-1 processing occurred in the presence of an MMPs, chymase, or neutral endopeptidase inhibitors, whereas ECE-1 inhibition decreased bigET-1 constriction in aged rats,

suggesting an increased contribution of ECE-1 in the processing of bigET-1 to ET-1.

Next, my focus was to assess the molecular mechanisms that could be associated with altered vascular function and poor pregnancy outcomes. As research revealed an increased ER and oxidative stress (via NOX) both in aging vasculature and with adverse pregnancy outcomes (132, 206), I speculated, ER and oxidative stress could be involved in mediating the observed vascular altered function and adverse pregnancy outcomes, therefore, quantified the expression of several ER stress proteins and NOX-2/ NOX-4 in systemic mesenteric arteries. The results demonstrated increased expression of GRP78, phospho-eIF2α, CHOP, sXBP-1, and NOX-4 in the aged group (non-pregnant and pregnant rats) compared to the young group (overall aging effect). Comparing between aged and young dams, a higher level of phospho-eIF2α, CHOP, and a trend in increased expression of NOX-4 was observed in aged pregnant rats compared to young pregnant rats without changes in aged groups (pregnant and non-pregnant rats). There were no changes in the levels of NOX-2 and ATF-6 among the groups. Overall, these data indicate that the age-related increase in ER stress and oxidative stress continues in pregnancy and may be one of the contributing factors to altered vascular function and adverse pregnancy outcomes observed in the current rat model.

After finding an increased ER stress in systemic mesenteric arteries, together with adverse pregnancy and altered vascular function, I designed an intervention study to improve pregnancy outcomes and altered vascular function. I used TUDCA which has been used as an ER stress inhibitor to improve vascular function in hypertension and diabetic animal studies (206, 207, 301). To the best of my knowledge, the Davidge lab is the first to assess the effect of TUDCA to improve pregnancy outcomes and vascular function and also its effect on the placenta in a rat model of advanced maternal age. Interestingly, TUDCA treatment decreased blood pressure, increased fetal weight, tended to improve uterine artery function, and reduced the expression of phospho-eIF2α

and CHOP in systemic arteries of aged dams in comparison with young dams. In addition, TUDCA reduced the expression of several ER stress proteins (GRP78, phospho-eIF2α, ATF-4, and CHOP) in the male placental labyrinth zone in aged dams compared to aged control.

In summary, my PhD thesis project emphasized that there is an increased risk of developing poor pregnancy outcomes with advancing age, and altered vascular pathways are associated with vascular adaptations to pregnancy in advanced maternal age in comparison to their younger counterparts. Furthermore, my research also illustrated the role of systemic vascular ER stress in aged rats and TUDCA as a novel therapeutic that may benefit pregnancy outcomes in advanced maternal age. Below Figures 6.1 and 6.2 summarize the key findings with or without TUDCA treatment in the current research project.

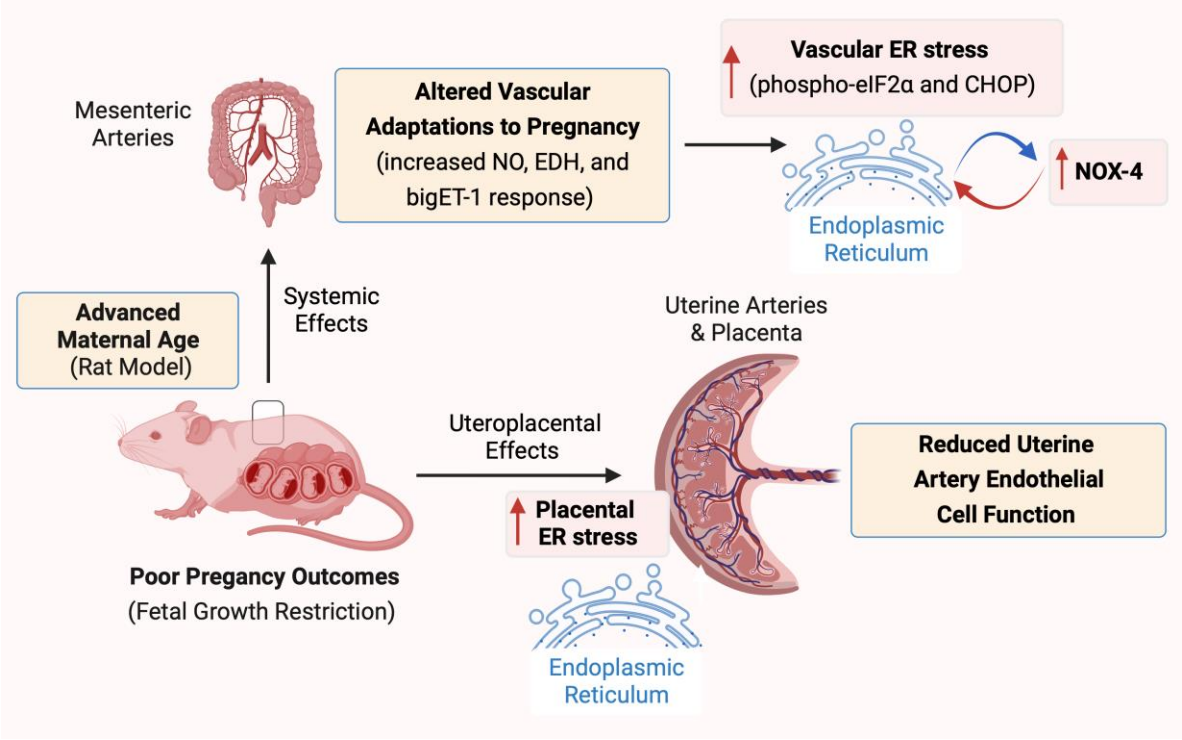

Figure 6.1 Summary of the effect of advanced maternal age on the systemic and uteroplacental unit in advanced maternal age rat model.

NO: nitric oxide, EDH: endothelium dependent hyperonization, bigET-1: big endothelin-1, ER: endoplasmic reticulum, ER stress proteins: phosphoEIF2 alpha and CHOP, NOX-4: NADPH oxidase-4. "Created with BioRender.com."

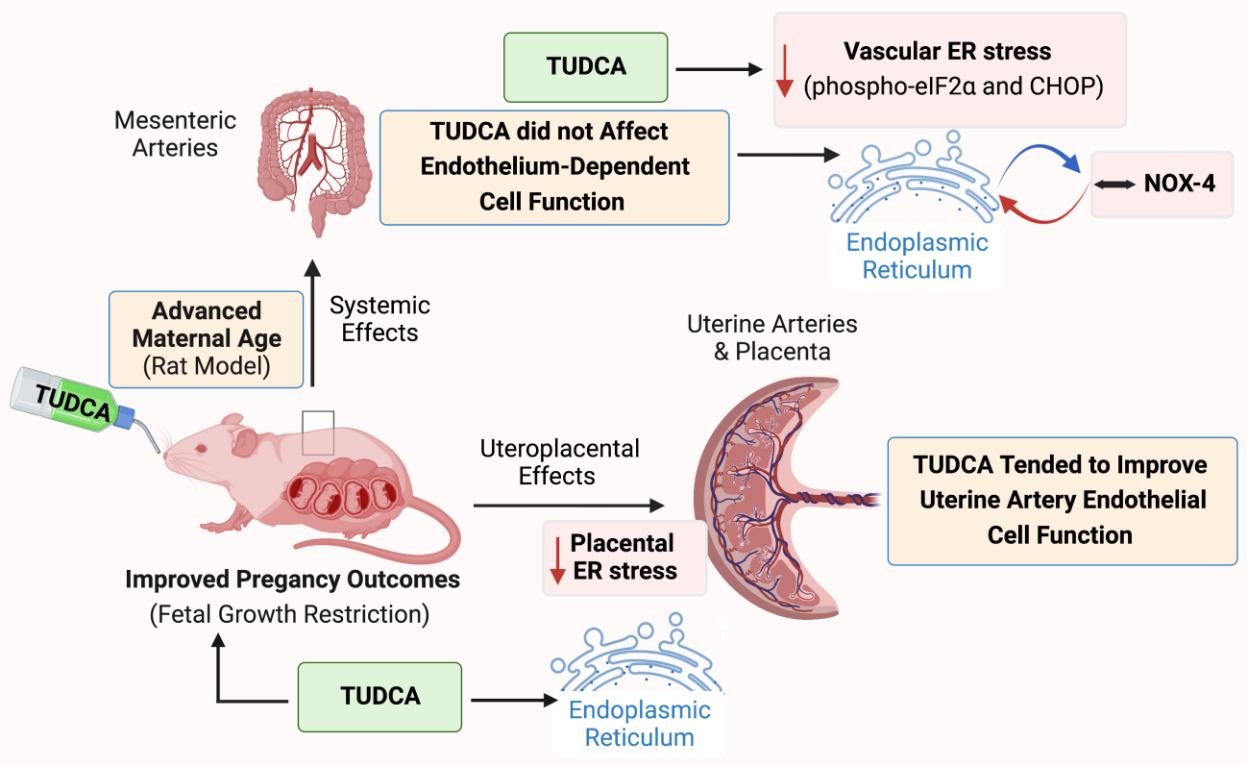

Figure 6.2 Summary of the effect of TUDCA treatment on the systemic and uteroplacental unit in advanced maternal age rat model.

TUDCA: tauroursodeoxycholic acid, ER: endoplasmic reticulum, ER stress proteins: phosphoEIF2 alpha and CHOP, NOX-4: NADPH oxidase-4. "Created with BioRender.com."

6.1.1 Advanced maternal age, pregnancy outcomes, and vascular pathways

Clinical studies have disclosed that advanced maternal age heightens the risk of pregnancy-related complications (low birth weight, gestational diabetes, hypertensive, preterm birth, and preeclampsia) (10, 12, 13, 18). Indeed, using a rat model of advanced maternal age, the Davidge

lab has demonstrated greater active myogenic responses in uterine and mesenteric arteries, and adverse pregnancy outcomes. The increased susceptibility to these complications may be, in part, due to inadequate maternal vascular adaptations during pregnancy (23, 24). Therefore, using a previously established advanced maternal age rat model first, I evaluated the pregnancy outcomes, blood pressure, and vascular pathways in mesenteric arteries using specific inhibitors (vasodilation; L- NAME for NOS, meclofenamate for PGHS, and apamin and Tram-34 for EDH) and (vasoconstriction; the processing of bigET-1 via MMPs, ECE-1, NEP, and chymase, and response to ET-1) that may be involved in the systemic vascular adaptations to pregnancy contributing to an increased risk of poor pregnancy outcomes. The results showed that the animal model used was suitable for this study, as it reproduced similar adverse pregnancy outcomes in aged dams vs young dams including fetal growth restriction phenotype (23, 288).

Higher blood pressure was observed in non-pregnant aged rats in comparison to the other groups. Although there might be several possible mechanisms that could have contributed to the observed increased blood pressure in aged non-pregnant rats (age-related vascular stiffness, inflammation, mitochondrial dysfunction, enhanced renin-angiotensin system, ER stress, etc.) (233, 255, 340, 341), I speculate, this may be due to enhanced ER and oxidative stress which was evident with increased expression of phospho-eIF2α, CHOP and NOX-4 proteins in aged non-pregnant rats compared to control rats (young non-pregnant) (discussed below). Interestingly, a significant decreased blood pressure was observed in aged dams vs aged non-pregnant rats, and it was similar to that of young pregnant rats. This finding is supported by a population-based cohort study, suggesting blood pressure differences appear to be the same between young and aged women during pregnancy i.e. within the normal physiological range (258). I also speculate that the

observed decreased blood pressure could be due to the increased contribution of NO and EDH-meditated endothelium-dependent vasodilator responses in aged pregnant rats (discussed below).

Overall endothelium-dependent vasodilation response to MCh in systemic mesenteric arteries was similar between young and aged groups (non-pregnant and pregnant rats). Pre-treatment with the L-NAME showed a significant decrease in maximum vasodilation response (E_{max}) in young and aged dams, and at the molecular level, increased phosphorylation of eNOS was observed in both aged and young dams compared to the young and aged non-pregnant groups. These key findings imply that NO-mediated vasodilation is increased during pregnancy, regardless of the aging effect to maintain pregnancy and support the developing fetus. This is in line with the pregnancy literature, demonstrating an enhanced contribution of NO during pregnancy in various vascular beds including systemic mesenteric arteries (39, 62, 64, 67, 68, 265-267). Apart from NO, the endothelium also produces other vasodilators such as PGI_2 and EDH contributing to the overall endothelium-mediated vasodilation. In the current study, there was an increased sensitivity to MCh was observed in the presence of meclofenamate in the young non-pregnant and pregnant rats, suggesting modulation of PGI_2 only in the young non-pregnant and pregnant rats. Indeed, studies have revealed an increased NOS- and PGHS-dependent vasodilation in uterine and mesenteries from young pregnant mice (12 weeks) (39), and upregulation of PGI_2 has been demonstrated in the uterine vasculature of pregnant compared to non-pregnant ewes (87). I speculate that the difference in response of PGI_2 depends on species/vascular bed or may be due to inhibition of a vasoconstrictor (TXA_2), a counterpart of PGI_2. In addition to NO, my data also showed an increased contribution of EDH (via SK_{Ca} and IK_{Ca} channels) but not via MEGJs to vasodilation in aged compared to young pregnant rats, and I speculate that this may serve as an additional endothelium-dependent vasodilation effect to compensate for increased vasoconstrictor state to

support pregnancy. An additional explanation could be that these were healthy aged dams that were able to carry the pregnancy were studied, so these animals may have reserve endothelium-dependent vasodilation to support pregnancy. Whereas those dams with reduced fertility (not able to get pregnant) could have more vascular complications in pregnancy if they had become pregnant. Though not in pregnancy, studies have demonstrated a loss of EDH-mediated vasodilation with aging and hypertension in rat mesenteric arteries, partly due to decreased synthesis/release of EDH (273, 274). Of note, NO is a major vasodilator in larger conduit arteries and EDH plays a role in smaller resistance arteries, and PGI_2 could maintain overall endothelium-dependent vasodilation under the conditions of reduced NO bioavailability (34, 41).

To understand the contribution of the endothelium vasoconstriction pathway, I evaluated the vasoconstriction responses to bigET-1 and ET-1. Though the ET-1 system is important in maintaining/regulating normal vascular tone, research has shown that an increased ET-1 activity is interrelated with pathophysiological conditions of aging leading to vascular dysfunction (141, 233, 277, 278). In the current research project, the results demonstrated an increased vasoconstriction response to bigET-1 in the aged non-pregnant and pregnant rats compared to the young non-pregnant and pregnant rats, independent of pregnancy, while no changes were observed with ET-1 sensitivity between the young and aged groups (vascular function), nor its expression levels (assessed via Western blotting). Since, I observed increased big-ET-1 response in the aged non-pregnant and pregnant rats, next I was curious to understand the contribution of various processing enzymes (MMPs, ECE-1, NEP, and chymase) responsible for converting bigET-1 to ET-1. The results showed only ECE-1 play a dominant role only in aged systemic mesenteric arteries, independent of pregnancy, whereas no contribution of other enzymes (MMPs, NEP, and chymase) was observed in processing big-ET-1 to ET-1 in young and age groups. This is in line

with the literature that has demonstrated an enhanced role of ECE-1 in converting bigET-1 to ET-1 in aging vasculature (283, 284).

Overall, my first part of the thesis demonstrated adverse pregnancy outcomes, reduced blood pressure, and in addition to NO, enhanced EDH-mediated vasodilation suggesting a compensatory beneficial adaptation in aged dams to support pregnancy. My next question was to understand molecular mechanisms that might be associated with adverse pregnancy outcomes and altered vascular function with advancing age.

6.1.2 Advanced maternal age, ER stress, and TUDCA intervention

It is documented that the aging process has been linked to several intricate cellular and molecular mechanisms. (142-148). I speculated loss of protein homeostasis could be one of the important molecular determinants, due to the fact that pregnancy is associated with increased nutritional demands of several proteins (peptide hormones, enzymes, and growth factors) required to regulate/maintain the growth of the fetus. ER is one of the most important organelles that assists in protein synthesis, and any disturbance (due to aging, hypoxia, Ca^{2+} imbalance, hypoglycemia, oxidative stress, etc.) could result in the accumulation of unfolded/misfolded proteins that trigger ER stress (149). Indeed, studies have demonstrated an increased ER stress linked to vascular abnormalities in aging and also in the pathogenesis of fetal growth restriction and preeclampsia (134, 150, 151). Further, research also suggests that ROS (via NOX) has been closely linked with ER stress, which can severely affect cellular homeostasis (134, 157, 159-161). Studies have delineated that NOX-induced oxidative stress is one of the key factors in the pathogenesis of reduced fetal growth and preeclampsia (155, 158, 166). Therefore, part of my Ph.D. thesis aimed to understand the contribution of ER and NOX-dependent ROS on vascular function and pregnancy outcome in an advanced maternal age rat model.

I quantified the various ER stress proteins and NOX isoforms in the systemic vasculature, and the results showed an increased expression of master regulator GRP78 in the aged non-pregnant and pregnant rats in comparison to the young non-pregnant and pregnant rats. In addition, higher levels of phospho-eIF2α, CHOP, and NOX-4 were observed in the aged non-pregnant and pregnant rats vs young non-pregnant and pregnant rats. The data also demonstrated an increased expression of phospho-eIF2α and CHOP, and a trend in increased expression of NOX-4 protein in aged dams in comparison to young dams suggesting that the age-related increased ER stress continues in pregnancy. Although not in pregnancy, studies have demonstrated a higher level of ER proteins (phospho-eIF2α, CHOP, and ATF6) and NOX-2 and NOX-4 in mesenteric arteries and aortas in p47phox-/-mice injected with an ER stress inducer (tunicamycin) (170), and in the NOX-4 knockout mice model (increased levels of IRE1α, and NOX-4) linked to endothelial dysfunction in aging (167). Therefore, based on my findings and together with the literature, I speculated that upregulation of ER stress and NOX-4 isoform in aged rats compared to young rats could have contributed to altered systemic vascular function.

Next, in order to link ER and oxidative stress (via NOX) to the observed poor pregnancy outcomes in aged dams, quantifying the various stress proteins in uterine arteries would have been ideal, but due to limited samples, I could not measure ER stress or NOX proteins. Literature shows that ER stress impairs uterine artery function, for example, Hu *et al.* in a pregnant sheep model acclimatized to high altitude disclosed that ER stress reduces Ca2+ sparks/STOCs and enhances myogenic tone in uterine arteries, and it was reversed using TUDCA/PERK inhibitor (GSK2606414). Furthermore, previously our lab has demonstrated increased apoptosis and oxidative stress in male and female placentas from advanced maternal-aged rats, and also literature support that increased ER stress in the placenta has been linked to reduced fetal growth and

preeclampsia (192, 193, 327). Therefore, I reasonably speculated the possibility of enhanced ER and oxidative stress in the uterine arteries and the placenta could have contributed to adverse pregnancy outcomes in aged dams. Subsequently, my next aim was to design an intervention study using TUDCA to improve altered vascular function and poor pregnancy outcomes in an advanced maternal age rat model.

TUDCA is a naturally occurring hydrophilic bile acid used to treat cholestasis (201, 205). TUDCA is known to act as an ER stress inhibitor, and antioxidant, and also has antiapoptotic properties (296, 332, 342, 343). In addition, TUDCA has been shown to improve vascular dysfunction in diabetic and hypertension mouse models and in obesity and Type 2 diabetes mellitus (clinical studies) (206, 207, 301, 344). In the current study, TUDCA was provided in regular drinking water throughout the pregnancy starting from GD0 to GD20 and was carefully observed for any adverse drug reaction.

One of the main objectives of the TUDCA intervention was to assess its effects on pregnancy outcomes in aged dams. Intriguingly, TUDCA treatment significantly increased fetal weight and tended to reduce the number of fetal resorption rates in aged dams in comparison to young dams, signifying its beneficial role in improving pregnancy outcomes. In general, several studies have demonstrated that placental insufficiency is the main cause of fetal growth restriction (304-309). Linking the placental insufficiency with ER stress, studies have shown that increased placental ER stress and ROS can negatively impact some of the nutrient sensors (O-GlcNAc transferase and mammalian targets of rapamycin pathways) that are involved in the glucose and amino-acid transport system, thus affecting the placental function and causing fetal growth restriction (310-312). Several studies have linked increased placental ER stress with adverse pregnancy outcomes in complicated pregnancies (159, 193, 194, 327). For example, increased

expression of GRP78 with decreased vascular endothelial growth factor protein in placentae of preeclamptic women (192), increased levels of ATF4 and ATF6β with reduced expression of placental growth factor in syncytiotrophoblast from preeclampsia (193), and prolonged ER stress (exposure to tunicamycin) induced fetal growth restriction and preterm birth with functional abnormalities in the placenta (changes in the placental labyrinth zone with increased GLUT3, but GLUT1 mRNA expression was higher) of pregnant mice (192). TUDCA, by reducing placental ER stress (discussed below), maybe indirectly control or improve the function of these key growth factors and nutrient sensors to improve fetal weight, however, further studies are warranted to support this concept.

It is well known that the placenta is a multifunctional organ that performs several key functions to support the normal growth and development of the fetus during pregnancy. The rat placenta has been divided into the labyrinth zone (the highly metabolic active part of the placenta), junctional zone (acts as the endocrine region), and metrial gland (important in placentation and fetal growth) (323-326). Previously we have demonstrated increased levels of placental oxidative stress in aged dams (both in male and female placentas) and increased apoptosis (only in the male placenta) in comparison to young dams, suggesting there might be sex differences in the mechanisms behind placental dysfunction (328). Thus, I quantified placental ER stress markers in the male and female offspring labyrinth and junction zones, which had not previously been investigated.

The results showed that TUDCA treatment decreased the levels of placental ER stress proteins only in aged dams (GRP78, phospho-eIF2α, ATF-4, and CHOP) in the male labyrinth zone and sXBP1 protein in male and female junctional zones. Although not in pregnancy, research highlights the cytoprotective effect of TUDCA by relieving ER stress (decreasing the levels of

GRP78, phospho-eIF2α, ATF-4, CHOP, IRE1α, and sXBP-1) and apoptosis (reduced CHOP and cleaved caspase 3 levels) (171, 329-333). Therefore, I speculate that TUDCA could be preventing excessive ER stress in the male labyrinth zone by keeping these proteins (GRP78, phospho-eIF2α, ATF-4, and CHOP) at basal levels in aged dams.

Moreover, the quantification of ER stress markers in the systemic mesenteric arteries showed that TUDCA decreased the levels of ER stress proteins including the NOX-4 isoform, and this could be linked to the observed reduced blood pressure with TUDCA treatment in aged dams, however, the specific mechanisms leading to this effect require further investigation. Although, not in pregnancy, research has revealed the effect of TUDCA improving endothelial dysfunction by reducing ER stress and NOX-2 and NOX-4 in systemic mesenteric and aortas in diabetic and hypertension animal models (164, 170, 171).

The vascular function data show no differences in the systemic mesenteric artery endothelium-dependent vasodilation responses to MCh, in addition, no contribution of NO (assessed using L-NAME) was observed between the young and aged control dams, however, in uterine arteries, a reduced E_{max} was seen in aged dams in comparison to young dams. Interestingly, one of the key findings was the effect of TUDCA treatment on uterine arteries, with a tendency to improve maximum vasodilation response. No changes in NO contribution (using L-NAME) were noticed with TUDCA treatment in uterine arteries suggesting that the improved maximum vasodilation responses in the uterine artery in aged dams were a NO-independent pathway. In contrast, studies have revealed an increased NO bioavailability with TUDCA treatment in systemic arteries and aorta using a chemically induced ER stress agent such as tunicamycin in db/db mice (non-pregnant). I speculate the possible explanation could be the effect of TUDCA may not be the same between pregnant vs non-pregnant state, as well as pathophysiological vs chemically induced

ER stress (171, 207, 288) (this notion needs further investigation).

Overall, my research findings highlight the complexity of cellular stress response in advanced maternal age, as well as the potential cytoprotective role of TUDCA by keeping ER stress proteins in the basal levels. Thus, our data show a potential beneficial effect of TUDCA treatment in the current rat model by reducing ER stress in mesenteric arteries and placenta, decreasing blood pressure, and increasing fetal body weight. Subsequently, TUDCA may be a novel and exciting therapeutic intervention in complicated pregnancies that warrants further research.

6.2 Project Limitation

I used a previously established rat model which recapitulated some of the common features of adverse pregnancy outcomes associated with advanced maternal age such as reduced fertility, fetal growth restriction, reduced litter size, and higher number of resorptions (23). Therefore, this animal model represents a consistent and reliable tool for our study, first to assess the vascular adaptation, then molecular mechanisms, and finally, allows us to study a possible therapeutic intervention to improve poor pregnancy outcomes. Despite the advantages, some of the limitations of this model are, clinically, aging is often accompanied by several co-morbidities such as obesity, diabetes, cardiovascular disease or hypertension. In the current study, aged rats were maintained in a controlled diet of 6 pellets/day to avoid age-related obesity and other issues as a confounding factor. However, because of this, the model may not completely mimic the clinical pathophysiology of advanced maternal age.

The other limitation is we only assess the mechanistic vascular pathways in the systemic mesenteric arteries (that regulate peripheral vascular resistance and control blood pressure), but not in the uterine arteries (that regulate uteroplacental blood flow). Although previously we have

demonstrated an increased myogenic tone in both mesenteric and uterine arteries, studying the contribution of vasodilation and vasoconstriction pathways in uterine arteries could have provided a better insight into the observed poor pregnancy outcomes in aged dams.

One of the key findings was reduced blood pressure in aged dams which was comparable to that of young dams. However, the question remains open, are the young dams the appropriate control to compare to? We did not notice any differences in the blood pressure between young dams and young control non-pregnant rats, whereas normally blood pressure decreases during normal pregnancy (primarily in the midgestation), thus, comparing the blood pressure of aged dams with the young dams may not represent a proper control. The fact that we did not observe a difference in blood pressure between pregnant and nonpregnant rats may be partly due to the reason that blood pressure was assessed on GD20 (i.e., late gestation) and CODA tail-cuff system was used; thus, missing the gestational window in which maximal differences in blood pressure are evident. Further, I speculate that replacing the tail-cuff method with the telemetric assessment of blood pressure throughout the pregnancy could have been more sensitive and may have shown differences between the groups, thus young dams could have been a proper control.

The study did not completely unmask the underlying vascular deficits in the current model, for example, we speculated there might be a more vasoconstrictive phenotype with increased levels of MMPs in the aged animals. However, no contribution of MMPs-mediated vasoconstriction response to bigET-1 was identified in systemic mesenteric arteries. Indeed, research has shown that aging is associated with enhanced MMPs activity (241, 278-280). We speculate that our advanced maternal-aged rat model is in fact "young" (representing ~35 years of age) and does not yet demonstrate age-related vascular dysfunctions. Therefore, including a "second hit" such as high salt or a high-fat diet may expose underlying vascular deficits, and thus could provide more

clinical insights.

We assessed the contribution of EDH using apamin and Tram-34 which inhibits SK_{Ca} and IK_{Ca}, but EDH is not a single factor, studies have highlighted more than one EDH possibly being active at any given time (apart from SK_{Ca} and IK_{Ca}), for example, epoxyeicosatrienoic acids, hydrogen peroxide, hydrogen sulfide, activation of smooth muscle channels (via inward-rectifying potassium channels, large-conductance calcium-activated potassium channels, and/or voltage-gated potassium channels), anandamide, C-type natriuretic peptide, etc. (345-351). Therefore, the observed vasodilation (due to SK_{Ca} and IK_{Ca}) in aged dams may not reflect a complete EDH-mediated vascular response.

In general, pressure myograph could have added advantage in evaluating the vascular function, as it allows to study under controlled and near physiologically relevant intraluminal pressures compared to conventional wire myography. We selected wire myography to assess the contribution of several vasoactive mediators simultaneously to study vasodilation and vasoconstriction pathways (i.e., multiple baths to incubate the inhibitors) whereas pressure myography would not be ideal (limited to 2 baths) to evaluate the proposed pathways.

At the molecular level, we assessed the ER stress proteins and NOX isoform only in the systemic mesenteric arteries, however, quantifying various ER stress and NOX proteins in the uterine vasculature and placentas could have provided a strong rationale for the intervention study.

To the best of my knowledge, we were the first to use TUDCA intervention to improve pregnancy outcomes in the current study. Therefore, there are several limitations to consider. We selected a fixed dose (150mg/kg/day) based on the literature to improve vascular dysfunction, ideally, a dose-response study should be performed before finalizing the target dose. We provided the TUDCA from GD0 and evaluated the pregnancy outcomes parameters on GD20, thus, missed

the gestational window to observe (if any) the effect of TUDCA on early fetal resorption rates. In addition, we did not measure the bioavailability of TUDCA, nor its metabolite taurine vs UDCA in the systemic circulation, or its direct effect on the placenta. Therefore, future studies need to be designed to test both the safety and efficacy of TUDCA intervention at various time points (early vs mid vs late gestation) and also need to evaluate the off-target effects of TUDCA on both mother and the developing fetus in advanced maternal age.

6.3 Future Directions

As highlighted in the project limitation section, maternal aging is frequently associated with co-morbidities, therefore, designing future studies that include a "second hit" could be interesting in the current rat model. For instance, exposing animals to high salt or a high-fat diet may reveal underlying vascular deficits, and may thus provide further clinically relevant insights. Vascular data with increased contribution of NO and EDH to endothelium-dependent vasodilation response, together with increased processing of bigET-1 to ET-1 via ECE-1 enzyme in systemic vasculature, implies a critical link between NO and EDH signaling and the bigET-1/ET-1 system. Thus, further research is warranted to understand how these mechanistic pathways could interact and contribute to vascular adaptations to pregnancy in the current animal model. Moreover, the contribution of NO and EDH in aged dams did not account for all of the endothelium-mediated vasodilation pathways, though there was no contribution of PGI_2, maybe its counterpart TXA_2 could have been upregulated thus, future experiments with or without TXA_2 inhibitor might provide a more complete endothelium-dependent response both in mesenteric and uterine arteries.

Since we observed fetal growth restriction phenotype in aged dams and studies have highlighted placental insufficiency as the major contributing factor (304-309), therefore, an important future direction would be designing and evaluating the placental functional study, for

example, studying the expression of the amino acid and glucose transporters system and their effect on key nutrient sensors such as mammalian targets of rapamycin and O-GlcNAc transferase pathways or growth factors. Moreover, studies have also demonstrated that superficial trophoblast invasion with deficient spiral artery remodeling has been linked with reduced fetal growth (352, 353), thus, studying in detail endovascular trophoblast invasion and associated remodeling of spiral arteries on GD17 (maximum invasion trophoblast occurs into the mesometrial triangle) could provide a possible mechanistic link contributing to poor pregnancy outcomes in a rat model of advance maternal age.

At the molecular level, the main focus was to understand the contribution of ER stress in vascular adaptation and adverse pregnancy outcomes with advancing age, however, emerging studies have revealed interconnections between ER stress, oxidative stress, and inflammation (such as NFκB, a key regulator of inflammatory response) pathways under several pathological conditions (including aging, cardiovascular-related disease obesity, and diabetes) (354-356). In addition, ER has been linked to mitochondria via mitochondria-associated membranes, regulating calcium homeostasis, mitochondrial function, autophagy, and apoptosis. Thus, further understanding these interconnecting pathways could lead to the design of a better novel therapeutic target.

Apart from the semiquantitative measurement of ER stress via the Western botting used in the current project, other techniques to quantity the ER stress might be useful in future research, for example, real-time redox measurements using eroGFP (redox-sensitive ER-targeted green fluorescence protein-GFP) or quantifying the ER stress in live cells using Thioflavin-T (a fluorescent compound exhibiting increased fluorescence when it binds to unfolded/misfolded/ protein aggregates).

Based on the encouraging results from the TUDCA intervention study, I strongly believe this drug has the potential to translate into clinical studies. However, before moving from bench side to bedside additional studies are required including evaluating the drug safety and toxicology studies; 1) acute (three months) and chronic studies (6-12 months) and 2) should be in two different animal species (could be in sheep or rat/mice). Second, drug pharmacokinetic studies (to assess absorption, metabolism, distribution, and excretion) are important to understand the accumulation of TUDCA in specific organs and or for any signs of its toxicity. Third, pharmacodynamic studies (drug-receptor interaction) to understand its mode of action. Fourth, as placental inefficiency is linked with fetal growth restriction, and TUDCA improved fetal body weight, further studies need to design to evaluate the various placental transport system with TUDCA intervention. In addition, assessing the effect of TUDCA on the offspring's health and their susceptibility to cardiovascular disease will be an advantage to understand its long-term effects. Nevertheless, more studies are needed, but preliminary animal research with TUDCA intervention is promising that may be a part of the effort to develop clinically evidence-based treatment options for pregnant women with complicated pregnancies.

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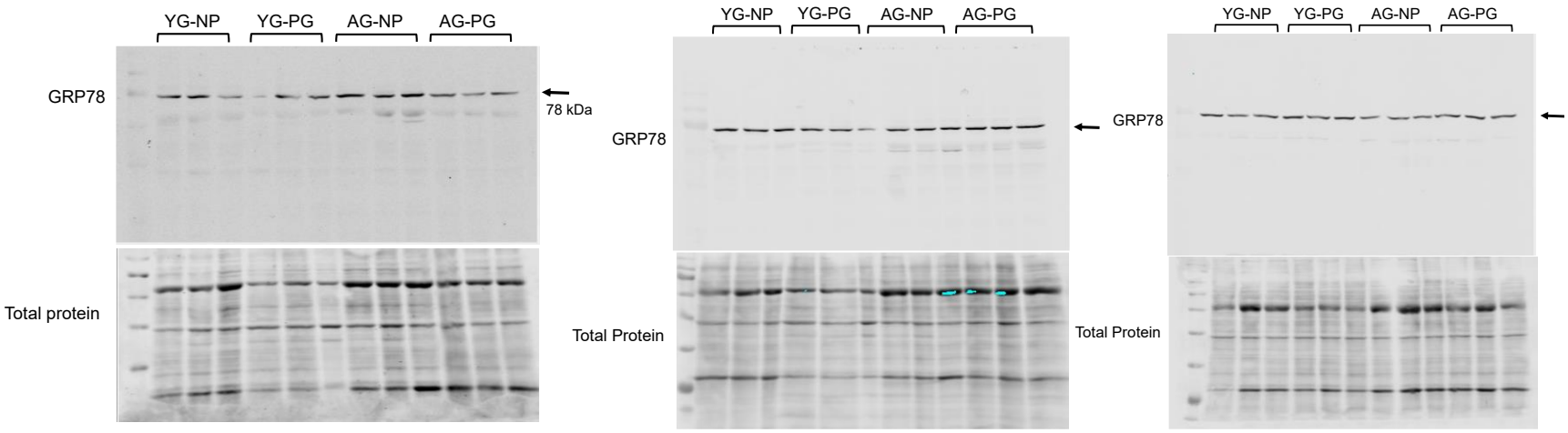
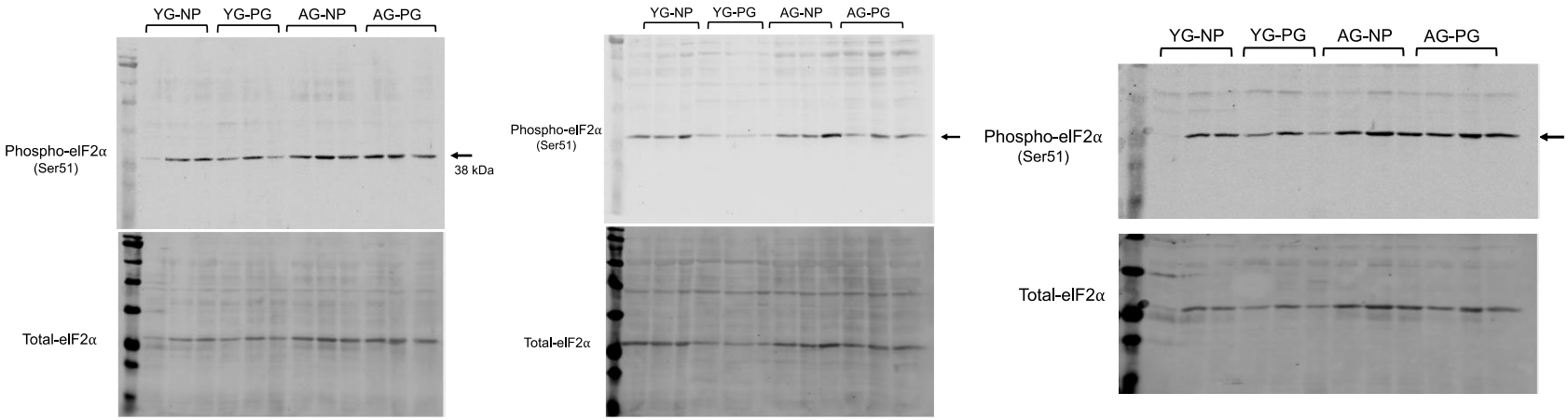
Appendices

Appendix A

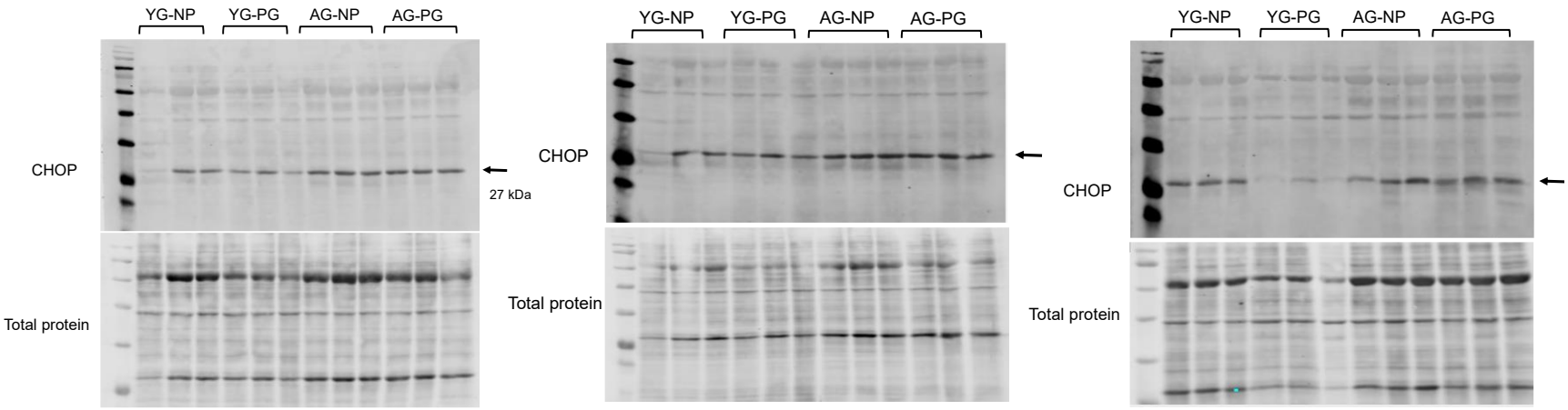
Data Supplement - Original Western Blots Chapter 4



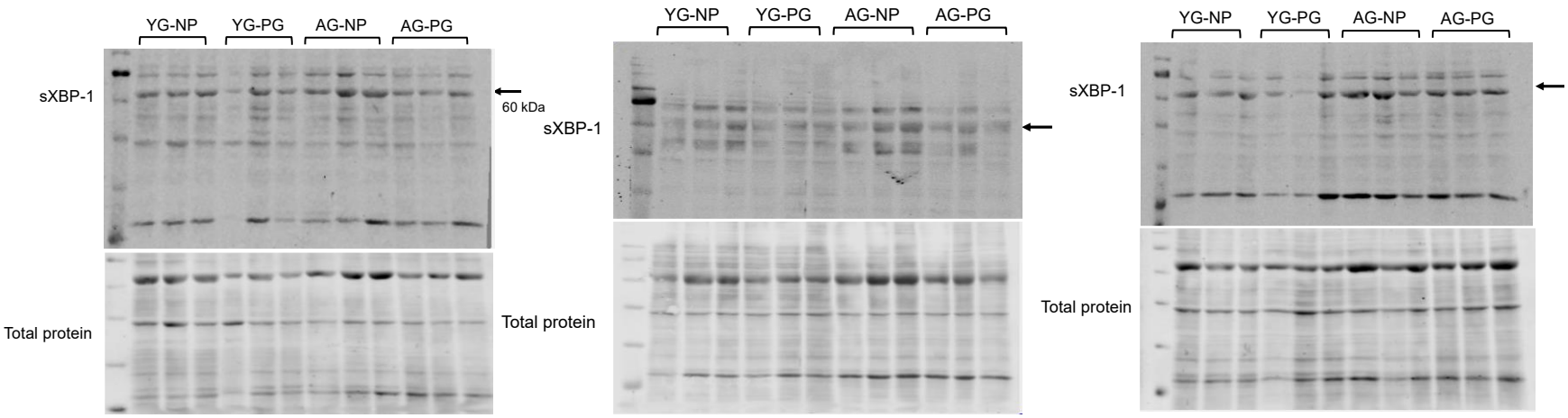
Supplemental Figure 4S1. Original Western blotting images and images of the total protein staining for GRP78 protein expression in mesenteric arteries. Analyzed data is shown in Figure 1. YG-NP=Young non-pregnant; YG-PG=Young pregnant; AG-NP=Aged non-pregnant; AG-PG= Aged pregnant.



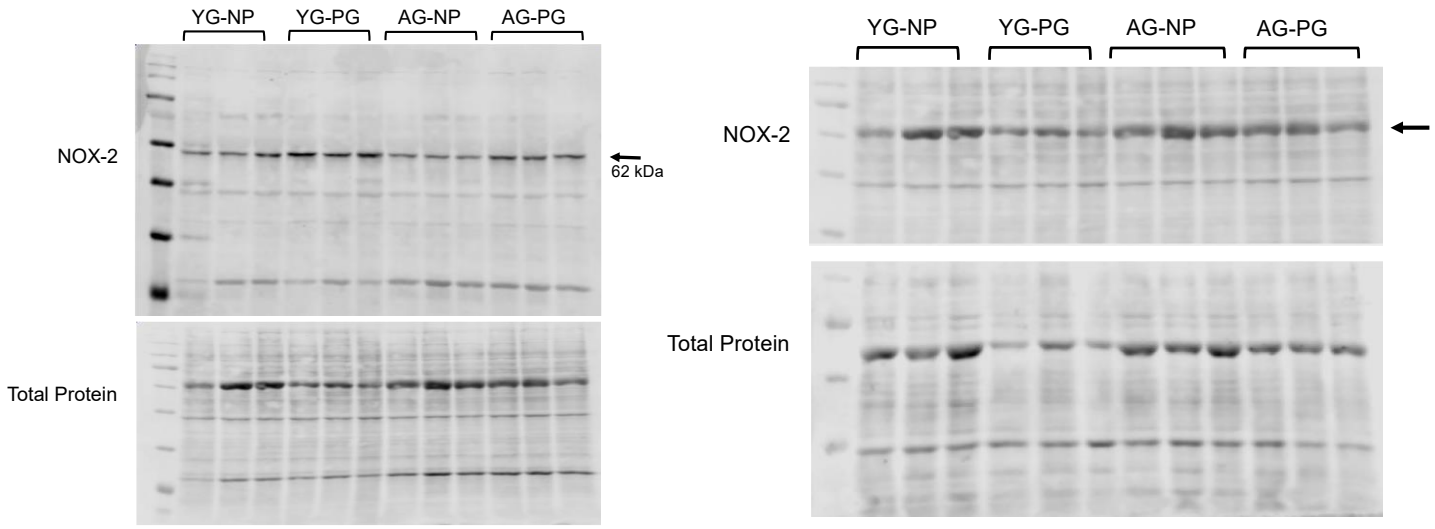
Supplemental Figure 4S2. Original Western blotting images of Phospho-eIF2α and Total-eIF2α protein expression in mesenteric arteries. Analyzed data is shown in Figure 1. YG-NP=Young non-pregnant; YG-PG=Young pregnant; AG-NP=Aged non-pregnant; AG-PG= Aged pregnant.



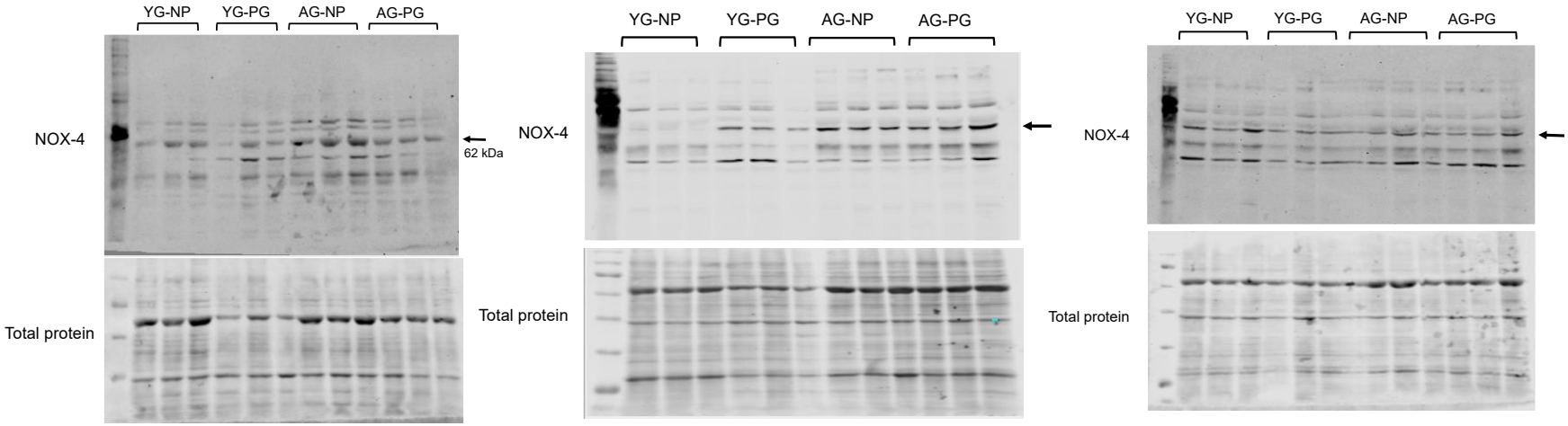
Supplemental Figure 4S3. Original Western blotting images and images of the total protein staining for CHOP protein expression in mesenteric arteries. Analyzed data is shown in Figure 1. YG-NP=Young non-pregnant; YG-PG=Young pregnant; AG-NP=Aged non-pregnant; AG-PG= Aged pregnant.



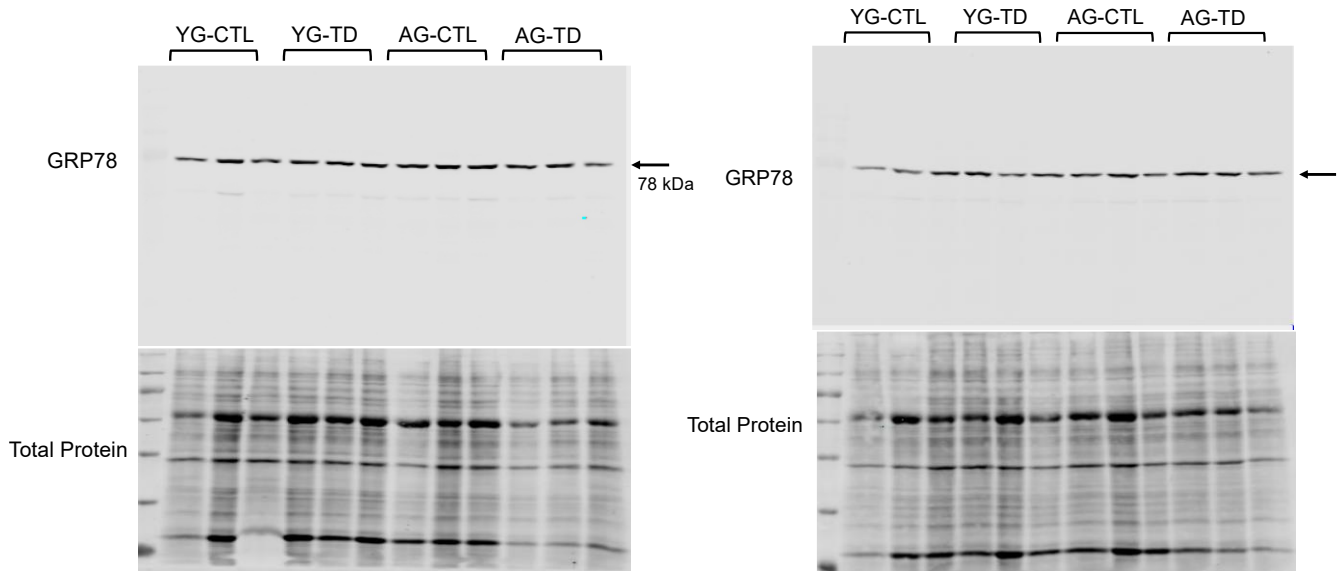
Supplemental Figure 4S4. Original Western blotting images and images of the total protein staining for sXBP-1 protein expression in mesenteric arteries. Analyzed data is shown in Figure 1. YG-NP=Young non-pregnant; YG-PG=Young pregnant; AG-NP=Aged non-pregnant; AG-PG= Aged pregnant.



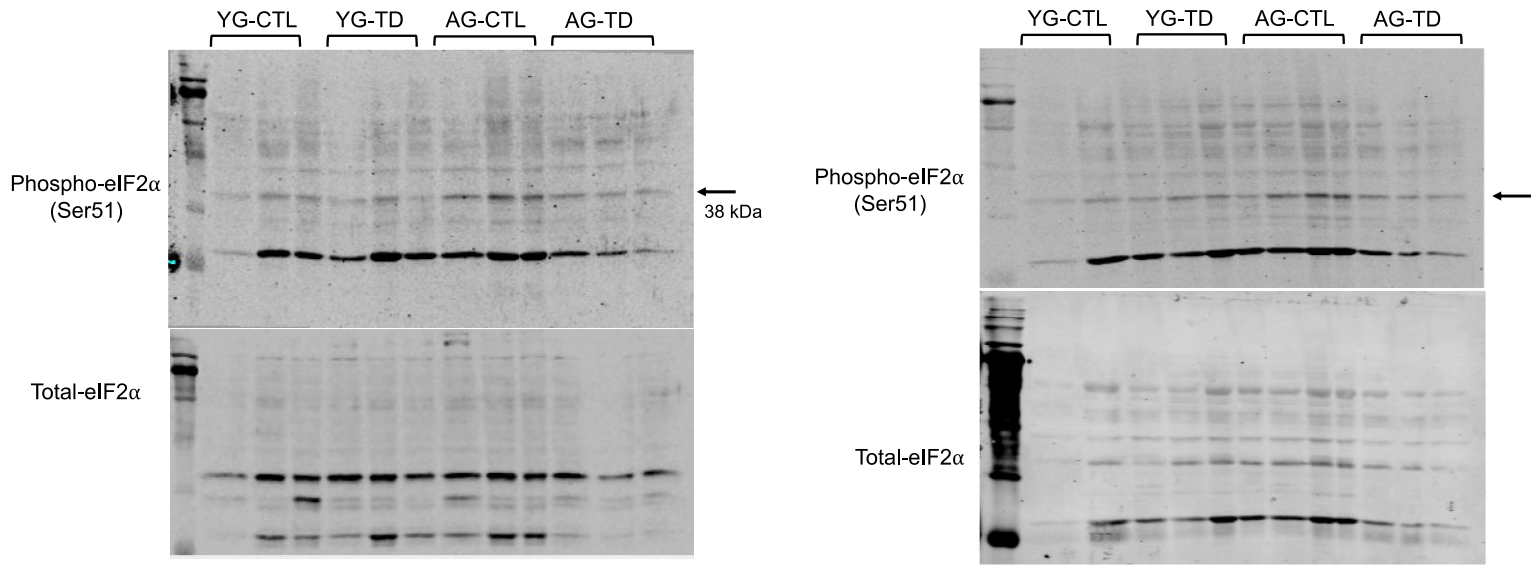
Supplemental Figure 4S5. Original Western blotting images and images of the total protein staining for NOX-2 protein expression in mesenteric arteries. Analyzed data is shown in Figure 1. YG-NP=Young non-pregnant; YG-PG=Young pregnant; AG-NP=Aged non-pregnant; AG-PG= Aged pregnant.



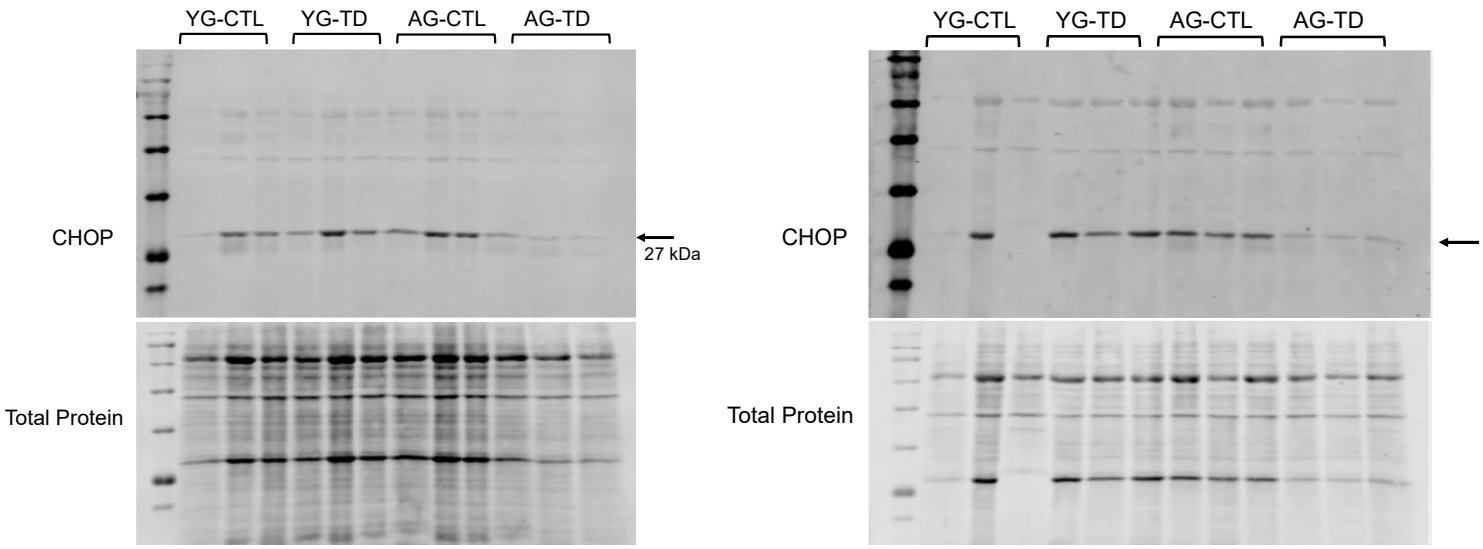
Supplemental Figure 4S6. Original Western blotting images and images of the total protein staining for NOX-4 protein expression in mesenteric arteries. Analyzed data is shown in Figure 1. YG-NP=Young non-pregnant; YG-PG=Young pregnant; AG-NP=Aged non-pregnant; AG-PG= Aged pregnant.



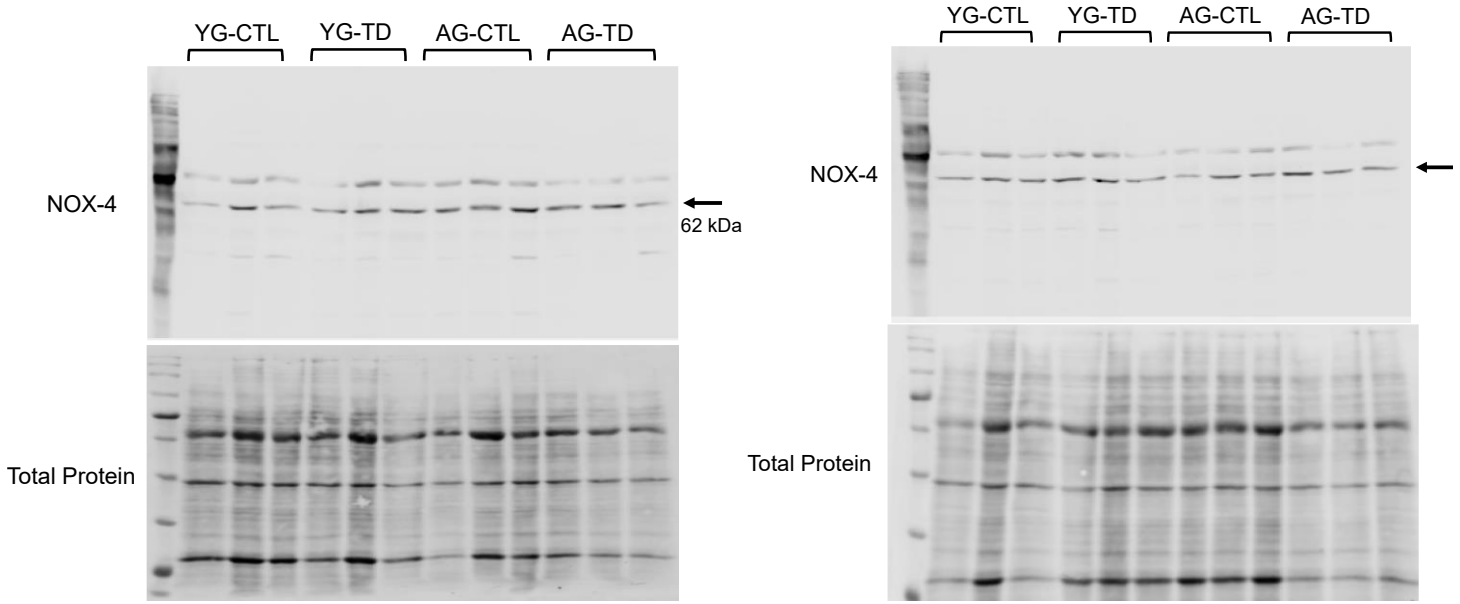
Supplemental Figure 4S7. Original Western blotting images and images of the total protein staining for GRP78 protein expression in mesenteric arteries. Analyzed data is shown in Figure 2. YG- CTL=Young control dams; YG-TD=Young TUDCA-treated dams; AG-CTL=Aged control dams; AG-TD=Aged TUDCA-treated dams.



Supplemental Figure 4S8. Original Western blotting images of Phospho-eIF2α and Total-eIF2α protein expression in mesenteric arteries. Analyzed data is shown in Figure 2. YG- CTL=Young control dams; YG-TD=Young TUDCA-treated dams; AG-CTL=Aged control dams; AG-TD=Aged TUDCA-treated dams.



Supplemental Figure 4S9. Original Western blotting images and images of the total protein staining for CHOP protein expression in mesenteric arteries. Analyzed data is shown in Figure 2. YG- CTL=Young control dams; YG-TD=Young TUDCA-treated dams; AG-CTL=Aged control dams; AG-TD=Aged TUDCA-treated dams.



Supplemental Figure 4S10. Original Western blotting images and images of the total protein staining for NOX-4 protein expression in mesenteric arteries. Analyzed data is shown in Figure 2. YG- CTL=Young control dams; YG-TD=Young TUDCA-treated dams; AG-CTL=Aged control dams; AG-TD=Aged TUDCA-treated dams.