Preeclampsia Link to Gestational Hypoxia

Wen Tong
Trinity College

This dissertation is submitted for the degree of
Doctor of Philosophy
August 2020
This thesis is dedicated to my parents

For their love, inspiration and encouragement
Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the acknowledgements and specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution.

I further state that no substantial part of my thesis has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar.

It does not exceed the prescribed word limit set by the Biology Degree Committee.

Wen Tong

31st August 2020
Preeclampsia Link to Gestational Hypoxia

Wen Tong

Pregnancy is a vulnerable period and complications can adversely affect mother and child. Pregnancy disorders, such as preeclampsia, contribute to significant morbidity and mortality worldwide, but underlying mechanisms remain uncertain, preventing effective diagnosis and treatment. Preeclampsia is a placental disorder originating from impaired spiral artery remodelling and resulting in increased placental vascular resistance, reduced uteroplacental perfusion, triggering placental hypoxia and oxidative stress. In turn, placental dysfunction is thought to be a common denominator between upstream adverse effects on the mother and downstream adverse effects on the fetus. Maternal adverse effects include the angiogenic imbalance that promotes maternal endothelial dysfunction, and adverse effects on the offspring include fetal growth restriction, which is not only a major driver of perinatal morbidity, but also increases cardiometabolic risk in later life. In this thesis, we used bespoke isobaric chambers to induce hypoxia in sheep for the last third of pregnancy and adopted an integrative approach, combining experiments in vivo with those at the cellular and subcellular levels to investigate whether we could recapitulate maternal, placental and fetal signatures associated with preeclampsia. The data reveal that hypoxic placentae showed increased oxidative stress, activation of the unfolded protein response, expansion of the endoplasmic reticulum, loss of cristae structure and size in the mitochondria, a shift in mitochondrial respiration away from fatty acid towards glucose metabolism, and increased expression of the anti-inflammatory cytokine tumour necrosis factor α and the anti-angiogenic factors soluble fms-like tyrosine kinase and soluble endoglin. Upstream adverse consequences on the hypoxic ewe included evidence of an angiogenic imbalance in maternal plasma, increased constrictor reactivity in isolated uterine and femoral arteries, impaired glomerular ultrafiltration, increased uterine artery pulsatility and a reduced ontogenic fall in uterine vascular resistance and in arterial blood pressure with advancing gestation. Downstream adverse consequences on the hypoxic fetus included growth restriction with evidence of brain sparing. Combined, therefore, the data show that chronic hypoxia during pregnancy provides a link between placental stress, fetal growth restriction and maternal cardiovascular dysfunction in adverse pregnancy, as in preeclampsia.
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>$\Delta P/\Delta t_{\text{max}}$</td>
<td>Maximum first derivate of left ventricular pressure change</td>
</tr>
<tr>
<td>$\Delta P/\Delta t_{\text{min}}$</td>
<td>Minimum first derivate of left ventricular pressure change</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>ABE</td>
<td>Acid-base excess</td>
</tr>
<tr>
<td>AC</td>
<td>Abdominal circumference</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AdipoQ</td>
<td>Adiponectin</td>
</tr>
<tr>
<td>AdipoR1</td>
<td>Adiponectin receptor 1</td>
</tr>
<tr>
<td>AdipoR2</td>
<td>Adiponectin receptor 2</td>
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<td>ADP</td>
<td>Adenine diphosphate</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>Ama</td>
<td>Antimycin A</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-dependent protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATF4</td>
<td>Activating transcription factor 4</td>
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<td>ATF5</td>
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<td>ATF6</td>
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</tr>
<tr>
<td>ATFS-1</td>
<td>Activating transcription factor associated with stress</td>
</tr>
<tr>
<td>Azd</td>
<td>Sodium azide</td>
</tr>
<tr>
<td>BIOPS</td>
<td>Biopsy preservation solution</td>
</tr>
<tr>
<td>BK$_{\text{Ca}}$</td>
<td>Large conductance calcium activated potassium channel</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BPD</td>
<td>Biparietal diameter</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBS</td>
<td>Cystathionine-$\beta$-synthase</td>
</tr>
<tr>
<td>CFR</td>
<td>Coronary flow rate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
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<td>Chem ROX</td>
<td>Non-mitochondrial residual oxygen consumption</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP homologous protein</td>
</tr>
<tr>
<td>ClpP</td>
<td>Caseinolytic protease proteolytic subunit</td>
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<tr>
<td>CO</td>
<td>Carbon monoxide</td>
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<tr>
<td>CORM-3</td>
<td>Carbon monoxide releasing molecule-3</td>
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<td>COX</td>
<td>Cyclo-oxygenase</td>
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<tr>
<td>CPT1</td>
<td>Carnitine palmitoyl transferase 1</td>
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<tr>
<td>CRL</td>
<td>Crown-rump length</td>
</tr>
<tr>
<td>CSE</td>
<td>Cystathionine-$\gamma$-lyase</td>
</tr>
<tr>
<td>dGA</td>
<td>Days gestational age</td>
</tr>
<tr>
<td>DNPH</td>
<td>2,4-dinitrophenyl-hydrazone</td>
</tr>
<tr>
<td>DRP1</td>
<td>Dynamin related protein 1</td>
</tr>
<tr>
<td>eIF2α</td>
<td>Eukaryotic initiation factor 2α</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ET</td>
<td>Uncoupled electron transport respiration (coupling control state)</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>F-pathway</td>
<td>β-oxidation pathway control state</td>
</tr>
<tr>
<td>FCCP</td>
<td>Carbonyl cyanide-4-((trifluoromethoxy) phenylhydrazone</td>
</tr>
<tr>
<td>FCR</td>
<td>Flux control rate</td>
</tr>
<tr>
<td>FGR</td>
<td>Fetal growth restriction</td>
</tr>
<tr>
<td>G6PDH</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>GLUT1</td>
<td>Glucose transporter 1</td>
</tr>
<tr>
<td>GLUT3</td>
<td>Glucose transporter 3</td>
</tr>
<tr>
<td>GMp</td>
<td>OXPHOS dependent on β-oxidation and complex I electron flux</td>
</tr>
<tr>
<td>GMS_E</td>
<td>Maximum uncoupled electron transport capacity</td>
</tr>
<tr>
<td>GMS_P</td>
<td>Maximum OXPHOS capacity</td>
</tr>
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<td>GRP78</td>
<td>Glucose-related protein 78</td>
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<tr>
<td>H2S</td>
<td>Hydrogen sulphide</td>
</tr>
<tr>
<td>HELLP syndrome</td>
<td>Haemolysis, elevated liver enzymes, low platelets</td>
</tr>
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<td>HIF1α</td>
<td>Hypoxia inducible factor 1α</td>
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<td>HO-1</td>
<td>Haem-oxygenase-1</td>
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<td>HR</td>
<td>Heart rate</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>Heat shock protein 60</td>
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<td>Heat shock protein 70</td>
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<tr>
<td>i.m.</td>
<td>Intramuscularly</td>
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<tr>
<td>i.v.</td>
<td>Intravenously</td>
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<td>IGF1</td>
<td>Insulin-like growth factor 1</td>
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<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<td>IRE1</td>
<td>Inositol-requiring enzyme 1</td>
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<tr>
<td>JNK</td>
<td>C-Jun N-terminal kinase</td>
</tr>
<tr>
<td>K_ATP channel</td>
<td>ATP-sensitive potassium channel</td>
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<tr>
<td>L-NAME</td>
<td>N(G)-Nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LEAK</td>
<td>Leak respiration (coupling control state)</td>
</tr>
<tr>
<td>LVDP</td>
<td>Left ventricular developed pressure</td>
</tr>
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<td>LVEDP</td>
<td>Left ventricular end diastolic pressure</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LVSP</td>
<td>Left ventricular systolic pressure</td>
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<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<td>MFN1</td>
<td>Mitofusin 1</td>
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<td>MFN2</td>
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<td>MiR05</td>
<td>Mitochondrial respiration medium 5</td>
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<tr>
<td>N-pathway</td>
<td>NADH electron transport-pathway control state</td>
</tr>
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<td>NAD</td>
<td>Nicotinamide adenine dinucleotide (oxidised)</td>
</tr>
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<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced)</td>
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<td>NaHS</td>
<td>Sodium hydrogen sulphide</td>
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<td>Norepinephrine</td>
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<td>NO</td>
<td>Nitric oxide</td>
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<td>OctM&lt;sub&gt;L&lt;/sub&gt;</td>
<td>Leak respiration</td>
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<td>OctM&lt;sub&gt;P&lt;/sub&gt;</td>
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<td>OPA1</td>
<td>Optic atrophy protein 1</td>
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<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
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<tr>
<td>P&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Oxygen tension, at which haemoglobin is 50% saturated</td>
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<td>pACC</td>
<td>Phosphorylated acetyl-CoA carboxylase</td>
</tr>
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<td>P&lt;sub&gt;A&lt;/sub&gt;CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Arterial partial pressure of carbon dioxide</td>
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<td>DL-propargylglycine</td>
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<td>pAKT</td>
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<td>Phosphorylated AMP-activated protein kinase</td>
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<td>P&lt;sub&gt;a&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>Pyruvate dehydrogenase</td>
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<td>Protein disulphide isomerase</td>
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<td>3-phosphoinositide-dependent protein kinase 1</td>
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<td>pERK</td>
<td>Phosphorylated extracellular signal-regulated kinase</td>
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<td>Protein kinase RNA-like ER kinase</td>
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<td>Placental growth factor</td>
</tr>
<tr>
<td>PM&lt;sub&gt;p&lt;/sub&gt;</td>
<td>OXPHOS dependent on β-oxidation and pyruvate</td>
</tr>
<tr>
<td>PO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor γ</td>
</tr>
<tr>
<td>pPDK1</td>
<td>Phosphorylated phosphoinositide-dependent protein kinase 1</td>
</tr>
<tr>
<td>r.p.m.</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>ROX</td>
<td>Residual oxygen consumption</td>
</tr>
<tr>
<td>RPL19</td>
<td>Ribosomal protein L19</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RUPP</td>
<td>Reduction of uteroplacental perfusion pressure</td>
</tr>
<tr>
<td>S-pathway</td>
<td>Succinate pathway control state</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>Description</td>
</tr>
<tr>
<td>---------------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>s.c.</strong> Subcutaneously</td>
<td>TGFβ Transforming growth factor β</td>
</tr>
<tr>
<td><strong>S.E.M.</strong> Standard error of the mean</td>
<td>TID1 Tumorous imaginal disc 1</td>
</tr>
<tr>
<td><strong>Sat Hb</strong> Percentage saturation of haemoglobin</td>
<td>TmE Uncoupled electron transport capacity dependent on complex IV</td>
</tr>
<tr>
<td><strong>SCR</strong> Substrate control rate</td>
<td>TNFα Tumour necrosis factor α</td>
</tr>
<tr>
<td><strong>S_E</strong> Uncoupled electron transport capacity</td>
<td>TXA2 Thromboxane A2</td>
</tr>
<tr>
<td><strong>sEng</strong> Soluble endoglin</td>
<td>Tau Left ventricular relaxation rate constant</td>
</tr>
<tr>
<td><strong>sFlt-1</strong> Fms-like tyrosine kinase-1</td>
<td>UPR Unfolded protein response</td>
</tr>
<tr>
<td><strong>SNP</strong> Sodium nitroprusside</td>
<td>UPR&lt;sub&gt;Cyt&lt;/sub&gt; Cytosolic unfolded protein response</td>
</tr>
<tr>
<td><strong>SUIT protocol</strong> Substrate-uncoupler-inhibitor</td>
<td>UPR&lt;sub&gt;ER&lt;/sub&gt; Endoplasmic reticulum unfolded protein response</td>
</tr>
<tr>
<td><strong>TBS</strong> Tris-buffered saline</td>
<td>UPR&lt;sub&gt;mt&lt;/sub&gt; Mitochondrial unfolded protein response</td>
</tr>
<tr>
<td><strong>TBS-T</strong> TBS containing 0.1% Tween 20</td>
<td>VEGF Vascular endothelial growth factor</td>
</tr>
<tr>
<td><strong>TBS-TT</strong> TBS containing 0.1% Tween 20 and</td>
<td>XBP1 X-box binding protein 1</td>
</tr>
<tr>
<td>0.1% Triton X-100</td>
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1. **General introduction**

1.1 **The burden of compromised pregnancy**

Pregnancy is a sensitive period for the mother and her unborn child, and complications can jeopardise both short and long-term maternal and fetal health. It is estimated that over 800 women die of preventable conditions related to pregnancy and childbirth every day, causing more than 6 million perinatal deaths each year (Say et al., 2014; Alkema et al., 2016). Fetal development *in utero* is vulnerable to perturbations by environmental stressors, and intrauterine complications may prompt the activation of adaptive mechanisms to protect the developing fetus at the expense of increasing risk of disease in later life (Gluckman et al., 2008; Eiríksdóttir et al., 2013; Usynina et al., 2016). Thus, adverse conditions *in utero* are not only major drivers of fetal and neonatal, but also of adolescent and adult morbidity and mortality, increasing the risk of developing a plethora of cardiometabolic diseases in later life (Gillman, 1995; Bernstein et al., 2000; Gluckman et al., 2008). While disorders of pregnancy present a substantial financial and scientific burden on public health worldwide, there is a lack of understanding of the underlying pathoaeiology, leading to a shortfall in effective prognostic, diagnostic and treatment options for some disorders, such as for pre-eclampsia (Hodgins, 2015). This is in part due to the multi-factorial nature of these syndromes, and in part due to the plethora of ethnic, social and economic confounding factors involved, calling for the development of improved animal models, in which to carry out more targeted research to isolate mechanisms, and thereby design potential therapeutic intervention (Ngan Kee, 2005; Say et al., 2014; Hodgins, 2015). In this thesis I will focus on chronic hypoxia in the last third of gestation in sheep as a preclinical model that recapitulates many pathophysiological outcomes of complicated pregnancy in humans, including preeclampsia.
1.1.1 Preeclampsia

Preeclampsia is a prevalent obstetrical disorder, affecting ca. 3-5% of pregnancies worldwide (Di Renzo, 2009). The clinical diagnosis currently endorsed by professional bodies recognises preeclampsia as new-onset maternal hypertension after 20 weeks of gestation accompanied by one or more symptoms, such as utero-placental dysfunction, proteinuria and/or fetal growth restriction (FGR; Brown et al., 2018). In addition, preeclampsia presents with thrombocytopenia, impaired liver function, renal insufficiency, pulmonary oedema and cerebral or visual impairment (American College of Obstetricians and Gynecologists, 2013). Maternal complications range from mild organ microangiopathy to seizures, strokes, renal failure, HELLP syndrome (Haemolysis, Elevated Liver enzymes and Low Platelets) and death (Sibai et al., 2005; Young et al., 2010). Offspring complications include preterm birth, FGR, developmental defects and increased risk of cardiovascular disease in later life (Poston, 1997; Lang et al., 2003; Gaillard et al., 2013).

Historically, preeclampsia was regarded to develop early in pregnancy due to a failure of spiral artery conversion, resulting in reduced uteroplacental perfusion (Steegers et al., 2010; Young et al., 2010). However, our understanding of the disease has grown and it is now accepted that preeclampsia encompasses a broader range of disorders, including late-onset preeclampsia, which is associated with normal uterine spiral artery conversion and low incidence of FGR (Huppertz, 2008; Burton et al., 2019). While the pathogenesis and classification of preeclampsia is widely debated, it is recognised that preeclampsia is a placenta-derived disease that presents with reduced uteroplacental perfusion that promotes placental hypoxia and placental oxidative stress (Steegers et al., 2010; Young et al., 2010). This is associated with placental
dysfunction and the release of pro-inflammatory cytokines, syncytiotrophoblast debris and anti-angiogenic factors into the maternal bloodstream (Benyo et al., 2001; Hung et al., 2004; Levine et al., 2004; Redman and Sargent, 2008; Maynard and Karumanchi, 2011; Tong and Giussani, 2019). These processes contribute to systemic cardiovascular and endothelial dysfunction due to the release of anti-angiogenic factors, such as soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng). This prohibits normal endothelial homeostasis by inhibiting vascular endothelial growth factor (VEGF), placental growth factor (PIGF) and transforming growth factor β (TGF-β) signalling (Venkatesha et al., 2006; Shibuya, 2012). This angiogenic imbalance contributes to the development of maternal cardiac afterload, hypertension, proteinuria and the increase in the uterine artery pulsatility index and cardiac dysfunction that occur in preeclampsia (VanWijk et al., 2000; Levine et al., 2004).

Despite great advances in the understanding of preeclampsia, the management of preeclampsia is currently limited to the management of maternal hypertension and the prevention of maternal seizures with magnesium sulphate (Cindrova-Davies, 2014). In most cases, delivering the placenta and the fetus preterm is the only effective treatment to ease maternal symptoms and to prevent further FGR (Townsend et al., 2016). Preeclampsia accounts for ca. 20% of all induced preterm births and, in combination with an increased risk of FGR, is associated with higher incidences of neonatal death, cerebral palsy and pulmonary disorders (Goldenberg and Rouse, 1998; Challis et al., 2001). Progress in research enhancing the understanding of preeclampsia has been hindered by limitations imposed by experimental settings and animal models (Szalai et al., 2015; Aouache et al., 2018). For instance, the natural experiment of high altitude pregnancy has been consistently linked with an increased
prevalence of preeclampsia and can mimic the effects of hypoxic stress \textit{in vivo} (Moore et al., 1982; Mahfouz et al., 1994; Palmer et al., 1999; Keyes et al., 2003). However, high altitude populations are heterogeneous and riddled with confounding factors, such as differences in socio-economic status, highland ancestry or underlying pathologies (Zamudio, 2007; Soria et al., 2013). On the other hand, \textit{in vitro} models are often not translational enough to apply to experimental animals or a human clinical setting, and artificial hypoxic stimuli \textit{ex vivo} may not mimic levels of oxygen deprivation \textit{in vivo}. Further, murine models of preeclampsia have been used, but they are problematic. When investigating the \textit{in vivo} interaction between mother, placenta and offspring, the maternal metabolic demand, the temporal profile of physiological development and access to longitudinal cardiovascular and endocrine measures are three important considerations. Rats and mice are litter bearing, and young are born highly immature (Marshall et al., 2018). In addition, rodent preclinical models do not permit longitudinal assessment of uterine blood flow via surgically implanted flow probes or serial long-term sampling of blood for endocrinology due to the limited blood volume.
1.2 Gestational hypoxia

Intrauterine hypoxia refers to a deficiency in oxygen supply to fetal, maternal or placental tissues and is one of the most common challenges in obstetric clinical practice (Fajersztajn and Veras, 2017). Early-onset and late-onset hypoxia often create markedly different outcomes, both in terms of placental function and in terms of fetal development. Thus, it is vital to consider not only the degree, but also the timing and duration of hypoxic exposure when discussing the effects of gestational hypoxia on pregnancy. Due to the importance of local oxygen tension in determining the onset of spiral artery conversion and the development of the haemochorial uteroplacental circulation at the end of the first trimester, trophoblast proliferation and placentation are highly vulnerable to disturbances in oxygenation, adversely affecting the morphological and functional maturation of the uteroplacental vascular bed (Genbacev et al., 1996; Kingdom and Kaufmann, 1997; Thompson et al., 2016). This has been linked to placental oxidative stress, reduced placental weight, FGR and defects in fetal cardiovascular and neurological development (Sharma et al., 2006; Ream et al., 2008; Tintu et al., 2009; Hauton, 2012; Phillips et al., 2017). On the other hand, many models of early-onset gestational hypoxia show experimental evidence of placental adaptations in response to adverse intrauterine oxygenation, salvaging at least part of the downstream consequences of fetoplacental hypoxia. These include endocrine adaptations, increased placental weight and increased placental vascularisation with greater capillary surface area for oxygen and nutrient exchange (Bacon et al., 1984; Higgins et al., 2016; Matheson et al., 2016; Sferruzzi-Perri and Camm, 2016). Thus, depending on the severity and duration of the hypoxic insult, early-onset gestational hypoxia may not necessarily lead to noticeable FGR (Higgins et al., 2016; Nuzzo et al., 2018). These observations are consistent with studies by our own laboratory, in
which we found that late-onset hypoxia for the last third of gestation leads to significant FGR, while early-onset hypoxic pregnancy leads to an adaptive increase in placental weight, cushioning adverse effects on fetal development and maintaining birth weight (Camm et al., 2010; Nuzzo et al., 2018). It is also important to note that the first ten weeks of intrauterine development in humans occurs under relatively hypoxic conditions before the haemochorial placenta is fully established. Cytotrophoblast cells are insensitive to hypoxic exposure before seven weeks of gestation with no effects on the placental invasion profile, at least *in vitro* (Rodesch et al., 1992; Genbacev et al., 1996). Hence, in human pregnancy, gestational hypoxia in the very first weeks of pregnancy may not adversely affect fetal organogenesis and development. On the contrary, premature onset of intervillous blood flow may disrupt the hypoxic *in utero* environment and promote placental oxidative stress, disrupting cellular differentiation and fetoplacental development (Watson et al., 1998; Jauniaux et al., 2000; Jauniaux et al., 2003; Hutter et al., 2010).

The degree and duration of hypoxic exposure used in the present thesis is based on a sheep model previously validated by our own laboratory, which induces a chronic and significant reduction in oxygenation to a degree beyond the level achieved by habitable high altitude (Brain et al., 2015). In this work, pregnant ewes were exposed to ca. 10% inspired fraction of oxygen for the last third of gestation from 105 to 138 dGA, while allowing concurrent measurement of maternal and fetal blood gases (Brain et al., 2015). Chronic hypoxia of this magnitude and duration induced a clinically significant level of fetal $P_{a}O_{2}$ and FGR independent of changes to maternal food intake or maternal and fetal stress hormones (Nicolaides et al., 1986; Soothill et al., 1986; Hecher et al., 1995; Brain et al., 2015). This model also recapitulated the profound fetal cardiomyopathy and endothelial dysfunction that is observed in offspring from
high-risk pregnancies in humans (Veille et al., 1993; Rizzo et al., 1995; Miyague et al., 1997; Mayhew et al., 1999; Brain et al., 2015; Brain et al., 2019). These changes persisted into adulthood and led to the development of hypertension by enhancing vasoconstrictor reactivity and impairing NO-dependent vasorelaxation (Brain et al., 2019). The programming effects of intrauterine exposure to hypoxia could be offset by maternal treatment with both Vitamin C and the mitochondrial antioxidant MitoQ (Brain et al., 2019; Botting et al., 2020). This work was performed in conjunction with studies on the chick embryo, which allowed the isolation of effects independent of maternal and placental influences (Giussani et al., 2007; Itani et al., 2018; Botting et al., 2020; Skeffington et al., 2020). Thus, while the effects of intrauterine hypoxia on the fetus in late gestation have been extensively investigated in our laboratory, maternal and placental aspects of gestational hypoxia have been relatively neglected. The work presented in this thesis therefore provides the first systematic approach to analyse maternal and placental cardiovascular and molecular changes in a sheep model that induces gestational hypoxia of appropriate severity, onset and duration to recapitulate clinically relevant fetal compromise.

### 1.2.1 Effects of gestational hypoxia on the fetus

Fetal hypoxia during gestation has been the focus of intensive scientific interest, and its effects on fetal growth and development have been extensively studied. Chronic fetal hypoxia can occur in a wide range of pregnancy complications, including preeclampsia, placental insufficiency, gestational diabetes, and in pregnancies at high altitude (Fajersztajn and Veras, 2017). For instance, high altitude pregnancy in Colorado was associated with decreased birth weight, independent of confounding factors, such as gestational length and socioeconomic status (Jensen and Moore,
It is generally accepted that birth weight decreases on average by ca. 100g for every 1,000 m above sea level, while this effect is attenuated in multigenerational inhabitants of high altitude, such as Tibetans and Andeans (Giussani et al., 2001; Moore et al., 2011; Soria et al., 2013). Both pregnancy at high altitude and experimental hypoxia disrupt transplacental nutrient and oxygen transfer and placental hormone synthesis, associated with asymmetric FGR and programming of cardiovascular disease in later life (Barker and Clark, 1997; Barker et al., 2002; Giussani and Davidge, 2013; Jang et al., 2015; Dimasuay et al., 2016). In addition, placental stress under conditions of hypoxia may cause oxidative stress to the fetus, such as protein or nucleic acid oxidation, protein synthesis inhibition or cell death, thus exacerbating FGR and worsening pregnancy outcome (Longini et al., 2007; Yung et al., 2012; Perrone et al., 2016). Despite in depth understanding of the causes and consequences of FGR, clinical practice is still lacking effective prevention and treatment against FGR. It is thus important to increase our understanding of the maternal and placental adaptations to gestational hypoxia, and how they may affect fetal development.

**1.2.2 Effects of gestational hypoxia on the placenta and the mother**

Hypoxic signalling at high altitude is associated with placental dysfunction and placental insufficiency, which is linked to the increased incidence of preeclampsia (Moore et al., 1982; Reshetnikova et al., 1996; Keyes et al., 2003). Despite these findings, the suitability of maternal systemic hypoxia induced by sustained reductions in the maternal inspired fraction of oxygen as a model to induce placental hypoxia and placental insufficiency has been questioned. This is, because maternal compensatory cardiorespiratory responses may be able to buffer the effects of systemic hypoxia on
placental oxygenation. However, both human studies and animal models of maternal hypoxia have confirmed that exposure to chronic hypoxia can lead to changes in placental structure and function, with an increase in uteroplacental vascular resistance and uteroplacental hypoxia (Zamudio et al., 2007b; Chang et al., 2009; Aljunaidy et al., 2016; Turan et al., 2017; Nuzzo et al., 2018). For instance, chronic maternal hypoxia alters proliferation patterns of the uteroplacental vasculature both in vivo and in vitro, and is associated with changes in the placental vascular phenotype (Rockwell et al., 2000; Mateev et al., 2003; Rockwell et al., 2006). This may underlie the diminished dilator and enhanced constrictor reactivity measured in the uteroplacental vascular bed of the hypoxic mother (Palmer et al., 1999; Postigo et al., 2009). Such changes will oppose the physiological increase in uteroplacental perfusion that occurs in healthy pregnancy, further compromising oxygen delivery to the hypoxic fetoplacental unit and promoting placental oxidative stress, thus triggering a vicious cycle (Mateev et al., 2003; Thaete et al., 2004; Julian et al., 2008; Zhou et al., 2013; Herrera et al., 2014; Matheson et al., 2016; Turan et al., 2017). These complications closely resemble the placental signature in preeclampsia in terms of placental molecular and vascular characteristics, and in terms of fetal and maternal pregnancy outcome (Zamudio, 2007; Zamudio et al., 2007b).

Adverse effects of gestational hypoxia on the placenta are closely linked to adverse upstream effects on maternal health (Zhou et al., 2013; Aljunaidy et al., 2016; Thompson et al., 2016). Healthy human pregnancy involves several maternal respiratory and cardiovascular adaptations to optimise pregnancy outcome of the growing fetus. For instance, by the third trimester, pregnant mothers show a significant increase in maternal ventilation, plasma volume and cardiac output (Palmer et al., 1992; Soma-Pillay et al., 2016). This is coupled with a decrease in maternal arterial
blood pressure, which follows a fall in uteroplacental and thus systemic vascular resistance (Palmer et al., 1992; van Oppen et al., 1996). This allows for the increase in uterine artery blood flow from 20 to 50 ml.min\(^{-1}\) in non-pregnant women to 450 to 800 ml.min\(^{-1}\) during pregnancy (Palmer et al., 1992; van Oppen et al., 1996). Vascular function relies on the complex interaction between an exhaustive list of angiogenic and vasoactive factors, cytokines and growth factors, and deregulation of vascular signalling often simultaneously affects the uteroplacental and the maternal systemic circulation, such as those involving oxidative stress and gasotransmitters (Vural, 2002; Wang et al., 2013; Xiao et al., 2013; Zhou et al., 2013; Al-Magableh et al., 2014). Any disruption of this intricate balance of circulating factors by the stressed placenta, as occurs in gestational hypoxia, may promote widespread endothelial dysfunction and vascular inflammation with detrimental effects on the maternal vasculature (Gilbert et al., 2008; Tissot van Patot et al., 2012; Shah and Khalil, 2015).

1.2.3 Signalling pathways involved in gestational hypoxia

1.2.3.1 Hypoxia and oxidative stress

Regulation of the balance between production of reactive oxygen species (ROS) and antioxidant capacity is vital for cellular signalling and tissue homeostasis. ROS are messengers in a multitude of signalling pathways, including initiation of immune responses, antioxidant signalling, initiation of DNA or protein repair responses and modulation of cellular survival and proliferation (Redza-Dutordoir and Averill-Bates, 2016; Li et al., 2017). Ultimately, the outcome of ROS signalling depends on its degree and duration, as well as on the co-activation of parallel signalling pathways. Under normal conditions, pro-oxidant generation of ROS by xanthine oxidase or uncoupled endothelial NO synthase (eNOS) is antagonised by continuous degradation of ROS.
General introduction

by antioxidant enzymes, such as superoxide dismutase, catalase or glutathione peroxidase, or by continuous free radical scavenging via antioxidant molecules, such as vitamin C, melatonin and hydrogen sulphide (H$_2$S; Valko et al., 2007). While ROS are vital second messengers required for modulation of redox-sensitive signalling pathways, including autophagy, cellular differentiation and inflammatory responses, excess generation of ROS or a decrease in cellular antioxidant capacity can lead to oxidative damage of lipids, proteins and nucleic acids (Valko et al., 2007; Zhang et al., 2016a). Hypoxia is a potent stimulus for the generation of ROS and downregulates antioxidant capacity in many tissues, including the placenta (Turrens, 2003; Berry and Hare, 2004; Martin et al., 2005; Dosek et al., 2007; Schoots et al., 2018). Hypoxia-induced oxidative stress is a potent trigger for oxidative protein damage, protein unfolding, fragmentation and aggregation, resulting in endoplasmic reticulum (ER) stress and activation of the unfolded protein response (UPR; Davies, 2005; Kamata et al. 2005; Malhotra and Kaufmann, 2007). This has widespread effects, including protein synthesis inhibition and activation of mitogen activated protein kinases (MAPKs), impairing trophoblast survival and proliferation. Measures of umbilical venoarterial differences in isoprostane, a measure of tissue peroxidation and oxidative stress, showed that the placenta is a major source of isoprostanes during pregnancy, which is elevated in the presence of placental abnormalities, contributing to the pathogenesis of preeclampsia (Chappell et al., 2002; Braekke et al., 2006). Placental oxidative stress as a function of impaired spiral artery conversion is now considered a key event in the development of preeclampsia (Redman and Sargent, 2000; Burton and Jauniaux, 2004). The resulting increase in uteroplacental resistance and placental stress response triggers the release of many factors into the maternal circulation that work in concert to induce a systemic inflammatory response and endothelial
dysfunction. Growing evidence points towards a role of oxidative stress and ROS in the regulation of placental and systemic vascular resistance. For example, increased ROS production by NADPH oxidase 2 in an ovine model of gestational hypoxia was responsible for increasing uterine artery myogenic tone, which was prevented by inhibiting NADPH oxidase 2 using apomycin (Xiao et al., 2013). In addition, the interaction between ROS and nitric oxide (NO) in the vascular endothelium has a vital role in vascular homeostasis, as discussed in detail in section 1.2.3.5. Thus, placental oxidative stress is another contributing factor to the increased prevalence of FGR and pregnancy complications at high altitude, which has prompted extensive studies into the use of antioxidant therapies in the treatment of gestational disorders, including preeclampsia (Poston et al., 2006; Rumbold et al., 2006; Villar et al., 2009; McCance et al., 2010; Roberts et al., 2010; Xu et al., 2010). However, the most recent metaanalyses on antioxidant supplementation in pregnancy, such as with vitamins C and E, have been unable to detect any beneficial effects on the relative risk of preeclampsia, FGR and preterm birth (Rumbold et al., 2008; Conde-Agudelo et al., 2011; Rumbold et al., 2015; Oh et al., 2020).

1.2.3.2 Hypoxia and endoplasmic reticulum stress
The ER is a cellular organelle composed of a network of membranous tubules, responsible for the synthesis, folding, post-translational modification and secretion of proteins. Disturbances to cellular protein homeostasis can occur under conditions of hypoxia, cellular energy depletion or excess free radical generation by causing oxidative protein damage or impairing the formation of high energy disulphide bonds (Berlett and Stadtman, 1997; Koumenis et al., 2007; Chipurupalli et al., 2019). The unfolding or misfolding of amino acid chains and the aggregation of misfolded proteins triggers the activation of a homeostatic endoplasmic reticulum UPR (UPR\textsuperscript{ER}), which is
conserved across many tissues and species, and governs a variety of signalling pathways in the ER, cytosol and the mitochondria (Schröder and Kaufman, 2005; Buchberger et al., 2010; Jovaisaite et al., 2014). Some of the downstream effects include protein synthesis inhibition, the expression of redox modulators, the increase in cellular folding capacity through expression of protein chaperones, the upregulation of protein degradation pathways and the modulation of angiogenesis- or apoptosis-related factors (Walter and Ron, 2011; Bouvier et al., 2012; Hetz, 2012; Ramnarayanan et al., 2016). UPR signalling is initiated by the ER transmembrane sensors protein kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1; Xu et al., 2005; Hetz, 2012). These sensors are normally kept inert by binding to the ER chaperone glucose-related protein 78 (GRP78). Under conditions of ER stress, GRP78 is sequestered to bind and stabilise unfolded or misfolded proteins that accumulate in the ER, and cleavage of ATF6 produces an active transcription factor, while IRE1 cleaves the X-box binding protein 1 (XBP1) mRNA to also become a transcription actor (Hetz, 2012). PERK phosphorylates the eukaryotic initiation factor 2α (eIF2α), which inhibits global protein translation while promoting expression of the activating transcription factor 4 (ATF4; Hetz, 2012). ATF6, XBP1 and ATF4 initiate separate but overlapping transcriptional responses, including the expression of chaperone proteins, antioxidant enzymes and cellular protein degradation machinery (Xu et al., 2005; Hetz, 2012). ER stress is strongly implicated in complicated pregnancies, including the development of FGR at high altitude and preeclampsia (Yung et al., 2007; Yung et al., 2012; Yung et al., 2014; Mizuuchi et al., 2016). While investigations into the use of ER chaperones as potential therapeutic intervention against complicated pregnancies have been limited, the preliminary evidence is promising. For example, treatment of placental explants...
undergoing hypoxia-reoxygenation with the ER chaperone TUDCA reduced levels of both endothelin-1 (ET-1) and sFlt-1 in these explants (Cindrova-Davies, 2014).

1.2.3.3 Hypoxia and mitochondrial stress

Placental mitochondria are vital in the regulation of placentation, placental energy metabolism and transplacental nutrient and oxygen delivery during pregnancy (Song et al., 2018). With advancing gestation, trophoblast cells increasingly rely on mitochondrial oxidative phosphorylation (OXPHOS) as oxygen tension rises with spiral artery remodelling, and as the energy demands of the growing fetus increase (Xie et al., 2014a; Matheson et al., 2016). Chronic hypoxia inhibits normal redox-sensitive mitochondrial respiration by reducing oxygen availability and disrupting electron flux along the electron transport chain (ETC), while increasing mitochondrial ROS production (Lushchak, 2014; Angelova and Abramov, 2016). Conceptually similar to the activation of the UPR$_{ER}$, accumulation of unfolded and misfolded proteins in the mitochondria in response to oxidative stress is potentially detrimental to mitochondrial and cellular function, and the mitochondrial UPR (UPR$_{mt}$) exists as a homeostatic mechanism to restore mitochondrial proteostasis (Hamanaka and Chandel, 2010). Compared to the UPR$_{ER}$, the UPR$_{mt}$ is relatively poorly investigated (Jovaisaite et al., 2014). In the canonical pathway, it is thought to be activated by the ClpP-mediated cleavage of damaged proteins into short peptides, which activate the UPR$_{mt}$ signal activator activating transcription factor (ATF5). ATF5 acts to increase the mitochondrial protein folding capacity, for example by upregulating mitochondrial chaperones and co-chaperones, such as heat shock protein 60 (HSP60), glucose-related protein 75 (GRP75) and tumorous imaginal disc 1 (TID1; Neupert and Hermann, 2007; Chacinska et al., 2009). Extensive crosstalk exists between the UPR$_{ER}$ and UPR$_{mt}$, and activation of one pathway in a cell is likely to trigger the other.
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(Senft and Ronai, 2015; Yung et al., 2019). For example, activation of C/EBP homologous protein (CHOP) downstream of ATF4 signalling can induce the expression of ATF5, HSP60, TID1 and ClpP, while eIF2α can regulate ClpP levels independently of ATF4 (Zhao et al., 2002; Teske et al., 2013; Yung et al., 2019). Thus, due to the functional interdependence of the ER and mitochondria, these two organelles are likely to be affected synergistically by adverse conditions, such as oxygen deprivation and energy depletion (Burton et al., 2017b). It is known that the high metabolic and synthetic activities of the trophoblast make it vulnerable to oxidative and ER stress. Recently, increasing research has focussed on the role of mitochondrial ROS production, placental mitochondrial stress and activation of the UPR\textsuperscript{mt} in trophoblast function and in disorders of pregnancy (Steegers et al., 2010). Placental hypoxia and hypoxia-reperfusion is associated with oxidative stress and mitochondrial dysfunction, which contribute to the development of FGR and preeclampsia through alterations in placental respiration and energy metabolism that are not clearly understood (Turrens, 2003; Myatt, 2006; Murray, 2012; Langston-Cox et al., 2020). It is known that placental hypoxia can trigger adaptive responses through remodelling of the ETC and activity of the citric acid cycle, ultimately affecting the efficiency of ATP synthesis, fusion and fission dynamics, biogenesis and protein expression (Aljunaidy et al., 2017; Fuhrmann and Brüne, 2017; Song et al., 2018). However, the source of mitochondrial ROS production, and which complexes are affected by hypoxia and ROS-mediated damage, has been widely debated (Dröse, 2013; Bleier et al., 2015; Vinogradov and Grivennikova, 2016; Fuhrmann and Brüne, 2017). In animal models of gestational hypoxia, placental mitochondria showed a reduction in complex-specific enzyme activity and protein levels independent of changes in mitochondrial density (Myatt et al., 2014; Song et al., 2018). These
changes became more apparent with advancing gestation and prolonged hypoxia, and were associated with an increase in hypoxia-induced nitration of tyrosine moieties by peroxynitrite and the formation of nitrotyrosine, which may impair enzyme activity and OXPHOS, decrease ATP production and further increase ROS generation (Song et al., 2018).

Mitochondrial hypoxia and oxidative stress can also affect mitochondrial morphology by altering fusion and fission dynamics (Twig et al., 2008; McCarthy and Kenny, 2016; Plecitá-Hlavatá and Ježek, 2016). The formation of mitochondrial tubular networks is mediated by mitofusins 1 and 2 (MFN1 and MFN2) and optic atrophy protein 1 (OPA1). The coupling of functional mitochondrial proteins and DNA is vital for mitochondrial health, and impaired fusion under conditions of hypoxia is associated with reduction in OXPHOS, mitochondrial mutations, and mitophagy (Knott and Bossy-Wetzel, 2008; Chen et al., 2010; Fuhrmann and Brüne, 2017). Increased mitochondrial fission mediated by dynamin related protein 1 (DRP1) is increased under conditions of hypoxia and seems to underlie disruption in mitochondrial cristae structures, triggering cytochrome c release and hypoxia-induced cell death (Lackner and Nunnari, 2009; Wu et al., 2016; Parra et al., 2017; Zhang et al., 2018).

There is increasing evidence to suggest that mitochondrial dysfunction and ROS production contribute to the placental stress observed in preeclampsia (Wang and Walsh, 1998; D'Souza et al., 2016). This is reflected in alterations of the placental mitochondrial metabolome in severe preeclampia, along with reduced ATP production, dyslipidaemia and reductions in β-oxidation (Bartha et al., 2012; Shi et al., 2013; El Khouly et al., 2016). Placental mitochondria from preeclamptic pregnancies also present with impaired mitochondrial fusion and biogenesis, and increased
mitochondrial fission and autophagy (Padmini et al., 2009; Yu et al., 2016; Zhou et al., 2017). These observations highlight the potential importance of mitochondrial stress in mediating hypoxia-induced dysfunction in the pathogenesis of preeclampsia.

1.2.3.4 **Hypoxia and HIF1α signalling**

Hypoxia inducible factors are a family of evolutionarily conserved oxygen-regulated transcription factors that mediate key processes during the cellular homeostatic response to hypoxia (Semenza and Wang, 1992; Wang et al., 1995). Under normoxic conditions, HIF1α is continually hydroxylated by prolyl hydroxylases and marked for proteasomal degradation through ubiquitination by the von Hippel-Lindau tumour suppressor (Maxwell et al., 1999). Under conditions of oxygen deprivation, the loss of function of prolyl hydroxylases rapidly stabilises HIF1α, which translocates to the nucleus and binds to hypoxia response elements, promoting the expression of HIF-regulated genes to modulate metabolic homeostasis and determine cell fate (Madan and Curtin, 1993; Firth et al., 1994; Ivan et al., 2001; Schofield and Ratcliffe, 2004). In addition, independent of hypoxia, HIF1α levels can be increased downstream of NF-κB signalling and by the presence of inflammatory cytokines, growth factors and mitochondrial ROS (Jung et al., 2003; Bárdos et al., 2004; Zhou and Brüne, 2006; Brüne and Zhou, 2007; Fuhrmann and Brüne, 2017). HIF1α and expression of HIF1α-regulated genes, such as VEGF-A and TGFβ, negatively correlate with placental efficiency and are associated with an increase in adverse clinical outcomes of gestational hypoxia at high altitude (Zamudio et al., 2007b). HIF1α also provides a powerful stimulus for sFlt-1 expression, and HIF1α-dependent sFlt-1 secretion can be induced experimentally in placental explants in vitro by hypoxia and oxidative stress (Nagamatsu et al., 2004; Cindrova-Davies et al., 2007). This is supported by the observation of increased circulating HIF1α in women suffering from preeclampsia,
which only decline following delivery of the placenta, suggesting that the pathogenesis of preeclampsia is at least partly driven by hypoxia-mediated signalling in the placenta (Caniggia and Winter, 2002; Rajakumar et al., 2003; Iwagaki et al., 2004).

It is now known that a number of HIF-regulated genes also directly or indirectly influence mitochondrial function, highlighting a complex interplay between the HIF-pathway and mitochondrial biology (Thomas and Ashcroft, 2019). Evidence suggests that HIF1α may even directly localise to mitochondria, which allow mitochondria to respond rapidly to changes in oxygen availability without the delay of transcriptional events in the nucleus (Briston et al., 2011). HIF-mediated mitochondrial adaptations have evolved to maintain metabolic homeostasis and to protect the cell from harmful effects of oxygen deprivation, such as mitochondrial ROS production (Thomas and Ashcroft, 2019). This generally leads to a an increase in anaerobic ATP production by shifting the conversion of pyruvate towards lactate and away from acetyl-CoA, thereby limiting substrates for the citric acid cycle and suppressing ETC activity (Papandreou et al., 2006; Fuhrmann and Brüne, 2017). HIF1α can also alter the structure and composition of the ETC itself, for instance, through expression of the hypoxia-responsive miRNA or “hypoxamir” miR-210 (Myatt, 2006; Chan et al., 2012; Muralimanoharan et al., 2012). miR-210-mediated suppression of the iron sulphur cluster assembly enzyme underlies the decrease in mitochondrial respiration by preventing the incorporation of iron-sulphur clusters into mitochondrial proteins, such as respiratory complex I (Favaro et al., 2010; Devlin et al., 2011; Chan et al., 2012; Muralimanoharan et al., 2012). MiR-210 can also stimulate the activity of calcium activated potassium channels in the uterine artery, impairing uteroplacental perfusion and exacerbating placental hypoxia (Colleoni et al., 2013; Hu et al., 2018). Both HIF1α and miR-210 are dysregulated in placentae during preeclampsia, as well as in plasma
collected from women suffering from preeclampsia (Rajakumar et al., 2003; Muralimananoharan et al., 2012; Akhilesh et al., 2013; Gan et al., 2017; Li et al., 2019).

1.2.3.5 Hypoxia and gasotransmitters

*Nitric oxide*

The gasotransmitter NO is an important modulator of vascular tone, and a key vasodilator in the uteroplacental and umbilical circulation, with additional vasoprotective and anti-atherosclerotic properties (Magness et al., 2001; Cindrova-Davies, 2014). Pregnancy leads to an increase in endothelial NO production, which may underlie the uterine artery vasodilatation and increase in uteroplacental blood flow that is associated with advancing gestation (Xiao et al., 1999; Nelson et al., 2000; Magness et al., 2001; Thakor et al., 2010a; Herrera et al., 2012; Aljunaidy et al., 2016). Under conditions of gestational hypoxia, NO-dependent uterine artery vasodilatation is reduced, at least partly due to decreased expression of enzymes involved in synthesis of NO, such as eNOS (Mateev et al., 2003; Thompson and Dong, 2005; Fish et al., 2010; Matsubara et al., 2015). Mice with a knockout for eNOS show significant FGR associated with increased uteroplacental vascular resistance, reduced uteroplacental perfusion, placental hypoxia, placental oxidative stress and reduced transplacental nutrient transport (Kusinski et al., 2012; Kulantavelu et al., 2013; Aljunaidy et al., 2016). Hypoxia and oxidative stress can also lead to free radical scavenging of NO in the oxidatively stressed uteroplacental vascular bed, forming peroxynitrite (Miller et al., 1996; Kossenjans et al., 2000; Farrow et al., 2008; Matsubara et al., 2015). Peroxynitrite can accumulate in the placenta and contributes to the pro-oxidant effects of ROS, capable of disrupting cytotrophoblast differentiation and uteroplacental vascular function (Miller et al., 1996; Kossenjans et al., 2000). In addition, altered NO bioavailability is increased in both the ischaemic placenta and the
systemic vasculature of women suffering from preeclampsia or diabetes (Myatt and Cui, 2004; Sandrim et al., 2008; Matsubara et al., 2015). Animal models with eNOS knockouts are also associated with maternal proteinuria and cardiovascular dysfunction, including reduced endothelium-dependent vasorelaxation and maternal hypertension (Duplain et al., 2001; Longini et al., 2007). While eNOS expression and activity is generally decreased in the vascular endothelium of preeclamptic women compared to healthy controls, the role of inducible NO synthase (iNOS) in pregnancy is more controversial (Bhavina et al., 2014; Du et al., 2017). iNOS is localised to the villous stroma and the extravillous trophoblast of the placenta, and is induced by a pro-inflammatory environment to temporarily increase NO bioavailability, potentially enhancing the formation of peroxynitrite and triggering apoptosis (Thomson et al., 1997; Nakatsuka et al., 1999). Studies have found conflicting evidence on alterations in iNOS levels in complicated pregnancies, making it difficult to pinpoint its contribution to the pathogenesis of preeclampsia (Thomson et al., 1997; Faxén et al., 2001; Schiessl et al., 2005; Du et al., 2017).

Many studies have investigated the therapeutic potential of altering NO bioavailability in preeclampsia, such as organic nitrate, S-nitrosothiols, L-arginine, sildenafil and other inhibitors of cyclic guanosine monophosphate (cGMP) breakdown (Johal et al., 2014). In a human case control study, treatment of women suffering from gestational hypertension with anti-hypertensive drugs in addition to NO donors and plasma volume expansion was more effective in reducing maternal hypertension and FGR compared to treatment with anti-hypertensive drugs alone (Valensise et al., 2008). The benefits of L-arginine administration have been observed in experimental animal models, as well as in human clinical trials. In a rat model of sFlt-1-induced preeclamptic syndrome, L-arginine administration improved hypertension and reduced ET-1
production, while its supplementation in a randomised controlled trial was associated with decreased risk of preeclampsia irrespective of pre-existing cardiovascular risk factors (Murphy et al., 2012; Dorniak-Wall et al., 2014). S-nitroglutathione administration in women with preeclampsia has been shown to reduce maternal endothelial dysfunction, hypertension and platelet aggregation and to improve uteroplacental perfusion in the absence of adverse side effects (de Belder et al., 1995; Lees et al., 1996; Johal et al., 2014).

ET-1 is a powerful antagonist of NO- and endothelium-dependent vasodilatation, and the two vascular mediators share a complex relationship by altering gene expression and ligand-receptor interactions (Yanagisawa et al., 1988; Khimji and Rockey, 2010; Bourque et al., 2011). The balance between ET-1 and NO signalling under physiological conditions is crucial for endothelial homeostasis and normal vascular function, while a disequilibrium between these two factors is implicated in many vascular pathologies, including several forms of hypertension (Alonso and Radomski, 2003; Bourque et al., 2011). This interplay is also involved in the increase in uteroplacental vascular resistance in hypoxia-induced FGR (Thaete et al., 2004; Julian et al., 2008). ET-1 has little effect on uteroplacental vascular tone under physiological conditions due to NO-dependent inhibition on ET-1 synthesis, receptor binding and downstream signalling (Takahashi et al., 2001). However, following the onset of hypoxia and HIF1α signalling, NO bioavailability is diminished, and ET-1 and its receptors are markedly upregulated (Takahashi et al., 2001; Moore et al., 2004; Bourque et al., 2011). Endothelin receptor A signalling is considered causative in impairing uteroplacental blood flow during chronic hypoxia and pregnancy at high altitude, and an increased ratio of ET-1 to NO shows a clear association with FGR (Thaete et al., 2004; Julian et al., 2008). ET-1 also has potent systemic vasoactive
effects on the maternal endothelium and is elevated in the plasma of preeclamptic pregnant women compared to healthy controls, likely to be one of the key mediators of maternal hypertension (Nova et al., 1991; Yamashita et al., 2001; Baksu et al., 2005). In addition, ET-1 is associated with endothelial dysfunction in animal models of placental hypoxia, mediating impaired uteroplacental perfusion, hypertension and proteinuria (Greenberg et al., 1997; George and Granger, 2011; Jain, 2012). In keeping with this, inhibition of endothelin receptor A in experimental animal models has been shown to prevent the ET-1-associated rise in blood pressure (LaMarca et al., 2008b; Murphy et al., 2010; Tam Tam et al., 2011; Jain, 2012). Interestingly, Andean populations show the presence of single nucleotide polymorphisms within the ET-1 gene, which is associated with a fall in plasma ET-1 levels with advancing gestation at high altitude (Moore et al., 2004). This does not occur in Europeans residing at the same altitude and supports the importance of hypoxia-mediated ET-1 signalling in vascular function. These seemingly adaptive responses may also explain the relative protection against high altitude-induced FGR in highland native populations, such as Andeans and Tibetans (Zamudio et al., 1993a; Giussani et al., 2001; Moore et al., 2004; Julian et al., 2009; Soria et al., 2013).

*Hydrogen sulphide*

Originally simply regarded as a toxic gas, H$_2$S has evolved into the forefront of the conversation regarding human physiology and vascular function. Deeper insight into the functions of H$_2$S has highlighted its role as a cytoprotective antioxidant, gasotransmitter and regulator of vascular function through vasodilatation and modulation of angiogenesis (Gadalla and Snyder, 2010; Wang, 2010; Forgan et al., 2017). More recently, the role of H$_2$S in the maintenance of placental vascular function during healthy and compromised pregnancy has been investigated. Different
mechanisms of action have been proposed to underlie the protective effects of H₂S on vascular function during pregnancy, including sequestration of ROS, regulation of potassium channels in vascular smooth muscle, modulation of the renin-angiotensin system and interference with anti-angiogenic factors, such as sFlt-1 (Bryan et al., 2011; Lu et al., 2017; Dongó et al., 2018). Most of the vasodilator properties of H₂S are likely mediated by ATP-sensitive potassium (K<sub>ATP</sub>) channel-dependent smooth muscle cell relaxation (Zhao et al., 2001). In addition, at least part of the proposed benefit of H₂S donors on vascular function in pregnant women with pre-eclampsia is thought to occur through upregulation of miR-133b, which in turn downregulates sFlt-1 release, increasing the bioavailability of PIGF and VEGF (Hu et al., 2017a). Cystathionine γ-lyase (CSE) is the rate-limiting enzyme for H₂S production in the vasculature and localises to the smooth muscle cells of placental stem villi, where it contributes to lowering uteroplacental vascular resistance and maintaining uteroplacental perfusion (Lu et al., 2017). CSE expression and activity are reduced in placentae under conditions of hypoxia and oxidative stress, possibly through miR-21-mediated downregulation of CSE associated with mitochondrial depolarisation, cytotrophoblast apoptosis and villous remodelling (Torry et al., 1999; Zhou et al., 2002; Tsatsaris et al., 2003; Li et al., 2005; Yang et al., 2012; Cindrova-Davies et al., 2013; Lu et al., 2017). CSE knockout mice and mice treated with CSE enzyme inhibitors show reduced levels of circulating H₂S, which is associated with increased sFlt-1 and sEng levels, reduced vasodilatation, significant maternal hypertension and FGR (Yang et al., 2008; Wang et al., 2013). In humans, women suffering from preeclampsia and from hypertension and pregnant women carrying growth restricted fetuses show lower circulating levels of H₂S compared to controls (Yang et al., 2012; Cindrova-Davies et al., 2013; Wang et al., 2013; van Goor et al., 2016). This has widespread adverse
effects on both uteroplacental and systemic vascular tone, and can exacerbate conditions of oxidative stress through the loss of H$_2$S-mediated antioxidant properties (Osmond and Kanagy, 2014; Dongó et al., 2018). Thus, deregulation of maternal H$_2$S biology is associated with impaired uteroplacental blood flow, uteroplacental hypoxia, FGR, maternal vascular dysfunction and renal damage, making H$_2$S a relevant candidate in bridging the gap between uteroplacental dysfunction and adverse fetal outcomes downstream as well as adverse maternal outcomes upstream. Accordingly, the therapeutic potential of using H$_2$S donors has been of increasing interest. Several animal models have demonstrated protective effects of H$_2$S against ischaemia-reperfusion injury, inflammation and apoptosis in tissues, such as the heart and the kidneys (Bos et al., 2009; Sodha et al., 2009). H$_2$S donors had potent K$_{ATP}$-dependent vasodilator effects on perfused placentae and reduced sFlt-1 levels, hypertension and proteinuria in animal models of gestational hypertension (Cindrova-Davies et al., 2013; Holwerda et al., 2014; Possomato-Vieira et al., 2016). In addition, treatment with sodium hydrogen sulphide (NaHS) and L-cysteine increased levels of VEGF both in vitro in placental explants and in vivo in the Dahl salt-sensitive rat model of preeclampsia (Hu et al., 2016; Possomato-Vieira et al., 2016). In contrast, a recent study in the same Dahl salt-sensitive rat model showed that treatment with sodium thiosulphate worsened proteinuria and decreased fetal-placental weight ratio, even though it had a favourable effect of reducing hypertension (Terstappen et al., 2020). This indicates that the use of H$_2$S donors could have beneficial effects in the treatment of preeclampsia, but that more detailed investigations are required to validate the effects of these agents while ruling out possible adverse side effects.

1.2.3.6 Hypoxia and angiogenic imbalance

The placental vasculature is a dynamic structure, regulated by a delicate balance of
pro-angiogenic and anti-angiogenic factors (Charnock-Jones et al., 2004). PIGF and VEGF are dimeric glycoproteins with pro-angiogenic properties and vital roles in maternal and placental endothelial function, promoting trophoblast survival, placental angiogenesis and vasculogenesis in the uteroplacental vascular bed (Torry et al., 1999; Zhou et al., 2002; Tsatsaris et al., 2003; Li et al., 2005). This ensures effective transplacental nutrient and oxygen delivery from the mother to the fetus (Charnock-Jones et al., 2004). The anti-angiogenic factor sFlt-1 is produced as a result of alternative splicing of VEGF receptor 1 and acts as a soluble decoy receptor to VEGF and PIGF, preventing their binding to VEGF receptors 1 and 2 (Shibuya, 2001; Tanbe and Khalil, 2010). The ratio of sFlt-1 to both angiogenic factors in the placenta and in maternal plasma provides a relevant reflection on placental and maternal angiogenic balance, respectively. In healthy pregnancy, sFlt-1 increases and PIGF decreases with advancing gestation, but these changes are more pronounced and occur earlier in complicated pregnancies (Levine et al., 2004). Early-onset and late-onset gestational hypoxia, placental oxidative stress and preeclampsia are all associated with decreased levels of PIGF and VEGF and an increased ratio of sFlt-1 to PIGF and VEGF in both the trophoblast and the maternal circulation compared to healthy control pregnancies (Maynard et al., 2003; Karumanchi and Bdolah, 2004; Li et al., 2005; Tam et al., 2011; Nevo et al., 2013; Appel et al., 2015). Pro-angiogenic growth factor expression is negatively regulated by the UPR transcription factors ATF6 and ATF4, and their concentration is decreased in women suffering from preeclampsia even before disease presentation (Taylor et al., 2003; Levine et al., 2004; Maynard and Karumanchi, 2011; Mizuuchi et al., 2016). In the early stages of gestation, the adverse effects of sFlt-1 signalling are mainly mediated by inhibiting cytotrophoblast invasion and differentiation, preventing the establishment of a mature haemochorial placenta.
General introduction

(Zhou et al., 2002; Fisher, 2004). In contrast, in the later stages of pregnancy, the inhibitory effect of sFlt-1 on PIGF and VEGF is implicated in the development of maternal endothelial dysfunction, hypertension and proteinuria in preeclampsia (Maynard et al., 2003; Karumanchi and Bdolah, 2004). In late gestation, administration of sFlt-1, systemic delivery of an adenoviral vector expressing sFlt-1, VEGF inhibition and PIGF knockout studies are associated with an increase in ROS in maternal tissues, hypertension, proteinuria, glomerular endotheliosis, as well as significant FGR (Maynard et al., 2003; Sugimoto et al., 2003; Eremina et al., 2008; Bridges et al., 2009; Bergmann et al., 2010; Parchem et al., 2018). This is, at least partly, attributed to the sFlt-1-mediated inhibition of VEGF receptor 2-dependent activation of eNOS and NO production, thus reducing NO bioavailability (Cindrova-Davies et al., 2007). These findings have raised questions about the suitability of sFlt-1 or the ratio of sFlt-1 to PIGF and VEGF as biomarkers for pregnancy complicated by placental insufficiency, such as preeclampsia (Herraiz et al., 2015; Saleh et al., 2016; Sovio et al., 2017). The development of validated commercial bioassays now allows these markers to be used as additional diagnostic tools to help the detection of preeclampsia (Verlohren et al., 2010; Zeisler et al., 2016). In contrast to their diagnostic use, the therapeutic use of VEGF or PIGF administration has been less well investigated, and the mechanism of action of potential protective signalling has not been fully elucidated. VEGF treatment of placental explants in vitro was associated with a reduction in ER stress markers, while administration of VEGF in vivo has been associated with adverse side effects, including oedema (Mochan et al., 2019). However, the administration of adenoviral vectors expressing VEGF shows potential as future therapy, which reduces circulating levels of sFlt-1 and alleviates both FGR and maternal symptoms of proteinuria, renal pathology, uteroplacental malperfusion and hypertension (Koyama et al., 2006;
Bergmann et al., 2010; Woods et al., 2011; Spencer et al., 2014). PIGF administration has also been found to reduce maternal blood pressure to healthy baseline levels in a rat of model of preeclampsia (Spradley et al., 2016; Zhu et al., 2016; Chau et al., 2017). These effects are likely mediated by improving levels of placental oxidative stress and promoting maternal endothelium-dependent vasodilatation by activating NO and cGMP-mediated pathways (Suzuki et al., 2009; Spradley et al., 2016; Zhu et al., 2016; Chau et al., 2017).

Another important factor in angiogenesis and placental vascular function is the cytokine TGF-β (Shao et al., 2009). TGF-β signalling in the placenta is involved in vascular development, regulation of trophoblast invasion, differentiation and placental hormone production (Jones et al., 2006; Xuan et al., 2007). The membrane-bound TGF-β coreceptor endoglin is a glycoprotein expressed by endothelial cells in the placenta and modulates downstream pro-angiogenic signalling (Goumans et al., 2002; Shao et al., 2009). The soluble form of endoglin sEng prevents TGF-β binding to its receptor and is associated with inhibition of endothelial tube formation and reduced TGF-β-dependent vasodilatation via eNOS and NO (Venkatesha et al., 2006). Preeclampsia is associated with an increase in sEng, even before the onset of the disease, and its concentration in plasma shows a positive correlation with the severity of the symptoms (Levine et al., 2006). Animal models treated with sFlt-1 and sEng have provided evidence that these two anti-angiogenic factors likely act in concert to amplify the symptoms of severe preeclampsia with HELLP syndrome, which is not observed when animals are treated with sFlt-1 alone (Venkatesha et al., 2006). However, in contrast to sFlt-1, there is only limited evidence that hypoxia mediates the upregulation of sEng expression in the trophoblast (Jeyabalan et al., 2008; Munaut et al., 2008).
1.2.3.7 Hypoxia and inflammatory cytokine signalling

A common downstream consequence of placental hypoxia, excess ROS production and oxidative damage to the endothelium is the release of inflammatory cytokines from the placental or the systemic maternal vasculature (Benyo et al., 1997; Gupta et al., 2012; Mittal et al., 2014). Compared to a healthy placenta, a hypoxic or oxidatively stressed placenta undergoes a shift in its inflammatory cytokine profile, showing reduced expression of anti-inflammatory cytokines, such as interleukin-10 (IL-10), and up-regulation of pro-inflammatory cytokines, such as tumour necrosis factor α (TNFα), interleukin-1β (IL-1β), interleukin-6 (L-6) and interleukin-8 (IL-8; Conrad and Benyo, 1997; Bowen et al., 2005, Casart et al., 2007; Cackovic et al., 2008; Royle et al., 2009; Shah and Khalil, 2015). For instance, altered NO bioavailability is associated with an increase in activated immunocytes in the ischaemic placenta and the systemic vasculature of women suffering from preeclampsia (Matsubara et al., 2015). This altered inflammatory state contributes to a systemic endovascular inflammatory state, which is detectable in the maternal circulation and the amniotic fluid prior to manifestation of preeclamptic symptoms (Pober and Cotran, 1990; Benyo et al., 1997; Conrad and Benyo, 1997; Coussons-Read et al., 2003). The levels of these inflammatory cytokines increase with advancing gestation and show positive correlations with the severity of hypertension and kidney disease in preeclampsia (Redman and Sargent, 2000; Pantham et al., 2015; Cuffe et al., 2017; Schoots et al., 2018). In addition, TNFα provides a potent stimulus for upregulation of ET-1-mediated vasoconstriction under hypoxia, and this relationship may, at least partly, underlie the maternal hypertension associated with an increase in circulating levels of TNFα (LaMarca et al., 2005; LaMarca et al., 2008a). Interestingly, endothelial cells are more susceptible to pro-inflammatory activation after treatment with sFlt-1 due to inhibition
of VEGF signalling, while TNFα itself can promote the release of sFlt-1, indicating that angiogenic imbalance and placental oxidative stress may act in concert to precipitate the preeclamptic syndrome (Parrish et al., 2010; Cindrova-Davies et al., 2011).
1.3 The pregnant ewe as an animal model of compromised pregnancy

Countless animal models of FGR and placental insufficiency have been developed to study compromised pregnancy, none of which truly recapitulate human pregnancy, each with unique advantages and disadvantages. For the purpose of this thesis, we investigated a sheep model of gestational hypoxia in order to relate uteroplacental hypoxia, not only to changes in fetal growth and molecular changes in the placenta, but also to maternal cardiovascular function.

Morphologically, there are striking differences between the synepitheliochorial cotyledonary ovine placenta and the human haemochorial discoid placenta. In the human, the trophoblast fully invades the uterine lining and comes into direct contact with maternal blood. In contrast, the ovine placenta is divided into numerous individual placentomes formed by interdigitation between the crypts of the maternal caruncles and the projecting villi of the fetal cotyledons (Stegeman, 1974; Ehrhardt and Bella, 1995). These placentomes can be classified into four types based on their morphological appearance, determined by the proportion of fetal trophoblast and maternal tissue, ranging from type A, with inverted fetal components entirely surrounded by maternal tissue, to type D, in which the fetal interface is exposed on top of the caruncle (Vatnick et al., 1991; Ward et al., 2006). Despite these differences, the villous tree formation within each placentome remains similar compared to the human placenta, which can also be functionally divided into cotyledons (Leiser et al., 1997). While rodent models benefit from haemochorial placentation, as in the human placenta, the rodent placenta is divided into the junctional and the labyrinth zone, which perform endocrine functions and nutrient transfer, respectively (Woods et al., 2018). This can be valuable for studying these elemental components of placental
function, but has significant consequences on molecular signalling pathways within these zones. For example, the labyrinth zone shows high levels of mitochondrial activity and is thus susceptible to mitochondrial stress, while ER stress only occurs in the junctional zone due to its synthetic and secretory activities (Yung et al., 2019). This means that in the rodent placenta UPR$^{ER}$ and UPR$^{mt}$ rarely occur in the same cell type, in contrast to the human syncytiotrophoblast, where extensive crosstalk exists between both pathways. In addition, the junctional zone is less well oxygenated than the labyrinth zone in healthy pregnancy, such that the response to gestational hypoxia will be markedly different depending on which zone is being investigated (Kusinski et al., 2012). Thus, this functional compartmentalisation may account for more differences compared to the human placenta than the morphological compartmentalisation of the sheep placenta.

Furthermore, there are several unique merits to sheep models of compromised pregnancy and the pregnant ewe has played a vital part in our current understanding of both normal and complicated pregnancies (Pardi and Cetin, 2006; Morrison et al., 2018; Beede et al., 2019). While rodent models allow easy handling and the generation of large sample sizes within a short period, ewes and sheep fetuses share comparable size and body weight compared to the human, with a relatively long gestational period and a high frequency of singleton pregnancies (Lesiński, 1962; Gardner et al., 2007; Barry and Anthony, 2008). Due to the comparable metabolic rate and the similar maternal to fetal mass ratio, the physiological challenge posed by pregnancy is similar in sheep compared to humans, allowing for a more accurate assessment of fetal development and maternal health and adding more clinical relevance to sheep models of compromised pregnancy (Kleiber, 1947; Morrison et al., 2018; Beede et al., 2019). Sheep are also suitable for the surgical instrumentation of
ewes and fetuses under general anaesthesia with catheters and flow probes for long-term recording of maternal and fetal cardiovascular parameters in conscious unrestrained animals, including blood pressure, pulsatile blood flow and vascular resistance (Barry and Anthony, 2008). In addition, the large blood volume allows for serial sampling from the mother and the fetus, facilitating chronic measurement of fetal and maternal blood gases, acid-base status, metabolic status and hormone levels (Barry and Anthony, 2008). Such techniques have highlighted important functional similarities between ovine and human placentae, showing that they share similar transplacental oxygen gradients and nutrient transporter expression (Wilkening et al., 1988; Wilkening and Meschia, 1992; Fujikura and Yoshida, 1996; Chung et al., 1998; Ma et al., 2011; Regnault et al., 2013). In addition, placental oxygen consumption rates are nearly identical in sheep and humans (37 vs. 34 ml.kg⁻¹.min⁻¹; Bonds et al., 1986).

Sheep models show particular clinical relevance for investigating in vivo cardiovascular function, as they go through comparable developmental stages of the respiratory and cardiovascular system as the human, with equivalent innervation by the autonomic nervous system and similar haemodynamic properties, including basal heart rate and cardiac output (McKibben and Getty, 1969; Sissman, 1970; Monie, 1976; Markovitz et al., 1989). Despite these advantages, there are several limitations to using sheep as experimental animals. Surgical instrumentation and maintenance of indwelling catheters and flow probes is technically demanding and costly and requires a high degree of daily maintenance. Due to these limitations, it is difficult to generate cohorts large enough to study sex differences. Sheep, as ruminants, also show great differences in digestion and energy metabolism. In contrast to humans, sheep use little glucose for cellular respiration and rely mainly on the use of volatile fatty acids (Bassett, 1978). Thus, sheep show lower levels of glucose tolerance and glucose uptake from
the blood by the liver compared to healthy humans, and the translational potential between ovine and human energy metabolism needs to be viewed with caution (Challiss and Ferré, 1988). However, glucose remains the primary substrate for the placenta and fetus in both humans and sheep, with the placenta as the main driver of increased glucose demands in the pregnant ewe (Meschia et al., 1980; Hay et al., 1983; Marconi et al., 1993; Bell and Bauman, 1997).
1.4 Aims and objectives

The overall aim of this PhD was to develop a preclinical model of improved human translational potential to investigate symptoms of preeclampsia in sheep. To achieve this, we exploited recently available technology to maintain pregnant sheep under highly controlled isobaric hypoxic conditions for long periods of gestation, while undergoing wireless recording of maternal cardiovascular function as the hypoxic pregnancy is occurring (Brain et al., 2015; Allison et al., 2016). Then, adopting an integrative approach, we combined experiments in vivo with those at the cellular and sub-cellular levels, to determine the inter-relationship between chronic hypoxia during pregnancy and maternal, placental and fetal outcomes, common in preeclampsia.

The aim was realised via three principal objectives:

1. To determine the effects of chronic hypoxia in the last third of gestation on markers of the unfolded protein response in the placenta;

2. To determine the effects of chronic hypoxia in the last third of gestation on placental mitochondrial function;

3. To determine the effects of chronic hypoxia in the last third of gestation on maternal cardiovascular function.
2. General Methods

2.1 Sheep husbandry

This study was carried out on Welsh Mountain sheep, carrying single fetuses of known gestational age, scanned for fetal sex. All procedures were performed at The Barcroft Centre of The University of Cambridge under the UK Animals Scientific Procedures Act 1986 and were approved by the Ethical Review Board of the University of Cambridge. The experimental design was conducted in accordance with the ARRIVE guidelines (Kilkenny et al., 2010). All ewes used for the current study were part of a contemporaneous cohort within the same breeding season in spring 2019.

2.1.1 Breeding cycles, sponging and dating of pregnancy

All ewes used for the current study were proven breeders and between 2 and 3 years of age. The ovarian cycles of the ewes were synchronised by inserting sponges containing progestogen (Chronogest CR; Intervet UK Ltd, UK) intra-vaginally for 12 to 14 days. Ewes were mated with one of four rams on site. The start date of pregnancy was approximated using the day that ewes presented with fresh raddle-ink marks on their rumps. The gestational period of Welsh Mountain sheep is ca. 145 days. Pregnancy with a single fetus was confirmed at ca. 80 days of gestation using abdominal ultrasonography (Toshiba Medical Systems Europe, Zoetermeer, Netherlands).

2.1.2 Management & maintenance

All sheep were held on open pasture or in stock holding pens with *ad libitum* access to hay and water. They were also fed approximately 200g pelleted concentrates daily (Sheep Nuts No.6. H & C Beart Ltd., UK).
2.2 Chronic hypoxia protocol

Pregnant Welsh Mountain ewes carrying singleton fetuses determined by ultrasound scan were weighed and randomly assigned to either long-term chronic normoxia or long-term chronic hypoxia for 1 month at 103 days gestational age (dGA; Figure 2.1). From 103 dGA, normoxic and hypoxic ewes were fed a daily bespoke maintenance diet consisting of concentrate and hay pellets, thereby facilitating the monitoring of food intake (Cambridge ewe diet: 40g nuts.kg\(^{-1}\) and 3g hay.kg\(^{-1}\); Manor Farm Feeds Ltd, UK; see Appendix for detailed composition).

![Diagram of chronic hypoxia protocol](image)

**Figure 2.1. Long-term chronic hypoxia protocol.**
Ewes were assigned to chronic normoxia (n=9) and chronic hypoxia (n=7) at 103 dGA and underwent daily controlled feeding. Blood samples were taken at 103, 105 and 138 dGA, and Doppler ultrasonography of uterine, femoral and carotid arteries was performed at 138 dGA prior to post-mortem.

2.2.1 Exposure to chronic hypoxia

At 103 dGA, ewes assigned to chronic normoxia were housed in individual floor pens.

Ewes assigned to the hypoxic cohort were moved into one of four bespoke isobaric
hypoxic chambers (Telstar Ace, UK; Figure 2.2) housed in a laboratory with a controlled dusk to dawn 12:12 hour light-dark cycle. The hypoxic chambers were supplied with controlled volumes of nitrogen and air via a bespoke air and nitrogen generating system (Domnick Hunter Gas Generation, UK), as previously described in detail (Brain et al., 2015; Allison et al., 2016; Shaw et al., 2018). In brief, compressed air (8-10 bar) was passed through an Air Pre-treatment Package with filters to remove oil, moisture and particulate. The air was then passed on to one of two continuously operated nitrogen generators for removal of oxygen, carbon dioxide and other trace gases by a molecular adsorption bed. The purity of the gas was constantly monitored and the system was automatically switched between the two nitrogen generators to ensure continuous supply of pure nitrogen gas, which was stored in a compressed nitrogen storage vessel. Compressed air and nitrogen were piped to the laboratory and gases were mixed to requirements via rotameter flow metres and pressure valves prior to entering the chambers. The chambers measured 3.8 m$^3$ (2.3m x 1.4m x 1.2m; same floor area as the individual floor pens) and the volume of gas in each chamber underwent a minimum of twelve changes per hour. The inspirate was passed via silencers able to reduce noise to levels below minimum exposure values of the Control of Noise at Work Regulations 2005 (80 dB(a) LEPd), providing a tranquil environment for the animal inside each chamber (63 dB(a)). All chambers were equipped with an electronic automatic humidity cool steam injection system (1100-03239 HS-SINF Masalles, Spain). Ambient partial pressures of oxygen and carbon dioxide (PO$_2$ and PCO$_2$), humidity and temperature within each chamber were monitored via sensors and values recorded continuously via the Trends Building Management System of the University of Cambridge.
Figure 2.2. Isobaric hypoxic chambers and CamDAS™.
Figures reproduced from Allison et al. 2016 (Copyright © 2016, Experimental Physiology) and Brain et al. 2015 (Copyright © 2015, Physiological Reports). Both panels: A specially designed nitrogen-generating system supplied variable amounts of compressed air and nitrogen to the bespoke isobaric hypoxic chambers housed in the hypoxic chamber laboratory. Each chamber was equipped with an electronic servo-controlled humidity cool steam injection system to return the appropriate humidity to the inspirate (i). Ambient PO₂, PCO₂, humidity, and temperature within each chamber were monitored via sensors (ii). For experimental procedures, each chamber had a double transfer port (iii) to internalise material and a manually operated sliding panel (iv) to bring the ewe into a position, where sampling of blood could be achieved through glove compartments (v). Each chamber incorporated a drinking bowl on continuous water supply and a rotating food compartment (vi) for determining food intake. A sealed transfer isolation cart could be attached to a side exit (vii) to couple chambers together for cleaning. (B) A separate cohort of ewes was instrumented with the CamDAS™ system during surgery to permit continuous monitoring of arterial blood pressure and uterine blood flow. The wireless CamDAS™ system was contained in two parts in a custom-made sheep jacket: the data acquisition system box (ix) on one side and a box containing the pressure connectors (x) on the other side. Cables (xi) provided connection between the two boxes and to two battery packs able to power the system for 24 hours. Measurements made using the CamDAS™ system were transmitted wirelessly via Bluetooth (xiii) to a laptop on the outside (xii), on which it was possible to continuously measure and record uterine blood flow and maternal arterial blood pressure during the experimental period.

Every chamber was equipped with a drinking bowl on continuous water supply and a removable, rotating food compartment to determine daily food intake. For blood sampling procedures within the chambers, materials could be introduced via a double transfer port. A sliding panel was then manually operated, encouraging the ewe to the front of the chamber, which permitted blood samples to be taken via glove compartments using sterile techniques and without losing the hypoxic exposure. Two chambers could be coupled together using a transfer isolation cart, which allowed ewes to transiently move between chambers maintained at the same atmosphere and facilitated weekly cleaning of the chambers. From 103 to 105 dGA, ewes were kept...
under normoxic conditions (11 L.sec\(^{-1}\) air, equating to 39.6 m\(^3\).h\(^{-1}\)). At 105 dGA, ewes assigned to the chronic hypoxia cohort were gradually subjected to hypoxia over 48 hours, reaching 10 ± 1% inspired oxygen (5 L.sec\(^{-1}\) air and 6 L.sec\(^{-1}\) N\(_2\)). We chose this level of maternal hypoxia based on previous studies of fetal surgical catheterisation in our own laboratory, in which we were able to sample daily from the fetal descending aorta. Thus, we were able to adjust maternal hypoxia in accordance to clinical relevance, decreasing fetal P\(_a\)O\(_2\) from a reference value of ca. 20 mmHg to between 12 and 13 mmHg in a highly controlled manner (Allison et al., 2016). This level of chronic fetal hypoxia is equivalent to that measured by cordocentesis in human growth restricted fetuses in preeclampsia and in pregnancies complicated by gestational hypoxia (Nicolaides et al., 1986; Soothill et al., 1986; Hecher et al., 1995; Najib et al., 2015).

### 2.2.2 Blood sampling regimen

In both normoxic and hypoxic ewes, venous samples (10 ml) were taken from the jugular vein at 103, 105 and 138 dGA. Samples were added to tubes containing the anti-coagulant EDTA (Sarstedt, Germany) and centrifuged at 3,000 r.p.m. for 5 minutes at 4 °C. They were then aliquoted into individual tubes of 200 µL for subsequent analysis, snap frozen in liquid nitrogen, and stored at -80 °C until further analysis.

### 2.2.3 Doppler ultrasonography

At 138 dGA, normoxic and hypoxic ewes were moved to a nearby ultrasound room. Ewes in the hypoxic group were transported to the ultrasound room and underwent all ultrasound procedures maintaining the hypoxic exposure of 10 ± 1% inspired oxygen via a customised respiratory hood in a mobile cart unit (Brain et al., 2015; Allison et
General Methods

The respiratory hood was constructed of clear polyethylene material to maintain visibility to other sheep in the room and connected to pressurised gas cylinders using plastic tubing and a one-way valve system. The gas mixture supplying the respiratory hood was the same ratio as the gas mixture supplying the chambers, adjusted to volume, and the level of hypoxia was monitored using an oxygen probe inserted into the respiratory hood and connected to a nearby monitor. This setup was well tolerated by all ewes and allowed experimental procedures to be carried out with as little as two researchers.

The maternal abdomen, medial surfaces of the hind limbs and neck were shaved and cleaned. Ultrasonography was performed using a Toshiba Powervision 7000 System with a convex 3.75 MHz Toshiba PVK-357AT transducer. Colour Doppler was used to identify the uterine, femoral and carotid arteries, and their waveforms were visualised using colour Doppler flowmetry (Figure 2.3).

![Figure 2.3. Doppler ultrasonography.](image)

Three consecutive waveforms were traced and averaged for calculation of the pulsatility index.
Training for the Doppler ultrasonography technique was provided by Dr. Caroline Shaw, M.D., a Specialty Registrar in Obstetrics and Gynaecology at Queen Charlotte’s and Chelsea Hospital (Imperial College Healthcare NHS Trust), who has extensive experience in working with large animals in a research setting.

Uterine artery Doppler was performed transabdominally by aiming the probe dorsomedially from behind the mammary glands, identifying an individual placentome and tracing back a branch of the uterine artery into the uterine myometrium. The femoral artery was identified by first holding the probe perpendicular to the medial surface of the hind limb at the femoral triangle and then turning it distally until both the femoral artery and vein could be visualised. The carotid artery was identified by placing the probe on the jugular groove facing dorsomedially and aiming the probe towards the mandibular angle. Three consecutive waveforms were traced and averaged for calculation of the pulsatility indices according to the following equation:

\[
Pulsatilty\ index = \frac{\text{Peak systolic velocity} - \text{End diastolic velocity}}{\text{Time averaged velocity}}
\]

The means of the right and left uterine, femoral and carotid pulsatility indices were used for analysis.

2.2.4 Post-mortem

At 138 dGA, normoxic and hypoxic ewes were weighed and moved to the post-mortem laboratory. Ewes in the hypoxic group remained hypoxic at 10 ± 1% inspired oxygen via a respiratory hood until euthanasia. Both normoxic and hypoxic ewes were killed humanely by an overdose of sodium pentobarbitone (0.4 ml.kg\(^{-1}\) i.v., Pentoject; Animal Ltd., UK). A blood sample (5 ml) was taken from the umbilical vein. Partial pressures of oxygen and carbon dioxide (PO\(_2\) and PCO\(_2\)), percentage saturation of haemoglobin
with oxygen (Sat Hb), and concentrations of haematocrit and haemoglobin were measured using an ABL9 blood gas analyser (Radiometer; Copenhagen, Denmark; all measurements corrected to 38 °C). The oxygen tension, at which haemoglobin is 50% saturated ($P_{50}$) was calculated by using the Hill equation describing the oxyhaemoglobin dissociation curve as follows (Doyle, 1997):

$$
P_{50}(\text{mmHg}) = PO_2(\text{mmHg}) \times \left( \frac{1 - \text{Sat Hb}}{\text{Sat Hb}} \right)^{\frac{1}{n}}
$$

*Hill exponent* $n = 2.711$

The fetus was delivered by Caesarean section, weighed, and fetal crown-rump length (CRL), abdominal circumference (AC), biparietal diameter (BPD) and hind limb lengths were measured. The hind limb length was divided into femoral, tibial and lower limb (calcaneal tuberosity until tip of phalanges) lengths. The fetal brain was isolated, weighed, and the left hemisphere was fixed while the right hemisphere was dissected into 3 mm thick slices frontodorsally before freezing. The fetal pituitary, heart, lungs, liver, kidneys, adrenals, perirenal fat depots, gonads, pancreas, thyroids and a piece of the biceps femoris were isolated, weighed and snap frozen in liquid nitrogen and stored at -80 °C or fixed in 4% paraformaldehyde (Sigma, UK), transferred to 70% ethanol after 24 hours and stored at 4 °C until further analysis. Fetal body mass index (BMI) and ponderal index (PI) were calculated according to the following equations:

$$\text{BMI} = \frac{\text{Fetal weight (kg)}}{\text{CRL (m)}^2}$$

$$\text{Ponderal index} = \frac{\text{Fetal weight (kg)}}{\text{CRL (m)}^3}$$

The maternal heart, lungs, liver, kidneys, adrenals, perirenal fat depots, pancreas,
ovaries and thyroids were isolated and weighed. The maternal heart was immediately placed in ice-cold Krebs-Henseleit bicarbonate buffer for Langendorff heart perfusion experiments. The left kidney was immediately taken for perfusion fixing. A 3rd order branch of the femoral artery was dissected into ice-cold Krebs solution for wire myography. All other tissues and a piece of the maternal biceps femoris were snap frozen in liquid nitrogen and stored at -80 °C or fixed in 4% paraformaldehyde (Sigma, UK), transferred to 70% ethanol after 24 hours and stored at 4 °C until further analysis.

Following hysterectomy, a 3rd order branch of the uterine artery was dissected into ice-cold Krebs solution for wire myography (section 2.6.1.1).

Maternal and fetal urine samples (5 ml) were taken with a syringe and needle via puncture directly from the bladder and aliquoted into individual tubes of 500 µL for subsequent analysis, snap frozen in liquid nitrogen, and stored at -80 °C.

2.2.4.1 Placentome classification
The ovine placenta is a conglomeration of 50 to 90 individual placentomes and each placentome is formed by interdigitation of the maternal caruncular endometrium with the villous trophoblast of the fetal cotyledon (Steven, 1975; Schneider, 1996). Following hysterectomy, individual placentomes were isolated, weighed and counted. Each placentome can be classified into different types from A to D based on its gross morphology, depending on the proportion of fetal compared to maternal tissue (Figure 2.4; Vatnick et al., 1991; Ehrhardt and Bella, 1995). In normal ovine pregnancies, gross placental morphology changes progressively throughout gestation, with an increase in type C and type D placentomes towards the end of gestation, which account for ca. 40% of the total placentome number at term (Alexander, 1964; Robinson et al., 1979). The distribution of placentome types is thought to shift more
towards type C and D placentomes in response to adverse conditions, including maternal undernutrition, carunculectomy and high altitude hypoxia (Alexander, 1964; Robinson et al., 1979; Vatnick et al., 1991; Heasman et al., 1998; Penninga and Longo, 1998; Heasman et al., 1999; Steyn et al., 2001; Osgerby et al., 2004; Vonnahme et al., 2006; Vonnahme et al., 2008; Zhang et al., 2016b). While it has been widely proposed that the morphological shift of the ovine placenta towards more “mature” type C and D placentomes may have adaptive functions, for example to increase placentome vascularity and thus transplacental nutrient delivery to the fetus, others have suggested that differences in umbilical perfusion pressure and the ontogenic rise in plasma cortisol levels could underlie these morphological changes (Alexander, 1964; Robinson et al., 1997; Bell et al., 1999; Gardner et al., 2002; Ward et al., 2006; Braun et al., 2011). Functional differences between the different placentome types has remained elusive, and many studies, including our own, have found no changes in placentome distribution in response to adverse pregnancy (Clarke et al., 1998; Gardner et al., 2002; Ward et al., 2006; Vonnahme et al., 2008). In the present study, we observed no significant changes to placentome weight or number distribution, and there were no differences in average placentome weight of any type. We further performed preliminary studies comparing the activation of placental stress pathways and mitochondrial function between type A vs. type D placentomes from the same ewes undergoing either control normoxic or chronic hypoxic pregnancies. Strikingly, we found no major differences between type A and type D placentomes related to placental oxidative stress and activation of the placental UPR or mitochondrial respiration, neither in normoxic nor hypoxic ewes. Thus, for clarity, and to control for placentome type, any of the molecular analyses performed in the present study are shown in type A placentomes only, unless otherwise specified.
Figure 2.4. Placentome classification.
Figure taken from Ward et al. 2006 (Copyright © 2006, Oxford University Press). Type A placentomes are round, usually small, with inverted fetal cotyledonary tissue surrounded by the maternal caruncle. Type D placentomes are flat and generally larger, with the fetal cotyledon everted across the maternal caruncle. Type B and type C placentomes are intermediate phenotypes.

Two representative type A placentomes were selected to provide samples for electron microscopy and for high resolution oximetry. Placentomes were cut twice close to the midline to attain a 1 mm thick slice, which was cut again close to the midline to attain a 1 mm thick strip. These were placed in ice-cold 2 mM calcium chloride in 0.05M Sodium cacodylate buffer at pH 7.4 containing 2% glutaraldehyde and 2% formaldehyde and in ice-cold biopsy preservation solution (BIOPS), respectively, until further processing. Representative examples of type A and type D placentomes were snap frozen in liquid nitrogen and stored at -80 °C or fixed in 4% paraformaldehyde (Sigma, UK), transferred to 70% ethanol after 24 hours and stored at 4 °C until further analysis.
2.3 Molecular analysis

2.3.1 Placental protein analysis

2.3.1.1 Preparation of placental protein lysates

Flash frozen type A and type D placentomes were homogenised to powder using pestle and mortar while keeping tissues frozen on dry ice. Homogenates were lysed in Lysing Matrix D tubes (MP Biomedicals UK, UK) containing 1.4 mm ceramic spheres with ice-cold cell lysis buffer (1 mL of buffer per 100 mg of tissue; Cell Signaling Technology, UK) containing protease inhibitors (cOmplete Mini, EDTA free; Roche Diagnostics, UK) 3 times for 20 seconds using a ribolyser (MagNA Lyser, Roche Diagnostics, UK). The samples were placed on ice and vortexed every 5 minutes for 20 minutes. They were then centrifuged for 5 minutes at 12,000 r.p.m. at 4 °C, and supernatants were collected in a fresh Eppendorf tube, followed by further centrifugation for 5 minutes at 15,000 r.p.m. at 4 °C. The supernatants were collected in fresh Eppendorf tubes and a fraction of the lysate was set aside to determine protein concentration using a commercially available bicinchoninic acid assay kit (23235, Thermo Fisher Scientific, UK). All plates, reagents and samples were warmed to room temperature (RT) before use. 100 µL of bovine serum albumin standards (BSA; Sigma, UK) or diluted placental protein lysate were added to duplicate wells on a 96 well plate. All wells were mixed with 100 µL of reaction mix containing bicinchoninic acid and copper sulphate. The reaction was incubated at 37 °C for 2 hours, and optical density was read at 570 nm absorbance using a microplate reader (BioTek ELx800 absorbance microplate reader). A standard curve was plotted in Microsoft Excel, and the concentration in each sample was determined by using the equation of a best-fit trendline. All measurements fell within the linear range. Protein lysates were stored at -80 °C until further processing.
The inter- and intra-assay coefficients of variation were <5.0% and <3.0%, respectively, and the lower limit of detection was 2 µg.ml⁻¹.

2.3.1.2 Western blotting

Western blotting was used to determine the relative levels of proteins of interest in protein lysates. A detailed composition of buffers and gels used for Western blotting can be found in Table 2.1.

Protein lysates were mixed with cell lysis buffer (Cell Signaling Technology, UK) containing protease inhibitors (cOmplete Mini, EDTA free; Roche Diagnostics, UK) and 4x SDS gel loading buffer to achieve a protein concentration of 2 µg.mL⁻¹. Gel loading samples were denatured for 5 minutes at 70 ºC prior to use.

Gels were set between two 8 cm x 9 cm glass plates sealed by a 1 mm thick gasket and held together by two clips either side. A 21 well plastic comb was inserted into the stacking gel prior to setting. Once set, clips and gaskets were removed, and the gels were placed into an electrophoresis chamber (ATTO AE-6500, Japan) filled with running buffer (Table 2.1). 4 µL of a molecular weight protein ladder (Thermo Scientific™ PageRuler™ Plus Prestained Protein Ladder, Thermo Fisher, UK) and 10-20 µg aliquots of protein were resolved on 8-14% SDS-PAGE agarose gels at 110V and 12 mA per gel for approximately 4 hours. For each protein of interest, all contemporaneous samples were run on the same gel in 16 consecutive lanes alongside the molecular weight marker for comparison.

The protein was then transferred from the agarose gel onto nitrocellulose membranes (Hybond® ECL™, Sigma-Aldrich, UK) using a semi-dry transfer apparatus (TE70 Semi-dry transfer unit, GE Life Sciences, UK). The agarose gel was removed from the glass plates, and the separating gel was placed in transfer buffer (Table 2.1) with a 7 cm x
8 cm nitrocellulose membrane for 5 minutes. The gel and nitrocellulose membrane were sandwiched between four pieces of filter paper soaked in transfer buffer on each side. A roller was gently used to remove air bubbles after each layer. The transfer was run at 40 mA per gel for 90 minutes, after which the nitrocellulose membrane was rinsed in deionised water and stained with Ponceau S solution (0.1% Ponceau S in 5% acetic acid; Sigma-Aldrich, UK). After rinsing in distilled water, the membrane was placed inside a plastic pouch and scanned (HP Officejet 4630, Hewlett Packard, UK). The Ponceau S stain was removed by washing the membrane in Tris-buffered saline containing 0.1% Tween 20 (TBS-T; Table 2.1). The membrane was incubated in blocking buffer (5% skim milk in TBS-T) for 1 hour at RT with gentle rocking to prevent non-specific binding. Primary antibodies were diluted in TBS-T with 0.5% sodium azide. A full list of primary antibodies, their host species, dilutions, incubation times and catalogue numbers can be found in Table 2.2. Membranes were then washed with TBS-T 3 times for 10 minutes and incubated with the relevant secondary antibodies conjugated with horseradish peroxidase against Rabbit IgG (Thermo Fisher, UK; diluted 1:10,000 in TBS-T with 2.5% skim milk) or against Mouse IgG (Thermo Fisher, UK; diluted 1:10,000 in TBS-T with 2.5% skim milk) for 1 hour at RT, where appropriate. Following further washing with TBS-T (4 times for 10 minutes), protein levels were visualised using an enhanced chemiluminescence kit (Pierce™ ECL, Thermo Fisher Scientific, UK) on film (Amersham™ Hyperfilm™ ECL, GE Healthcare, UK).

Protein band densities were quantified using ImageJ software (NIH; RRID:SCR_003070) and normalised against total protein expression determined by Ponceau S staining, which has been found to be a reliable loading control similar to ß-actin quantification (Romero-Calvo et al., 2010).
### General Methods

<table>
<thead>
<tr>
<th>Buffer/Gel</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x SDS gel loading buffer</td>
<td>200 mM trisaminomethane base (pH 6.8)</td>
</tr>
<tr>
<td></td>
<td>400 mM dithiothreitol</td>
</tr>
<tr>
<td></td>
<td>8% sodium dodecyl sulphate</td>
</tr>
<tr>
<td></td>
<td>40% glycerol</td>
</tr>
<tr>
<td></td>
<td>A few crystals of bromophenol blue</td>
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<tr>
<td>Separating gel</td>
<td>8%-14% acrylamide</td>
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<tr>
<td></td>
<td>37.5 mM trisaminomethane base (pH 8.8)</td>
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<tr>
<td></td>
<td>1% sodium dodecyl sulphate</td>
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<td></td>
<td>0.005% tetramethylethylenediamine</td>
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<td></td>
<td>0.05% ammonium persulphate</td>
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<td>Stacking gel</td>
<td>5% acrylamide</td>
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<td>12.5 mM trisaminomethane base (pH 6.8)</td>
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<td>0.05% ammonium persulphate</td>
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<td>Running buffer</td>
<td>2.5 mM trisaminomethane base</td>
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<td></td>
<td>250 mM glycine</td>
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<td></td>
<td>0.5% sodium dodecyl sulphate</td>
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<td>Transfer buffer</td>
<td>48 mM trisaminomethane base</td>
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<td>39 mM glycine</td>
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<td></td>
<td>0.037% sodium dodecyl sulphate</td>
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<td></td>
<td>20% methanol</td>
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<tr>
<td>Tris-buffered saline (TBS)</td>
<td>20 mM trisaminomethane base</td>
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<tr>
<td></td>
<td>0.15 mM sodium chloride</td>
</tr>
<tr>
<td></td>
<td>pH adjusted to 7.5 with hydrogen chloride</td>
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**Table 2.1. Buffer and gel compositions for Western blotting.**

All reagents from Sigma, UK.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host species, dilution, incubation time</th>
<th>Catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-phosphoinositide-dependent protein kinase 1 (PDK1)</td>
<td>Rabbit, 1:1,000, overnight at 4°C</td>
<td>Cell Signaling Technology Cat# 3062, RRID:AB_2236832</td>
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<td>Aceto-acetyl carboxylase (ACC)</td>
<td>Rabbit, 1:1,000, overnight at 4°C</td>
<td>Cell Signaling Technology Cat# 3676, RRID:AB_2219397</td>
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<td>Activating transcription factor 4 (ATF4)</td>
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<td>Cell Signaling Technology Cat# 11815, RRID:AB_2616025</td>
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<td>Activating transcription factor 5 (ATF5)</td>
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<td>Abcam Cat# ab184923, RRID:AB_2800462</td>
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<tr>
<td>Activating transcription factor 6 (ATF6)</td>
<td>Mouse, 1:1,000, overnight at 4°C</td>
<td>Abcam Cat# ab11909, RRID:AB_298691</td>
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### General Methods

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Incubation</th>
<th>Vendor and Cat#</th>
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<td>AMP-activated protein kinase (AMPK)</td>
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<td>B-cell lymphoma 2 (BCL2)</td>
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<td>BD Biosciences Cat# 610539, RRID:AB_397896</td>
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<tr>
<td>Bcl2-associated X protein (BAX)</td>
<td>Rabbit</td>
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<td>overnight at 4°C and 1 hour at RT</td>
<td>BioRebyt Cat# orb4655, RRID:AB_10919579</td>
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<td>C-Jun N-terminal kinase (JNK)</td>
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<td>overnight at 4°C</td>
<td>Cell Signaling Technology Cat# 9252, RRID:AB_2250373</td>
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<tr>
<td>C/EBP homologous protein (CHOP)</td>
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<td>Caseinolytic protease proteolytic subunit (ClpP)</td>
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<tr>
<td>Citrate synthase</td>
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<tr>
<td>Cystathionine-β-synthase (CBS)</td>
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<td>Sigma-Aldrich Cat# HPA001223, RRID:AB_1846112</td>
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<td>Cystathionine-γ-lyase (CSE)</td>
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<td>Dynamin related protein 1 (DRP1)</td>
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<td>Endothelial nitric oxide synthase (eNOS)</td>
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<td>Heat shock protein 70 (HSP70)</td>
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<td>Hypoxia inducible factor 1α (HIF1α)</td>
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<td>1:1,000</td>
<td>overnight at 4°C and 4 hours at RT</td>
<td>R and D Systems Cat# MAB1935, RRID:AB_2279622</td>
<td></td>
</tr>
<tr>
<td>Mitofusin 2 (MFN2)</td>
<td>Rabbit</td>
<td>1:1,000</td>
<td>overnight at 4°C and 4 hours at RT</td>
<td>Cell Signaling Technology Cat# 9482, RRID:AB_2716838</td>
<td></td>
</tr>
<tr>
<td>Optic atrophy protein 1 (OPA1)</td>
<td>Rabbit</td>
<td>1:1,000</td>
<td>overnight at 4°C and 4 hours at RT</td>
<td>Cell Signaling Technology Cat# 80471, RRID:AB_2734117</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2. Primary antibodies used in Western blotting.
Primary antibodies, their host species, dilutions, incubation times and catalogue numbers used in Western blotting. All primary antibodies were previously validated by running ovine placentome samples alongside control human trophoblast samples to ensure specificity of antibody binding, and then optimised by adjusting dilution factors and incubation times.

2.3.1.3 Measurement of protein carbonylation
To determine post-translational protein carbonylation as a result of oxidative damage, an OxyBlot™ analysis was performed using an OxyBlot™ detection kit, according to the manufacturer’s instructions (Millipore Ltd., UK). 10 µg of protein were denatured in 5% sodium dodecyl sulphate (Sigma, UK). Each sample was treated with 10 µL of 2,4-dinitrophenylhydrazine solution to derivatise carbonyl groups to 2,4-dinitrophenyl-
hydrazone (DNPH) moieties (Figure 2.5A), and a negative control sample was treated with 10 µL of a derivatisation-control solution.

**Figure 2.5. OxyBlot™ detection of protein carbonylation.** (A) Figure adapted from Weber et al. 2015 (Copyright © 2015, Elsevier B.V.). Peptides containing an oxidised lysine residue (carbonyl group) were derivatised using a 2,4-dinitrophenyl hydrazine solution to form lysine DNPH moieties. These were detected by Western blotting (B).

After 15 minutes, the reactions were stopped through addition of 7.5 µL of neutralisation solution. All samples were separated on a 12% SDS-PAGE agarose gel and then transferred onto a nitrocellulose membrane (Hybond® ECL™, Sigma-Aldrich, UK), as previously described in section 2.3.1.2. Non-specific binding was inhibited by blocking the membrane in 2.5% BSA (Sigma, UK) in TBS-T for 1 hour at RT. The nitrocellulose membrane was incubated with a primary anti-DNPH antibody (Chemicon Oxyblot™; diluted 1:200) for 1 hour at RT. Membranes were then washed with TBS-T and incubated with a secondary antibody conjugated with horseradish peroxidase against Rabbit IgG (Thermo Fisher, UK; diluted 1:10,000) for 1 hour at RT. Following further washing with TBS-T, levels of protein carbonylation were visualised.
(Figure 2.5B) using an enhanced chemiluminescence kit (Pierce™ ECL, Thermo Fisher Scientific, UK).

Densities of each lane were quantified using ImageJ software (NIH, RRID:SCR_003070) and normalised against Ponceau S staining.

### 2.3.2 Placental transcript analysis

#### 2.3.2.1 RNA and miRNA extraction

Placental RNA and miRNA were extracted from flash frozen type A placentomes using a Qiagen miRNeasy® Mini Kit (Qiagen, UK). The tissue was homogenised using an electrical homogeniser (Polytron PT1200, Kinematica, UK) and disrupted by shaking the sample vigorously with 700 µL QIAzol Lysis Reagent Solution and 140 µL chloroform for 15 seconds. All tissue lysates were incubated at RT for 3 minutes and then centrifuged at 12,000 r.p.m. for 15 minutes at 4 °C. The supernatant was collected in fresh Eppendorf tubes. After addition of 525 µL 100% ethanol and thorough mixing, 700 µL of each sample was added to miRNeasy® Mini purification columns inside 2 mL collection tubes and centrifuged at 8,000 r.p.m. for 15 seconds at RT. The flow through was discarded. This was repeated with the rest of the sample, and then repeated with 700 µL of RWT buffer and 500 µL of RPE buffer. The spin column was then transferred to a new collection tube, and 50 µL of RNase-free water was added to the spin column membrane. The tubes were centrifuged at 8,000 r.p.m for 1 minute at RT to elute the RNA and miRNA. Total RNA concentration was determined using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, UK).

The ratio of absorbance between 260 nm/280 nm for all samples was over 2. RNA samples were stored at -80 °C until further analysis.
2.3.2.2 mRNA transcript analysis

Reverse transcription of mRNA

RNA was reverse transcribed into cDNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, UK). 20 ng of RNA sample, 1 µL of Oligo (dT)\textsubscript{18} primer, 4 µL of 5x reaction buffer, 1 µL of RiboLock RNase inhibitor (20 U.µL\textsuperscript{-1}), 2 µL 10mM dNTP mix and 1 µL of RevertAid M-MulVRT (200 U.µL\textsuperscript{-1}) were added together in a nuclease-free tube on ice, and each sample was adjusted with RNase free water to achieve a total volume of 20 µL. Each sample was gently mixed and centrifuged, and placed into a thermocycler (S1000, BioRad, UK) to undergo the cDNA synthesis reaction. Samples were heated to 25 °C for 5 minutes and then to 42 °C for 60 minutes. The reaction was terminated by heating to 70 °C for 5 minutes and then cooled to 4 °C. cDNA was stored at -20 °C until further analysis.

Quantitative real-time PCR

Forward and reverse primers were diluted to 100 µM stock solutions with nuclease-free water. A full list of primer sequences can be found in Table 2.3.

Each primer pair was tested on pooled cDNA to ensure amplification efficiency over 90%. Primer efficiencies were obtained by plotting Ct values of various amounts of pooled cDNA against log[cDNA] and calculated using the following equation:

\[
Primer \text{ efficiency} (%) = \left(10^{-\text{Slope}} - 1\right) \times 100
\]

The presence of a single amplification product was determined by gel electrophoresis (Figure 2.6).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adiponectin (AdipoQ)</td>
<td>Forward</td>
<td>ATCAAACTCTGGAGACCTCTATCTAC</td>
<td>(Kasimanickam, 2016)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTGCATTGCAGGCTCAAG</td>
<td></td>
</tr>
<tr>
<td>Adiponectin receptor 1 (AdipoR1)</td>
<td>Forward</td>
<td>GCTCCGGCTAGCAACAGGGC</td>
<td>(Kasimanickam, 2016)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCTGCAGGGGCACTGAGGC</td>
<td></td>
</tr>
<tr>
<td>Adiponectin receptor 2 (AdipoR2)</td>
<td>Forward</td>
<td>CCCAGTACCAGGGGGGCTGAGA</td>
<td>(Kasimanickam, 2016)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGAAGTGGACGAAACGCAA</td>
<td></td>
</tr>
<tr>
<td>Carnitine palmitoyl transferase 1 (CPT1)</td>
<td>Forward</td>
<td>CGACTGGTGAGGAGGAATACA</td>
<td>(Sharma et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TGCCTCTGAAAGCAGGATG</td>
<td></td>
</tr>
<tr>
<td>Cystathionine-β-synthase (CBS)</td>
<td>Forward</td>
<td>TGAGATTGGAGGAGGAGCcAC</td>
<td>(Lechuga et al., 2015)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCACACTGCTGAGGATCTC</td>
<td></td>
</tr>
<tr>
<td>Cystathionine-γ-lyase (CSE)</td>
<td>Forward</td>
<td>TTGTATGGATGATGTATGAGGAAGG</td>
<td>(Lechuga et al., 2015)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCAAAACAGCTTGTGTTCTGAGT</td>
<td></td>
</tr>
<tr>
<td>Endothelial NO synthase (eNOS)</td>
<td>Forward</td>
<td>TGGGCGGCGATCCAGTG</td>
<td>(Kasimanickam, 2016)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAACATCTCCTGTGCTGAGCTG</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase (G6PDH)</td>
<td>Forward</td>
<td>TGACCTATGGCAACGATACAA</td>
<td>(Vorachek et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCGCAAAAGACATCCAGGAT</td>
<td></td>
</tr>
<tr>
<td>Glucose transporter 1 (GLUT1)</td>
<td>Forward</td>
<td>ATCGTGGCCATCTCTGTTCTGAGT</td>
<td>(Botting et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTGGAAGCACAGTGGCAGCAGAA</td>
<td></td>
</tr>
<tr>
<td>Glucose transporter 3 (GLUT3)</td>
<td>Forward</td>
<td>AGAGTATGCGATGGAGAAGC</td>
<td>(Botting et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CACCGATAAGTGGGCAGGACC</td>
<td></td>
</tr>
<tr>
<td>Inducible NO synthase (iNOS)</td>
<td>Forward</td>
<td>AAGGCAGCCTGTGAGACATT</td>
<td>(Botting et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAGATTCTGCTGCGATTTGA</td>
<td></td>
</tr>
<tr>
<td>Insulin-like growth factor 2 (IGF2)</td>
<td>Forward</td>
<td>CTGTCGGACACCCCTCAGTT</td>
<td>(Kasimanickam, 2016)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCCGGAAACCGGTCGTA</td>
<td></td>
</tr>
</tbody>
</table>
General Methods

Table 2.3. Primers used in qRT-PCR.
All reagents from Sigma, UK.

For running experimental samples, 5 ng of sample cDNA, 300nM of each primer and 5 µL SYBR® Green PCR master mix (Thermo Fisher Scientific, UK) were added together in duplicate wells on a 96 well PCR Plate (MicroAmp™ Optical 96 well

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward</th>
<th>Reverse</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin 1β (IL-1β)</td>
<td>TCACAGGAATGAGCCGAGAA</td>
<td>CAGCTGCAGGGTGGTGATAT</td>
<td>(Kasimanickam, 2016)</td>
</tr>
<tr>
<td>Interleukin 6 (IL-6)</td>
<td>ACACCACCCAAGCAGACTACT</td>
<td>CCCAGATTGGAAGCATCCAT</td>
<td>(Kasimanickam, 2016)</td>
</tr>
<tr>
<td>Interleukin 8 (IL-8)</td>
<td>GCCAGAAGAAACCTGACAAAAAG</td>
<td>GCAGTGTGGCCACTCTCA</td>
<td>(Kasimanickam, 2016)</td>
</tr>
<tr>
<td>Peroxisome proliferator-activated receptor γ (PPARγ)</td>
<td>ATGTCTCATAATGCCATCAGGTT</td>
<td>GATAAAACGCGATTTGCTGTC</td>
<td>(Kasimanickam, 2016)</td>
</tr>
<tr>
<td>Placental growth factor (PIGF)</td>
<td>GCCCTTTCTTGAGGATGA</td>
<td>GTATCACCCGCACCTTTTG</td>
<td>(Ma et al., 2010)</td>
</tr>
<tr>
<td>Ribosomal protein L19 (RPL19)</td>
<td>AGCCTGTGACTGTCATCC</td>
<td>ACGTTACCTTCGCGC</td>
<td>(Vorachek et al., 2013)</td>
</tr>
<tr>
<td>Soluble endoglin (sEng)</td>
<td>GGACACAGGATAAGGCCCAAG</td>
<td>GCTTGGATGCCTGGAGAGTC</td>
<td>(Sunderland et al., 2011)</td>
</tr>
<tr>
<td>Soluble fms-like tyrosine kinase (sFlt-1)</td>
<td>GCCACGCCTGAATCTACCA</td>
<td>GGCCTTGAG CCTGGAGAGTC</td>
<td>(Kasimanickam, 2016)</td>
</tr>
<tr>
<td>Tumour necrosis factor α (TNFα)</td>
<td>GACCCTCCTC ATCCCTTTCT</td>
<td>AGCCCACCATGTCAAGGT</td>
<td>(Kasimanickam, 2016)</td>
</tr>
<tr>
<td>Vascular endothelial growth factor A (VEGF-A)</td>
<td>CGAAAGTCTGGAGTAGTGTC</td>
<td>TATGTGCTGGCTTTTG</td>
<td>(Ma et al., 2010)</td>
</tr>
</tbody>
</table>
reaction plate, Thermo Fisher, UK) and the volume in each well was adjusted to 10 µL with nuclease-free water. The plate was sealed and centrifuged for 1 minute at 1,000 r.p.m. The 7500 Fast Real-Time PCR machine (Applied Biosystems, UK) was used to run the amplification reaction by completing a hold stage of 5 minutes at 95 °C and 40 cycles of 15 seconds at 95 °C for denaturation and 1 minute at 60 °C for annealing and extension. After the reaction, the PCR plate was heated to generate a melt curve of the reaction products. Threshold and Ct values were determined automatically by the PCR machine. Samples were excluded, if duplicate samples presented Ct values with greater than 0.5 standard deviation difference, if the machine was unable to determine a Ct value due to insufficient amplification product detection, or if there were multiple peaks present in the melt curve.

![Gel electrophoresis of PCR amplification products.](image)

**Figure 2.6. Gel electrophoresis of PCR amplification products.**
Single bands indicate a single amplification product. Ladder (left) indicates size of the amplification product.

Expression levels of each sample were analysed by using the threshold cycle ΔΔCt method (Livak and Schmittgen, 2001). A total of six validated ovine reference genes, including 14-3-3 protein zeta/delta, succinate dehydrogenase complex flavoprotein subunit A, glyceraldehyde 3-phosphate dehydrogenase, glycophorin C, 60S ribosomal
protein L19 (RPL19) and glucose-6-phosphate dehydrogenase (G6PDH) were tested for efficiency, specificity and stability between normoxic and hypoxic groups (Vorachek et al., 2013). The Ct values of each gene of interest was compared to the Ct values of the two best-performing housekeeping genes, RPL19 and G6PDH, in order to control for differences in loading, extraction, RNA quality and RT efficiency, yielding the ΔCt. The differences in Ct values between the gene of interest and the housekeeping genes (ΔCt) were then compared to the mean ΔCt value of the control normoxic cohort, yielding the ΔΔCt. The value for expression fold change vs. control was then calculated using the following equation:

$$fold\ change\ (vs.\ control) = 2^{-\Delta\Delta C_t}$$

Neither RPL19 nor G6PDH placental transcript levels were affected by exposure to chronic hypoxia.

2.3.2.3 miRNA transcript analysis

Reverse transcription of miRNA

miRNA was reverse transcribed into cDNA using a miRCURY® LNA® RT Kit (Qiagen, UK). 10 ng of RNA sample, 2 µL of 5x miRCURY RT reaction buffer and 1 µL of miRCURY RT enzyme mix were added together in a nuclease-free tube on ice and each sample was adjusted with RNase free water to achieve a total volume of 10 µL. Each sample was gently mixed and centrifuged, and placed into a thermocycler (S1000, BioRad, UK) to undergo the cDNA synthesis reaction. Samples were heated to 42 °C for 60 minutes. The reaction was terminated by heating to 95 °C for 5 minutes and then cooled to 4 °C. cDNA was stored at -20 °C until further analysis.
Quantitative real-time PCR

The miRNA primer assays for miR-21 and miR-133b (miRCURY® LNA® PCR Assay, Qiagen, UK) were suspended in 220 µL nuclease-free water and incubated for 20 minutes at RT. Samples of cDNA were diluted 1:60 in nuclease-free water. 3 µL sample cDNA, 1 µL assay mix, 1 µL of nuclease-free water and 5 µL SYBR® Green PCR master mix (Thermo Fisher Scientific, UK) were added together in duplicate wells on a 96 well PCR Plate (MicroAmp™ Optical 96 well reaction plate, Thermo Fisher, UK). The plate was sealed and centrifuged for 1 minute at 1,000 r.p.m. The 7500 Fast Real-Time PCR machine (Applied Biosystems, UK) was used to run the amplification reaction by completing a hold stage of 2 minutes at 95 °C and 40 cycles of 10 seconds at 95 °C for denaturation and 1 minute at 56 °C for annealing and extension. After the reaction, a melt curve was generated and threshold values for each sample were obtained and analysed as previously described in section 2.3.2.2. The Ct values of the miRNA of interest were compared to the Ct values in the reference miRNAs miR-26b and let-7f-5p (miRCURY® LNA® PCR Assay, Qiagen, UK), and the value for expression fold change vs. control was calculated as previously described. Neither reference gene was affected by exposure to chronic hypoxia.

2.3.3 Placental enzyme activity analysis

For placental enzyme activity analysis, tissue lysates were prepared from flash frozen type A placentomes. Tissue was homogenised to powder using pestle and mortar while keeping tissues frozen on dry ice and then further homogenised in Eppendorf tubes with homogenisation buffer (300 µL per 10 mg of tissue; Table 2.4) using a plastic pestle. The samples were centrifuged for 30 seconds at 2,000 r.p.m. at 4 °C, and supernatants were collected in fresh Eppendorf tubes. Protein concentration was
determined as previously described in section 2.3.1.1. All lysates were diluted to reach a protein concentration of 2 µg.µL⁻¹ and stored at -80 °C until further processing.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Homogenisation buffer</strong></td>
<td>20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid 1 mM ethylenediaminetetraacetic acid 0.1% Triton X-100 50 mM sodium fluoride 10 mM dichloroacetate pH adjusted to 7.2 with 10N potassium hydroxide</td>
</tr>
<tr>
<td><strong>Citrate synthase assay buffer</strong></td>
<td>20 mM trisaminomethane base (pH 8.0) 0.1 mM 5,5′-dithiobis-(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td><strong>Triethanolamine – hydrogen chloride buffer</strong></td>
<td>0.5 M triethanolamine pH adjusted to 8.0 with 10N hydrogen chloride 5mM ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td><strong>Hexokinase assay buffer</strong></td>
<td>40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid pH adjusted to 7.0 with 10N hydrogen chloride 10 mM magnesium chloride 8.7 mM ATP</td>
</tr>
<tr>
<td><strong>Pyruvate dehydrogenase assay buffer</strong></td>
<td>50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid 1 mM magnesium chloride 0.08 mM egtazic acid 1 mM dithiothreitol pH adjusted to 7.2 with 10N potassium hydroxide</td>
</tr>
<tr>
<td><strong>Lactate dehydrogenase assay buffer</strong></td>
<td>50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid pH adjusted to 7.0 with 10N potassium hydroxide</td>
</tr>
<tr>
<td><strong>Hydrogen sulphide production assay buffer</strong></td>
<td>2 mM L-cysteine 2 mM pyridoxal phosphate 100 mM potassium phosphate</td>
</tr>
</tbody>
</table>

**Table 2.4. Buffer compositions for enzyme activity assays.**

All reagents from Sigma, UK.

**2.3.3.1 Citrate synthase activity assay**

Citrate synthase activity was measured at 37°C using an Evolution 220 spectrophotometer (Thermo Fisher Scientific, USA) at 412 nm, as previously described (Horscroft et al., 2019). The assay uses saturating concentrations of substrates and cofactors and detects the transfer of sulphhydryl groups to 5,5′-
dithiobis(2-nitrobenzoic acid), according to the following reaction:

\[
\text{acetyl coASH} + \text{oxaloacetate} + H_2O \rightarrow \text{coASH} + \text{citrate}
\]

\[
\text{citrate synthase}
\]

\[
\text{coASH} + \text{DTNB} \rightarrow \text{TNB} + \text{coSSTNB}
\]

In brief, 20 µL of tissue lysate, 50 µL of 10 mM oxaloacetate and 905 µL of citrate synthase assay buffer (Table 2.4) were added to a UV quartz cuvette (Sigma, UK) with a stirrer. The cuvette was placed inside the spectrophotometer to determine a blank value and allowed to warm for 3 minutes. After absorbance was measured for 3 minutes, 25 µL of 12 mM acetyl coA were added to start the reaction, and after 10 seconds, absorbance was measured for another 3 minutes. Citrate synthase enzyme activity was calculated using the Beer-Lambert Law:

\[
\Delta \text{Absorbance} = \epsilon \times \Delta \text{concentration} (M) \times \text{Length of light path (cm)}
\]

Molar extinction coefficient for DTNB \( \epsilon_{412} = 13,600 \, M^{-1} \, cm^{-1} \)

### 2.3.3.2 Hexokinase activity assay

Hexokinase activity was measured at 37°C using an Evolution 220 spectrophotometer (Thermo Fisher Scientific, USA) at 340 nm, as previously described (Horscroft et al., 2019). The assay uses saturating concentrations of substrates and cofactors and detects the production of nicotinamide adenine dinucleotide (reduced; NADH), according to the following reaction:

\[
\text{glucose} + \text{MgATP} \rightarrow \text{G6P} + \text{ADP}
\]

\[
\text{hexokinase}
\]

\[
\text{G6P} + \text{NAD} \rightarrow \text{6PGL} + \text{NADH}
\]

\[
\text{G6PDH}
\]
In brief, 8 µL of 250 mM nicotinamide adenine dinucleotide (oxidised; NAD), 2 µL of 5.67 U·µL⁻¹ G6PDH, 25 µL of 200 mM glucose and 890 µL of hexokinase assay buffer (Table 2.4) were added to a UV quartz cuvette (Sigma, UK) with a stirrer. The cuvette was placed inside the spectrophotometer to determine a blank value and allowed to warm for 10 minutes. After absorbance was measured for 5 minutes, 75 µL of tissue lysate were added to start the reaction, and after 10 seconds, absorbance was measured for another 5 minutes. Hexokinase enzyme activity was calculated using the Beer-Lambert Law:

\[
\Delta \text{Absorbance} = \varepsilon \times \Delta \text{concentration} \times \text{Length of light path (cm)}
\]

Molar extinction coefficient for DTNB \( \varepsilon_{340} = 6,220 \text{ M}^{-1}\text{cm}^{-1} \)

### 2.3.3.3 Pyruvate dehydrogenase activity assay

PDH activity was measured at 30°C using an Evolution 220 spectrophotometer (Thermo Fisher Scientific, USA) at 340 nm. The assay uses saturating concentrations of substrates and cofactors and detects the production of nicotinamide adenine dinucleotide (reduced; NADH) according to the following reactions:

\[
\text{pyruvate} + \text{coA} + \text{NAD} \rightarrow \text{acetyl coA} + \text{NADH} + \text{CO}_2
\]

PDH (TPP, Mg²⁺)

In brief, 8 µL of 0.5 mM rotenone, 2 µL of 1 U·µL⁻¹ LDH, 25 µL of 4 mM coenzyme A, 10 µL of 20 mM thiamine pyrophosphate and 925 µL of PDH assay buffer (Table 2.4) were added to a UV quartz cuvette (Sigma, UK) with a stirrer. The cuvette was placed inside the spectrophotometer to determine a blank value and allowed to warm for 5 minutes. After absorbance was measured for 2 minutes, 30 µL of tissue lysate were added to start the reaction, and after 10 seconds, absorbance was measured for
another 2 minutes. PDH enzyme activity was calculated using the Beer-Lambert Law:

$$\Delta \text{Absorbance} = \varepsilon \times \Delta \text{concentration (M)} \times \text{Length of light path (cm)}$$

Molar extinction coefficient for NADH $\varepsilon_{340} = 6,220 \, M^{-1} \, cm^{-1}$

### 2.3.3.4 Lactate dehydrogenase activity assay

LDH activity was measured at 37°C using an Evolution 220 spectrophotometer (Thermo Fisher Scientific, USA) at 340 nm, as previously described (Horscroft et al., 2019). The assay uses saturating concentrations of substrates and cofactors and detects the disappearance of NADH according to the following reaction:

$$\text{pyruvate} + \text{NADH} \rightarrow \text{lactate} + \text{NAD}$$

$\text{LDH}$

In brief, 30 μL of tissue lysate, 20 μL of 15 mM NADH and 946 μL of LDH assay buffer (Table 2.4) were added to a UV quartz cuvette (Sigma, UK) with a stirrer. The cuvette was placed inside the spectrophotometer to determine a blank value and allowed to warm for 4 minutes. After absorbance was measured for 3 minutes, 4 μL of 50 mM pyruvate were added to start the reaction, and after 10 seconds, absorbance was measured for another 3 minutes. LDH enzyme activity was calculated using the Beer-Lambert Law:

$$\Delta \text{Absorbance} = \varepsilon \times \Delta \text{concentration (M)} \times \text{Length of light path (cm)}$$

Molar extinction coefficient for NADH $\varepsilon_{340} = 6,220 \, M^{-1} \, cm^{-1}$

### 2.3.3.5 Hydrogen sulphide production assay

The level of placental $H_2S$ production in placental tissue was determined using a zinc acetate assay according to the following reactions:
General Methods

\[ \text{zinc acetate} + \text{hydrogen sulphide acid} \rightarrow \text{zinc sulphide} + \text{acetic acid} \]

\[ \text{zinc sulphide} + N,N-,\text{diimethyl}-4-\text{phenylenediamine} \rightarrow \text{methylene blue} + \text{NAD} \]

\[ \text{ferric chloride} \]

In brief, 200 µL of sample or sodium H\(_2\)S standards of known concentration ranging from 0 to 100 µM were mixed with 800 µL of H\(_2\)S production assay buffer (Table 2.4) in an Eppendorf tube while kept on ice. The Eppendorf was sealed with Parafilm® (Sigma-Aldrich, UK) to prevent gas from escaping, and then incubated for 1 hour at 37 °C. The tubes were returned to ice to stop the reaction. 500 µL of 10% zinc acetate was injected into the tubes using a needle and syringe and the samples were agitated for 20 minutes at 4 °C. After removing the Parafilm®, the tubes were centrifuged at maximum speed at 4 °C for 10 minutes, and the supernatant was decanted. The remaining pellet of zinc sulphide was resuspended in 500 µL ferric chloride and 500 µL dimethyl-4-phenylenediamine. The tubes were covered in aluminium foil to protect from light and incubated for 40 minutes at RT, after which the absorbance was measured at 670 nm in a spectrophotometer (Thermo GENESYS™ 10 UV, Thermo Fisher Scientific, UK). The standard curve was plotted in Microsoft Excel, and the concentration in each sample was determined by using the equation of a best-fit trendline. All measurements fell within the linear range.

2.3.4 Tissue metabolite analysis

For tissue metabolite analysis, tissue lysates were prepared from flash frozen type A placentomes, maternal liver and maternal skeletal muscle. Tissue was homogenised to powder using pestle and mortar while keeping tissues frozen on dry ice. Homogenates were further homogenised in Eppendorf tubes with ice-cold deionised water (200 µL per 10 mg of tissue) using a plastic pestle. The samples were incubated
for 10 minutes at 100 °C to inhibit enzyme activity. Samples were centrifuged for 5 minutes at maximum speed at 4 °C, and supernatants were collected in fresh Eppendorf tubes. All lysates were stored at -20 °C until further processing. Concentrations of glucose and lactate in tissue lysates were measured using a Yellow Springs 2700D Select Biochemistry Analyser (YSI Ltd., UK). Placental and skeletal muscle samples showed no detectable levels of tissue glucose. Concentration of glycogen in tissue lysates was measured using a commercially available colorimetric glycogen assay kit (AB169558, Abcam, UK) according to manufacturer's instructions. In the assay, glycogen is hydrolysed into glucose, which is further oxidised to form an intermediate. This intermediate reduces a colourless probe to a coloured product, the absorbance of which can be measured at 450 nm. Up to 50 µL of standards or tissue lysates were added to duplicate wells on a 96 well plate. All wells were volume adjusted to 50 µL using glycogen hydrolysis buffer. 2 µL of hydrolysis enzyme mix were added to standards and samples, but not to background controls, and the plate was incubated at RT for 30 minutes. 2 µL of development enzyme mix and 2 µL of the probe were added to each well, and all wells were volume adjusted to a total of 100 µL using glycogen development buffer. The plate was incubated at RT for 30 minutes and the optical density was read at 450 nm absorbance using a microplate reader (BioTek ELx800 absorbance microplate reader). A standard curve was plotted in Microsoft Excel, and the concentration in each sample was determined by using the equation of a best-fit trendline. All measurements fell within the linear range. Background control measurements from liver samples were subtracted from measurements of hydrolysed samples to control for endogenous glucose levels. The inter- and intra-assay coefficients of variation were <10.0% for both, and the lower limit of detection was 4 µg.ml⁻¹.
2.3.5 Plasma and urine biochemical analysis

Maternal plasma samples taken on 103, 105 and 138 dGA and maternal urine samples taken on 138 dGA were analysed for the following components:

2.3.5.1 Angiogenic factors

Plasma sFlt-1 (Cat. No. MBS736347), sEng (Cat. No. MBS039643), VEGF-A (Cat. No. MBS7700771) and PI GF (Cat. No. MBS736570) were measured by commercially available ELISA kits (MyBioSource, USA). Up to 100 µL of standards, previously unthawed EDTA treated plasma samples or phosphate buffered saline were added to duplicate wells coated with antibodies on a 96 well plate. All wells were volume adjusted to 100 µL using sample diluent and mixed with 100 µL horseradish peroxidase (HRP) conjugate. The plates were sealed using a plastic membrane and incubated at 37 °C for 60 minutes. The contents of the plate were discarded by gently tapping the plate on a paper towel and the wells were washed with wash buffer 3 times. 50 µL each of chromogen solutions A and B containing 3,3',5,5'-tetramethylbenzidine were added to the wells and mixed. The colour developing reaction was incubated for 15 minutes at 37 °C, after which it was stopped by adding 50 µL of stop solution to each well. The optical density was read at 450 nm absorbance using a microplate reader (BioTek ELx800 absorbance microplate reader). A standard curve was plotted in Microsoft Excel, and the concentration in each sample was determined by using the equation of a best-fit trendline. All measurements fell within the linear range. Measurements from plasma samples taken on 103 and 105 dGA before the onset of chronic normoxia or hypoxia were averaged as baseline measurements.

For sFlt-1, the inter- and intra-assay coefficients of variation were <12.0% and <10.0%, respectively, and the lower limit of detection was 0.1 ng.ml⁻¹. For sEng, the inter- and
intra-assay coefficients of variation were <15.0% for both, and the lower limit of detection was 0.1 ng.ml\(^{-1}\). For PIGF, the inter- and intra-assay coefficients of variation were <10.0% for both, and the lower limit of detection was 1.0 pg.ml\(^{-1}\). For VEGF, the inter- and intra-assay coefficients of variation were <15.0% and <10.0%, respectively, and the lower limit of detection was 1.0 pg.ml\(^{-1}\) units.

### 2.3.5.2 Creatinine and estimated glomerular filtration rate

Plasma and urine concentrations of creatinine were measured using a commercially available colourimetric kit (AB65340, Abcam, UK). 50 µL of standards, 10 µL of previously unthawed EDTA plasma samples or 20 µL of previously unthawed urine samples were added to duplicate wells on a 96 well plate. All wells were volume adjusted to 50 µL using assay buffer. Standard and sample wells were mixed with 50 µL of reaction mix containing assay buffer, creatinase, creatininase, enzyme mix and creatinine probe, while blank control wells were mixed with 50 µL of background control mix containing assay buffer, creatinase, enzyme mix and creatinine probe. The reaction was incubated at 37 °C for 60 minutes. The optical density was read at 570 nm absorbance using a microplate reader (BioTek ELx800 absorbance microplate reader). A standard curve was plotted in Microsoft Excel, and the concentration in each sample was determined by using the equation of a best-fit trendline. All measurements fell within the linear range.

The inter- and intra-assay coefficients of variation were < 5.0% for both, and the lower limit of detection was 2.0 µg.ml\(^{-1}\).

Glomerular filtration rate was estimated from the predicted clearance of creatinine from measurements of plasma concentrations of creatinine according to the Cockcroft-Gault equation, which is used routinely to approximate renal function in a clinical
setting (Cockcroft and Gault, 1976):

\[
Creatinine clearance (\frac{ml}{min}) = \frac{(140 - age) \times body \ weight \ (kg)}{72 \times serum \ creatinine (\frac{mg}{dl})}
\]

The main source of inaccuracy while estimating glomerular filtration rate arises from differences in body composition (Chudleigh et al., 2008). The Cockcroft-Gault equation was deemed the most appropriate calculation in this instance, as it takes into account the body weight of the subject, which is absent in other estimations, such as in the Modification of Diet in Renal Disease equation (Cockcroft and Gault, 1976; Levey et al., 2000). However, it is important to note that the Cockcroft-Gault equation has not been validated to estimate glomerular filtration rate in sheep and needs to be investigated further through 24-hour urine collection in the future.

2.3.5.3 Total protein

Urine concentrations of total protein were measured using a commercially available bicinchoninic assay kit (23235; Thermo Fisher, UK). 100 µL of BSA standards (Sigma, UK) or diluted urine were added to duplicate wells on a 96 well plate. All wells were mixed with 100 µL of reaction mix containing bicinchoninic acid and copper sulphate. The reaction was incubated at 37 °C for 2 hours. The optical density was read at 570 nm absorbance using a microplate reader (BioTek ELx800 absorbance microplate reader). A standard curve was plotted in Microsoft Excel, and the concentration in each sample was determined by using the equation of a best-fit trendline. All measurements fell within the linear range.

The inter- and intra-assay coefficients of variation were <5.0% and <3.0%, respectively, and the lower limit of detection was 2 µg.ml⁻¹.
2.4 Placental and renal histology

2.4.1 Kidney perfusion fixation

The left maternal kidney was perfusion fixed immediately post-mortem. The renal capsule was carefully removed, and the kidney was placed in a container with 0.012% papaverine hydrochloride (Sigma, UK) in saline (0.9% sodium chloride; Sigma, UK). A catheter connected to a gravity-fed perfusion system was inserted into the renal artery and secured using silk ties. The kidney was perfused with saline (0.9% sodium chloride; Sigma, UK) at 80 mmHg, which is equivalent to mean arterial blood pressure in pregnant ewes at the end of gestation (Metcalfe and Parer, 1966). 0.1 ml of 1.2% of papaverine hydrochloride (Sigma, UK) was injected to prevent arterial constriction. After all blood was cleared from the kidney, the perfusion solution was switched to 4% paraformaldehyde (Sigma, UK), and the kidney was perfused until it turned stiff and pale. It was then placed in a container filled with 4% paraformaldehyde (Sigma, UK), transferred to 70% ethanol after 24 hours and stored at 4 °C until further analysis.

2.4.2 Paraffin embedding and sectioning

Type A placentomes were cut in half, while kidneys were cut sequentially into halves until they were able to fit into histological cassettes (Thermo Scientific™ Shandon™ Tissue Cassettes I, Fisher Scientific, UK). Tissues were dehydrated in a series of ethanol solutions (70%, 90%, 100%), followed by Histo-Clear™ (Fisher Scientific, UK) and placed in warm paraaffin (Agar Scientific, UK) overnight in a tissue processor (Leica TP 1020, Leica Biosystems, UK). Tissues were embedded in paraaffin blocks using an embedding machine (Leica EG 1150, Leica Biosystems, UK). Each block was then sectioned at 7 µm thickness on an ultramicrotome (Leica RM 2235, Leica Biosystems, UK), floated in a water bath at 40 °C, mounted onto glass slides (Superfrost Plus™
adhesion slides, Fisher Scientific, UK) and dried overnight at 37 °C.

2.4.3 Immunohistochemistry

In the present study, Western blotting was used to quantify markers of interest in whole placentomal lysates from normoxic and hypoxic pregnancies. Due to the anatomy of the ovine placentome, separation of maternal, trophoblast and fetal tissues at post-mortem is not feasible. Thus, to complement the quantitative Western blotting data, sectioned placentomes were immunostained to localise the signal activation of the ER, cytosolic and mitochondrial unfolded protein response pathways (UPR_{ER}, UPR_{Cy} and UPR_{mt}) to fetal, maternal or trophoblastic components of the placentome (shown on a haematoxylin and eosin stain in Figure 2.7) and to subcellular compartments, such as the nucleus or the mitochondria. Immunohistochemistry was only used to pinpoint the localisation of the signal, but not to quantify the strength of the signal. In many cases, differences in protein levels could not be visualised using global staining methods.

Figure 2.7. Placentome morphology.
Haematoxylin and eosin showing general placentome morphology. (A) Overview showing fetal cotyledon with branching villi (v) invading the maternal caruncle. The placentome is surrounded by uterine myometrium (mm) and endometrial glands (eg). Scale bar = 5 mm. (B) 40x magnification of a fetal stem villus (sv) surrounded by trophoblast (t) containing binucleate cells (b) and syncytial giant cells (syn). Maternal uterine stroma (m) is adjacent covered in uterine epithelium and invaded by smaller fetal villi (v). Scale bar = 50 µm.
Sections were rehydrated and incubated in 3% hydrogen peroxide (Fisher Scientific, UK) for 15 minutes to block endogenous peroxidase activity. After rinsing in distilled water, the sections were incubated in Tris-buffered saline containing 0.1% Tween 20 and 0.1% Triton X-100 (TBS-TT) for 30 minutes. After rinsing in TBS, slides were blocked in 5% BSA in TBS for 1 hour and then incubated overnight in primary antibody diluted in 5% BSA. A full list of primary antibodies, their host species, dilutions, and catalogue numbers is found in Table 2.5.

### Table 2.5. Primary antibodies used in immunohistochemistry.

Primary antibodies, their host species, dilutions, incubation times and catalogue numbers used in immunohistochemistry.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Properties</th>
<th>Catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Rabbit, 1:200, overnight at 4°C</td>
<td>Abcam Cat# ab184923, RRID:AB_2800462</td>
</tr>
<tr>
<td>(ATF5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activating transcription factor 6</td>
<td>Mouse, 1:200, overnight at 4°C</td>
<td>Abcam Cat# ab11909, RRID:AB_298691</td>
</tr>
<tr>
<td>(ATF6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystathionine-β-synthase (CBS)</td>
<td>Rabbit, 1:200, overnight at 4°C</td>
<td>Sigma-Aldrich Cat# HPA001223, RRID:AB_1846112</td>
</tr>
<tr>
<td>Cystathionine-γ-lyase (CSE)</td>
<td>Rabbit, 1:200, overnight at 4°C</td>
<td>Proteintech Cat# 12217-1-AP, RRID:AB_2087497</td>
</tr>
<tr>
<td>Heat shock protein 60 (HSP60)</td>
<td>Rabbit, 1:200, overnight at 4°C</td>
<td>Abcam Cat# ab46798, RRID:AB_881444</td>
</tr>
<tr>
<td>Heat shock protein 70 (HSP70)</td>
<td>Rabbit, 1:200, overnight at 4°C</td>
<td>Enzo Life Sciences Cat# ADI-SPA-812-J, RRID:AB_11177999</td>
</tr>
<tr>
<td>Proliferating cell nuclear antigen</td>
<td>Rabbit, 1:200, overnight at 4°C</td>
<td>Abcam Cat# ab34747, RRID:AB_777130</td>
</tr>
<tr>
<td>(PCNA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein disulphide isomerase (PDI)</td>
<td>Rabbit, 1:200, overnight at 4°C</td>
<td>Abcam Cat# ab3672, RRID:AB_303990</td>
</tr>
</tbody>
</table>

The following day, sections were washed using TBS-TT and then incubated in biotinylated secondary antibody against Rabbit IgG (Vector Laboratories, Canada; diluted 1:200) in 5% BSA. After washing in TBS-TT, staining was visualised using the VECTASTAIN avidin-biotin complex method (Vector Laboratories, Canada) by adding metal DAB (Thermo Fisher Scientific, UK) as the chromogen for 2 to 5 minutes.
Staining was stopped by rinsing in distilled water. Sections were dehydrated and cover slips were mounted using DPX Mountant (Sigma-Aldrich, UK). Slides were left to dry for 48 hours before scanning (NanoZoomer 2.0 RS, Hamamatsu, UK) and visualising (NDP.view 2 viewing software U12388, Hamamatsu, UK).

2.4.3.1 Analysis of cell proliferation

PCNA staining was used to quantify the number of proliferating cells in placental sections. 20 images per section were taken at 40x magnification through systematic random sampling of the trophoblast area of whole placentomal cross sections (Figure 2.8). The number of stained nuclei in each image was counted and averaged across all images for each placentome.

![Figure 2.8. PCNA immunostain.](image)

(A) Overview of placentome immunostained with PCNA antibody. Scale bar = 5 mm. (B) 40x magnification of proliferating nuclei stained with PCNA. Scale bar = 50 µm.

2.4.4 Periodic acid-Schiff stain

Periodic acid-Schiff staining was used to visualise the general tissue morphology of maternal kidney sections. Sections were rehydrated and covered in 0.5% periodic acid (Sigma-Aldrich, UK) solution for 5 minutes, rinsed in distilled water and then covered in Schiff’s reagent (Sigma-Aldrich, UK) for 15 minutes. Sections were washed under running tap water for 5 minutes and then counter-stained in haematoxylin (Sigma-
Aldrich, UK) for 2 minutes. After further washing, sections were dehydrated and cover slips were mounted using DPX Mountant (Sigma-Aldrich, UK). Slides were left to dry for 48 hours before scanning (NanoZoomer 2.0 RS, Hamamatsu, UK) and visualising (NDP.view 2 viewing software U12388, Hamamatsu, UK; Figure 2.9).

![Figure 2.9. Periodic acid-Schiff stain of kidney section.](image)

(A) Overview showing renal cortex (c) and medulla (m). Scale bar = 5 mm. (B) 20x magnification of a glomerulus (g) surrounded by Bowman’s space (B) and proximal tubules (pt). Scale bar = 100 µm. (C) 40x magnification of the outer medulla showing proximal tubules (pt) with a brush border, thin segments of the loop of Henle (t), distal tubules (dt) and collecting ducts (cd). Scale bar = 50 µm

The cortical region of each kidney section was viewed through systematic random sampling at 20x magnification for 25 fields of view. Measurements for renal corpuscle area and glomerular area were taken, and total diameter and lumen diameter of 2 non-adjacent proximal tubules were measured. Urinary space and proximal tubule wall thickness were calculated according to the following equations:

\[
\text{Urinary space (mm}^2\) = \text{renal corpuscle area (mm}^2\) – \text{glomerular area (mm}^2\)
\]

\[
\text{Proximal tubule wall thickness (µm) =} \frac{\text{total diameter (µm) – lumen diameter(µm)}}{2}
\]
Values were averaged across all images for each section. Each sampled glomerulus was also scored for glomerulosclerosis ranging from Stage I to Stage IV (Figure 2.10) and the glomerulosclerosis index was calculated across all images for each section.

![Stage I](image1.png) ![Stage II](image2.png) ![Stage III](image3.png) ![Stage IV](image4.png)

**Figure 2.10. Stages of glomerulosclerosis.**
Glomerulosclerosis is scarring of renal glomeruli and evident through thickening of the glomerular basement membrane and capillary tuft (stains dark purple). The stage of glomerulosclerosis depends on the amount of scar tissue present ranging from Stage I (<25%) to Stage IV (>75%).

### 2.4.5 Picrosirius red stain

Picrosirius red staining was used to visualise collagen in maternal kidney and placentome sections. Sections were rehydrated and covered in 0.1% Direct Red 80 in saturated picric acid (both from Sigma-Aldrich, UK) for 1 hour and then washed in 0.5%
acetic acid (Sigma-Aldrich, UK) twice. Sections were dehydrated and cover slips were mounted using DPX Mountant (Sigma-Aldrich, UK). Slides were left to dry for 48 hours before scanning (NanoZoomer 2.0 RS, Hamamatsu, UK) and visualising (NDP.view 2 viewing software U12388, Hamamatsu, UK; Figure 2.11).

![Figure 2.11. Picrosirius red stain of placental and kidney sections.](image)

(A) Overview of type A placentome. Scale bar = 5 mm. (B) 20x magnification of type A placentome. Scale bar = 100 µm. (C) Overview of kidney section. Scale bar = 5 mm. (D) 20x magnification of renal cortex showing glomerulus. Scale bar = 100 µm. (E) 20x magnification of renal medulla. Scale bar = 100 µm.

20 images per section were taken at 20x magnification through systematic random sampling of the trophoblast area of whole placental and whole renal cross sections, of the renal cortex and of the renal medulla. The area fraction of collagen was determined using the green channel colour threshold function on ImageJ (NIH, RRID:SCR_003070) and averaged across all images for each section.
2.4.6 Transmission electron microscopy

Tissue processing, embedding and sectioning for transmission electron microscopy was performed by the Cambridge Advanced Imaging Centre. Training for transmission electron microscopy imaging was provided by the Cambridge Advanced Imaging Centre.

In brief, small pieces of type A placentomes were fixed immediately post-mortem by immersion in ice-cold 2 mM calcium chloride in 0.05M sodium cacodylate buffer at pH 7.4 containing 2% glutaraldehyde and 2% formaldehyde, provided by the Cambridge Advanced Imaging Centre.

The tissues were then transported to the Cambridge Advanced Imaging Centre, where they were fixed overnight at 4°C and osmicated in 0.05M sodium cacodylate buffer at pH 7.4 containing 1% osmium tetroxide and 1.5% potassium ferricyanide for three days at 4°C. They were then treated with 0.1% thiocarbohydrazide for 20 minutes in the dark at RT and osmicated a second time in 2% osmium tetroxide and stained in 0.05 M maleate buffer at pH 5.5 containing 2% uranyl acetate for three days at 4°C. The tissues were dehydrated in ascending concentrations of ethanol to 100% ethanol and then in 100% dry acetone and dry acetonitrile. They were embedded in Quetol epoxy resin over the course of 11 days. 80 nm sections were cut on an Ultracut UCT (Leica, Germany) and mounted onto 400 mesh bare copper grids.

Transmission electron microscopy was performed at the Cambridge Advanced Imaging Centre on an FEI Tecnai G2 transmission electron microscope run at 200 keV accelerating voltage and using a 20 µm objective aperture to improve contrast. Images were taken using an AMT camera (Figure 2.12).
Figure 2.12. Transmission electron microscopy.

(A) Figure taken from Das, 2014 (Copyright © 2014, The Author). Schematic diagram of transmission electron microscopy. A beam of electrons is transmitted through a specimen to

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General Methods
form an image, which is then magnified and focussed onto a fluorescent screen. (B) The FEI Tecnai G2 transmission electron microscope at the Cambridge Advanced Imaging Centre (Copyright © 2020, University of Cambridge) (C) Electron micrograph of the feto-maternal microvillous junction (ju) in the ovine placenta taken at 2,500x magnification. The trophoblast (t) shows mitochondria (mt) in its apical portion while the maternal uterine epithelium (ut) contains membrane-bound vesicles (v) close to the microvillous junction. A syncytial nucleus (nu) can be seen on the bottom right. The cytoplasm of a binucleate cell (bn) can be seen on the top left, which is more electron-dense than the surrounding trophoblast.

2.5 High-resolution tissue oxygraphy

At post-mortem, small strips of type A and type D placentomes were placed in ice-cold BIOPS preservation solution (Table 2.6). A dissecting microscope (Stemi 2000, Zeiss, UK) and forceps were used to loosen up the tissue, which was then placed in an ice-cold BIOPS solution containing 0.4 M saponin (Sigma-Aldrich, UK) and incubated with gentle agitation for 20 minutes on ice to permeabilise the cell membrane, while leaving mitochondrial membranes intact. The tissue was then washed with ice-cold mitochondrial respiration medium (MiR05, Table 2.6) 3 times for 5 minutes to remove the cytosol and endogenous substrates. The tissue was blotted on filter paper and weighed before placing in a chamber filled with MiR05. All respirometry was run in duplicate in an Oxygraph-2K (Oroboros Instruments, Austria; Figure 2.13A) at 37 °C.

The substrate-uncoupler-inhibitor titration protocol (SUIT protocol; Figure 2.13C) used was adapted from previously described methods, and optimised for ovine placental tissue in collaboration with Dr. Andrew Murray (Pesta and Gnaiger, 2012; Horscroft et al., 2019). It was developed to assess mitochondrial respiratory control in the three coupling control states leak respiration (LEAK), OXPHOS and electron transport (ET) in a sequence of pathway control states (Gnaiger, 2012).
Table 2.6. Buffers and solutions for high resolution oxygraphy.

All reagents from Sigma, UK.

The respiratory medium was hyperoxygenated periodically throughout the experiment by lifting the stopper of the chamber slightly, injecting pure oxygen into the gas phase using a syringe and needle, and resealing the chamber with the stopper once an oxygen concentration of 400 µM was reached. Thus, oxygen concentration within the chambers could be maintained between 200 and 400 µM to control for differences associated with oxygen diffusion.

Unit-specific oxygen consumption allows internal normalisation to maximum ET capacity ($G_{MS_E}$) following the addition of FCCP. This provides a measure of oxygen consumption per mitochondrial unit present in the chamber, independent of mitochondrial content, and was calculated as follows:

$$\text{Unit specific oxygen consumption} = \frac{\text{Mass specific oxygen consumption}}{\text{Mass specific } G_{MS_E}}$$
Flux control ratios (FCRs) normalised to maximum flux in the same coupling control state express respiratory activity independent of mitochondrial content. They were calculated as internal controls according to the following equations:

\[
FCR_F = \frac{OctM_p}{GMS_p}
\]

\[
FCR_N = \frac{GM_p}{GMS_p}
\]

\[
FCR_S = \frac{S_E}{GMS_E}
\]

Substrate control ratios (SCRs) express relative change of flux in response to a transition between pathway control states due to a change in substrate availability at the same mitochondrial coupling control state. They were calculated according to the following equations:

\[
SCR_{FA} = \frac{OctM_p}{PM_p}
\]

\[
SCR_{P} = \frac{PM_p}{GM_p}
\]

Coupling efficiencies express OXPHOS or ET capacities corrected for LEAK, conceptualised as “free” OXPHOS or ET capacities. They were calculated according to the following equations:

\[
OXPHOS \ coupling \ efficiency = 1 - \frac{OctM_L}{GMS_p}
\]

\[
ET \ coupling \ efficiency = 1 - \frac{OctM_L}{GMS_E}
\]
Figure 2.13. High resolution tissue oxygraphy.

(A) Oroboros Instruments O2k-FluoRespirometer for high-resolution respirometry (Copyright
© 2020, Oroboros Instruments GmbH). 10-20 mg of permeabilised placental tissue was monitored for oxygen consumption in duplicate. (B) Mitochondrial respiratory control and pathway control states in the ETC (Gnaiger, 2012). Glycolysis, β-oxidation and the citric acid cycle reduce NAD* to NADH and FAD to FADH₂, which donate electrons to the ETC via the N-pathway through complex I and the F-pathway through electron-transferring flavoprotein complex, respectively. Succinate supports electron flux via the S-pathway to FADH₂ bound to complex II. The F-pathway, N-pathway and S-pathway converge through electron flow at the Q-junction and further downstream via complex III, cytochrome c and complex IV to oxygen, the final electron acceptor during aerobic respiration. In the absence of ADP, oxygen consumption occurs due to proton leak across the inner mitochondrial membrane (LEAK). In the presence of saturating concentrations of ADP, ATP synthase (complex V) activity supports OXPHOS. In the presence of a protonophore, ET and proton flux occur uncoupled from ATP production. (C) SUIT protocol for analysis of mitochondrial function of preserved placentome tissue. LEAK respiration (OctMₐ) was measured after addition of 0.2 mM octanoyl carnitine and 2mM malate. OXPHOS capacity via the fatty acid pathway control state (F-pathway) dependent on β-oxidation (OctMₚ) was measured after the addition of 5 mM ADP. Additional OXPHOS capacity dependent on electron flux through the NADH pathway control state (N-pathway) via complex I was measured after the addition of 5 mM pyruvate (PMₚ) and of 10 mM glutamate (GMₚ) as substrates. Additional OXPHOS capacity dependent on electron flux through the succinate pathway control state (S-pathway) via complex II (GMSₚ) was measured after the addition of 10 mM of succinate as a substrate. To assess the integrity of the outer mitochondrial membrane, 10 µM of cytochrome c was added to ensure that OXPHOS was not limited by cytochrome c leakage. Uncoupled maximum ET capacity (GMSₑ) through convergent electron flow via the Q-junction was measured by titrating up to 0.25 µM of the protonophore carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP). ET capacity limited to complex II (Sₑ) was measured after the addition of 0.5 µM of rotenone to inhibit ET at complex I. Residual oxygen consumption (ROX) was measured after titration of up to 2.5 µM of antimycin A (Ama) to inhibit ET at complex III. ET capacity of complex IV (Tmₑ) was measured after the addition of 2mM ascorbate, immediately followed by addition of 0.5 mM N′-tetramethyl-p-phenylenediamine (AsTm). Non-mitochondrial respiration (Chem ROX) was measured after the addition of 200 mM sodium azide (Azd).
2.6 Ex vivō experiments

2.6.1 Myography

*In vitro* wire myography experiments were performed by Mr. Qiang Lyu on maternal uterine arteries and by Dr. Tess Garrud on maternal femoral arteries.

3rd order branches of the maternal uterine or femoral arteries of approximately 300 μm diameter were dissected immediately post-mortem into ice-cold Krebs buffer (Table 2.7). 2 mm sections of the vessels were carefully dissected and cleaned of perivascular connective tissue, and 2 platinum wires of 40 μm diameter were fed through the arterial lumen. The vessels were then mounted onto individual channels of a small vessel wire myograph (Muti-wire Myograph System 610M and 620M, DMT, UK; Figure 2.14) gassed with 95:5 oxygen:carbon dioxide. Vessels were allowed to warm up to 37°C, after which they were normalised by exposing the vessels to high potassium solutions at different diameters. This step was used to determine the resting diameter, at which the potassium-induced constriction was maximal. Vascular tension was recorded throughout experiments on Labchart 7 Pro (Version 7.2.4, AD Instruments, Australia).

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krebs buffer</td>
<td>120 mM sodium chloride</td>
</tr>
<tr>
<td></td>
<td>4.7 mM potassium chloride</td>
</tr>
<tr>
<td></td>
<td>1.2 mM magnesium sulphate heptahydrate</td>
</tr>
<tr>
<td></td>
<td>1.2 mM potassium phosphate</td>
</tr>
<tr>
<td></td>
<td>25 mM sodium bicarbonate</td>
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<tr>
<td></td>
<td>2.8 mM glucose</td>
</tr>
<tr>
<td></td>
<td>2.5 mM calcium chloride dihydrate</td>
</tr>
</tbody>
</table>

Table 2.7. Buffers for wire myography experiments.

All reagents from Sigma, UK.
Figure 2.14. Small vessel wire myography.
All images provided by Dr. Tessa Garrud. (A) DMT 620M wire myograph with four separate channels. (B) A 2mm segment of vessel attached to the jaw via 2 40µm platinum wires. One side was connected to a micrometer, so that the diameter between jaws could be adjusted to stretch or relax the vessel. The other side was connected to a force transducer, which recorded tension generated by the vessel in mN. (C) and (D) Examples of dose response curves. In (C), vessels were pre-constricted with 10 µM norepinephrine (NE), then exposed to increasing doses of sodium nitroprusside (SNP) to calculate % relaxation. In (D), vessels were exposed to increasing doses of PE, and the absolute constriction at each dose was measured.

2.6.1.1 Uterine artery myography
Vessels were exposed to 125 mM potassium chloride to ensure vascular integrity.

Vasoconstrictor properties (Figure 2.15) were assessed by exposing the vessel to increasing concentrations of serotonin (5-HT). Vessels were then treated with 10 µM of the Rho-kinase inhibitor Y27632 to assess the Rho-kinase-dependent component of the 5-HT-induced contraction. Vessels were also treated with increasing concentrations of the Rho kinase inhibitor Y27632 to measure the contribution of Rho kinase-dependent vascular smooth muscle tone under baseline conditions. Contractile responses to the thromboxane analogue U46619 were also assessed.
Vascular smooth muscle constriction is induced by neurocrine, paracrine and endocrine signalling via 5-HT, ET-1, 5-HT-induced thromboxane A2 (TXA2) production, epinephrine and NE. Vasoconstriction is mediated by modulation of myosin light chains. For example, Rho kinase can directly phosphorylate myosin light chain or inhibit the activity of myosin light chain phosphatase. Activity of myosin light chain kinase is increased by α1-adrenergic activation of phospholipase C, leading to production of inositol triphosphate and Ca\(^{2+}\) release from the sarcoplasmic reticulum, and by α2-adrenergic inhibition of adenylyl cyclase and cyclic adenosine monophosphate (cAMP) production. U46619 is a thromboxane analogue and Y27632 is a Rho kinase inhibitor.

To assess vasodilator function (Figure 2.16), vessels were pre-constricted submaximally with 10 µM 5-HT and then exposed to increasing concentrations of acetylcholine (ACh) to measure endothelium-dependent relaxation and the NO donor SNP to measure NO- and smooth-muscle-dependent relaxation. Pre-constricted vessels were also exposed to increasing concentrations of NaHS. They were then treated with 10 µM of the eNOS inhibitor N(G)-Nitro-L-arginine methyl ester (L-NAME) to assess the NO-dependent component of the NaHS response, or with 10 µM of the
large conductance calcium activated potassium channel (BK$_{Ca}$) inhibitor iberiotoxin to measure the potassium-dependent component of the NaHS response. Between each dose-response curve vessels were allowed to return to baseline tension for up to 2 hours and washed repeatedly with Krebs buffer to remove any residual drug from the bathing solution.

**Figure 2.16. Vasodilator pathways.**
Vascular smooth muscle relaxation is induced by neurocrine, paracrine and endocrine signalling via ACh, NO production by eNOS, H$_2$S production by CSE and CO production by haem-oxygenase-1 (HO-1). Vasodilatation is mediated by guanylyl cyclase production of cGMP and by hyperpolarisation of vascular smooth cells through potassium channels. SNP is an NO donor, L-NAME is an inhibitor of eNOS, DL-propargylglycine (PAG) is an inhibitor of CSE and iberiotoxin is an inhibitor of BK$_{Ca}$ channels.

**2.6.1.2 Femoral artery myography**
Vessels were then exposed to 125 mM potassium chloride, 100 µM phenylephrine (PE) and 100 µM ACh to ensure endothelial and vascular smooth muscle integrity. To assess vasoconstrictor function (Figure 2.15), vessels were exposed to increasing concentrations of PE and ET-1 at 2-minute intervals. To assess vasodilator function
(Figure 2.16), vessels were pre-constricted submaximally with 10 µM NE and then exposed to increasing concentrations of SNP to measure NO and smooth muscle-dependent relaxation and of NaHS to measure H₂S-dependent relaxation. Pre-constricted vessels were also exposed to increasing concentrations of ACh to measure endothelium-dependent relaxation. They were then treated with 10 µM of L-NAME to assess the NO-dependent component of the ACh response, and treated with 10 µM of the CSE inhibitor PAG to assess the H₂S-dependent component of the ACh response. Between each dose-response curve vessels were allowed to return to baseline tension for 40 minutes and washed repeatedly with Krebs buffer to remove any residual drug from the bathing solution.

### 2.6.2 Langendorff heart perfusion

Langendorff heart perfusion experiments were performed on the isolated maternal heart by Dr. Youguo Niu, as previously described (Niu et al., 2013). The heart was placed into ice-cold Krebs-Henseleit bicarbonate buffer (Table 2.8) immediately post-mortem.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krebs-Henseleit bicarbonate buffer</td>
<td>120 mM sodium chloride</td>
</tr>
<tr>
<td></td>
<td>4.7 mM potassium chloride</td>
</tr>
<tr>
<td></td>
<td>1.2 mM magnesium sulphate heptahydrate</td>
</tr>
<tr>
<td></td>
<td>1.2 mM potassium phosphate</td>
</tr>
<tr>
<td></td>
<td>25 mM sodium bicarbonate</td>
</tr>
<tr>
<td></td>
<td>10 mM glucose</td>
</tr>
<tr>
<td></td>
<td>1.3 mM calcium chloride dihydrate</td>
</tr>
</tbody>
</table>

**Table 2.8. Buffers for Langendorff heart perfusion experiments.**

All reagents from Sigma, UK.

The heart was mounted onto a Langendorff apparatus (Figure 2.17) and perfused with the same recirculating Krebs-Henseleit bicarbonate buffer.
Figure 2.17. Langendorff heart perfusion apparatus.

Figure reproduced from Niu et al. 2013 (Copyright © 2013, The Journal of Physiology). Mounted hearts were perfused at 70 mmHg with a recirculating Krebs-Henseleit bicarbonate buffer solution, which was filtered through a 5 µm thick cellulose nitrate filter (Millipore Ltd., USA) and gassed with 95:5 oxygen:carbon dioxide at 38 °C. Arrows show the direction of buffer flow. Pulmonary arteriotomy was performed and a small flexible non-elastic balloon was inserted into the left ventricle through the left atrium. The balloon was filled with distilled water and attached to a rigid water-filled catheter, which was connected to a calibrated pressure transducer (Argon Medical Devices, USA). The balloon volume was set at 40 mL, at which control hearts from pilot studies generated LVEDP recordings of 5 to 10 mmHg, mimicking physiological LVEDP.

After 15 minutes of stabilization, basal heart rate, left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), systolic duration and diastolic duration were recorded (Figure 2.18). The maximum and minimum first derivates of...
left ventricular pressure changes ($\Delta P/\Delta t_{\text{max}}$ and $\Delta P/\Delta t_{\text{min}}$) were calculated using an MPAQ data acquisition system (Maastricht Programmable AcQuisition System, the Netherlands).

![Diagram of cardiac cycle parameters](image)

**Figure 2.18. Parameters of the cardiac cycle.**
LVEDP represents the lowest pressure reached during diastole and LVSP represents the highest pressure reached during systole, while LVDP is calculated to represent the amplitude of left ventricular contraction. Cycle duration is calculated as the time between the peak of each contraction. $\Delta P/\Delta t_{\text{max}}$ and $\Delta P/\Delta t_{\text{min}}$ are the maximum and minimum first derivatives of the left ventricular contraction curve, respectively.

The coronary flow rate (CFR) was calculated by timed collections of the perfusate. Left ventricular developed pressure (LVDP), contractility index and the left ventricular relaxation rate constant ($\tau$) were calculated as follows:

$$LVDP \ (\text{mmHg}) = LVSP \ (\text{mmHg}) - LVEDP \ (\text{mmHg})$$

$$\text{Contractility index} \ (s^{-1}) = \frac{\Delta P}{\Delta t_{\text{max}}} \frac{(\text{mmHg})}{s} \frac{\text{Pressure (mmHg) at } \Delta P}{\Delta t_{\text{max}}}$$

$$\tau (s) = \frac{\Delta P}{\Delta t_{\text{min}}} \frac{(\text{mmHg})}{s} \frac{\text{Pressure (mmHg) at } \Delta P}{\Delta t_{\text{min}}}$$
2.7 Longitudinal cardiovascular measurements

A second cohort of pregnant ewes was surgically prepared with catheters and flow probes to permit continuous monitoring of arterial blood pressure and uterine blood flow in normoxic and hypoxic groups via the CamDAS™ wireless data acquisition system (Fletcher et al., 2002; Jellyman et al., 2004; Allison et al., 2016; Shaw et al., 2018). In this second cohort, recording during chronic normoxia or chronic hypoxia occurred for 10 days, from 125 to 135 dGA (Figure 2.19). This work was performed by Dr. Beth J. Allison.

![Diagram](image)

**Figure 2.19. CamDAS™ short-term chronic hypoxia protocol.**

Ewes underwent surgery at 116 dGA. Between 120 and 135 dGA, ewes underwent daily controlled feeding and blood sampling and continuous CamDAS™ recording. From 125 dGA until post-mortem at 135 dGA ewes underwent chronic normoxia (n=5) or chronic hypoxia (n=5), as previously described.

2.7.1 Surgical preparation

Animals were fasted for 24 hours prior to surgery with ad libitum access to water to minimise bloating and regurgitation of reticulo-rumen contents during surgery. On the day of surgery, animals were transferred to the pre-operative room for induction using
Alfaxan (1.5-2.5 mg.kg\(^{-1}\) alfaxalone, i.v.; Jurox Ltd., UK) into the jugular vein. The ewe was placed in a dorsal recumbent position for intubation with a cuffed endotracheal tube (Portex\textregistered cuffed endotracheal tube; Smiths Medical International Ltd., UK) using a laryngoscope and then maintained under general anaesthesia using 1.5-2.0% isoflurane (IsoFlo\textregistered; Abbott Laboratories Ltd., UK) in 60:40 oxygen:nitrogen using a positive pressure ventilator (Datex-Ohmeda Ltd., UK). Following induction, the maternal abdomen, flanks and medial surfaces of the hind limbs were shaved and cleaned, and an antibiotic (30 mg.kg\(^{-1}\) procaine benzylpenicillin i.m.; Depocillin; Intervet UK Ltd., UK) and an analgesic (1.4 mg.kg\(^{-1}\) carprofen s.c.; Rimadyl; Pfizer Ltd., UK) were administered. The ewe was then transferred to the surgical theatre and secured to the operating table using ropes, while general anaesthesia was maintained, as before. Shaved and cleaned surfaces were cleaned again, using a mixture of alcohol and water, followed by hibitane (Hibitane Plus\textregistered 5% chlorhexidine gluconate; Regent Medical Ltd., Manchester, UK; mixed with alcohol and water) and a concentrated iodine solution (Providone-Iodine; Seton Healthcare Group PLC, UK). From that point onwards, the entire surgery was performed under strict aseptic conditions. All researchers present during the surgery wore facemasks, surgical hats and sterile surgical gowns. All instruments and equipment were either sterilized by autoclave or by gas-sterilisation using ethylene oxide (Anprolene; H.W. Anderson Products Ltd., UK).

### 2.7.2 Surgery

Welsh Mountain ewes carrying singleton fetuses underwent laparotomy at 116 ± 1 dGA for instrumentation with the wireless data acquisition system under general anaesthesia, as previously described (Allison et al., 2016). The animal was covered
with sterile drapes (Buster Opcover; Buster, Kruuse, Denmark) and sterile surgical linen drapes, exposing only the abdomen, and a midline abdominal incision was made. An approximately 10 cm long incision was made using a scalpel blade, while avoiding the mammary veins. The abdominal cavity was opened along the linea alba to expose the uterus. A Transonic flow probe (MC2RS-JSF-WC120-CS12-GCP, Transonics, UK) was positioned around the maternal uterine artery, as before, and then exteriorised through a keyhole incision in the ewe’s right flank for connection to the wireless data acquisition system. The maternal peritoneum was closed with thick linen and the abdominal skin was sutured together (Ethilon 2-0; Ethicon Ltd., UK). For catheterisation, maternal femoral artery and vein were exposed and ligated distally. Catheters were inserted via a small hole in the maternal femoral vein (inner diameter 0.86 mm, outer diameter 1.52 mm; Critchly Electrical Products, Australia) into the maternal inferior vena cava, and via the maternal femoral artery (inner diameter 1.00 mm, outer diameter 1.60 mm; Altec, UK) into the maternal descending aorta. Both catheters were flushed with heparinised saline (Heparin sodium, 80 i.u.ml\(^{-1}\) in 0.9% sodium chloride; Wockhardt, UK and Sigma, UK) to test their patency and subsequently plugged with brass pins. Catheters were then exteriorised through a keyhole incision in the maternal left flank and connected to the wireless data acquisition system. The maternal skin incisions for insertion of catheters and for exteriorisation were closed using sutures and, while still under general anaesthesia, the ewe was fitted with a bespoke jacket housing the wireless data acquisition system. After the end of anaesthesia, the ewe continued to be ventilated until spontaneous respiratory movements were observed.
2.7.3 CamDAS™ System

The wireless data acquisition system (Figure 2.2) has been previously described in detail (Allison et al., 2016). In brief, the CamDAS™ (Maastricht Instruments, the Netherlands) consisted of a pressure box attached to one side of the ewe containing pressure transducers (COBE; Argon Division, Maccim Medical, USA) connected to catheters, and a miniaturised flow module on the other side connected to Transonic flow probes. The pressure and flow boxes were powered by Lithium batteries housed within the same jacket, allowing continuous wireless transmission and recording of maternal uterine blood flow and maternal arterial blood pressure beat-by-beat onto a laptop computer via Bluetooth technology. The weight of the CamDAS™ system is less than 2 kg (akin to a ewe carrying a twin pregnancy).

2.7.4 Post-surgical recovery

Ewes were allowed to recover in a floor pen adjacent to other sheep with free access to hay and water with a controlled dusk to dawn 12:12 hour light-dark cycle. Ewes were fed concentrates once a day (200g sheep nuts no. 6; H & C Beart Ltd., UK) and, generally, normal feeding patterns were resumed within 24 to 48 hours of post-operative recovery. Antibiotic (30 mg.kg⁻¹ procaine benzylpenicillin i.m.; Depocillin; Intervet UK Ltd., UK) and analgesic (1. mg.kg⁻¹ carprofen s.c.; Rimadyl; Pfizer Ltd., UK) agents were administered to the ewe for five days following surgery. Catheters were flushed daily with heparinised saline (Heparin sodium, 100 IU.mL⁻¹ in 0.9% sodium chloride; Wockhardt, UK and Sigma, UK) and blood gas analysis was performed daily on a maternal arterial blood sample (0.3 mL).
2.7.5 Experimental procedures

From 120 dGA ewes were fed the daily maintenance diet (5g hay kg\(^{-1}\) and 40g sheep nuts kg\(^{-1}\); Manor Farm Feeds Ltd., Oakham, UK) and pregnancies were randomly assigned to chronic normoxia or chronic hypoxia cohorts, as before. Ewes assigned to the hypoxic cohort were transferred to the hypoxic chambers to acclimatize under normoxic conditions, while ewes assigned to chronic normoxia were housed in individual floor pens occupying the same floor area, as before. Five days after surgery, at 125 dGA, ewes in the hypoxic cohort were gradually subjected to hypoxia, reaching 10 ± 1 % inspired oxygen over 48 hours, as before (Figure 2.2). Exposure to chronic hypoxia in these ewes lasted 10 days, until 135 dGA. Continuous CamDAS™ recording of maternal arterial blood pressure and uterine blood flow were converted into minute averages off-line. Uterine vascular resistance was calculated according to Ohm’s principle (Jellyman et al., 2004):

\[
\text{Vascular resistance (mmHg/ml/min)} = \frac{\text{Blood pressure (mmHg)}}{\text{Blood flow (ml/min)}}
\]

In both normoxic and hypoxic ewes, arterial blood samples were taken daily to measure maternal blood gases, ABE and metabolic status, as previously described (Allison et al., 2016). Arterial partial pressures of oxygen and carbon dioxide (\(P_{\text{a}}O_2\) and \(P_{\text{a}}CO_2\)), arterial pH, arterial ABE and arterial bicarbonate concentration were measured using an ABL5 blood gas analyser (Radiometer; Copenhagen, Denmark; maternal measurements corrected to 38 °C). Values for haemoglobin and oxygen saturation (Sat Hb) were determined using a haemoximeter (OSM3; Radiometer, UK). The oxygen tension, at which haemoglobin is 50% saturated (\(P_{50}\)) was calculated, as previously described in section 2.2.4.
Maternal arterial oxygen content was calculated as follows:

$$O_2 \text{ content} \left( \frac{ml}{dl} \right) = \left( 1.36 \times Hb \left( \frac{g}{dl} \right) \times Sat Hb \% \right) + \left( 0.0031 \times P_a O_2 (mmHg) \right)$$

Arterial blood glucose and lactate concentrations were measured using a Yellow Springs 2300 Stat Plus Biochemistry Analyser (YSI Ltd., UK). Values for haematocrit were obtained in duplicate using a microhaematocrit centrifuge (Hawksley, UK). At the end of in vivo experiments, normoxic and hypoxic ewes were transported to the post-mortem laboratory and humanely killed as before (section 2.2.4), and the positions of the uterine Transonic flow probe and maternal catheter tips were verified.
2.8 Data and statistical analysis

Our predicted n was based on power calculations derived from past data and preliminary experiments to determine the minimum sample size required to achieve significance with a confidence interval of 95% (α=0.05, p<0.05). Calculations were based on the variable with the minimum detectable difference and greatest variance between means allowing for multiple comparisons, where appropriate, for each type of experimental outcome: in vivo, isolated organ, cellular and molecular experiments.

Data are expressed as mean ± standard error of the mean (S.E.M.) unless otherwise specified. Statistical outliers were removed using the Grubb’s test. All variables were statistically analysed using the Student’s t-test when comparing the effects of normoxic vs. hypoxic exposure, using the two-way analysis of variance (ANOVA) with or without repeated measures, as appropriate, when comparing between the effects of oxygenation and gestational age, dose concentration, placentome type or vitamin C treatment. When a statistical significance using two-way ANOVA was detected (p<0.05), the Šidák’s or Tukey’s post hoc tests were used to control for multiple comparisons and isolate the differences, as appropriate. All analysis and graphs were generated in Prism 8 (GraphPad, USA).
3. Chronic hypoxia and the placental unfolded protein response

3.1 Introduction

Pregnancy is a highly vulnerable period for the mother and her unborn child, and adverse conditions in utero are not only major drivers of maternal and fetal, but also of adolescent and adult morbidity and mortality (Gillman, 1995; Bernstein et al., 2000; Gluckman et al., 2008). While disorders of pregnancy present a substantial financial and scientific burden on public health care worldwide, there is a lack of effective prognostic, diagnostic and treatment options for some disorders, such as for chronic fetal hypoxia and for pre-eclampsia (Hodgins, 2015).

Fetal hypoxia during gestation can occur in a multitude of pregnancy complications, and is one of the most common challenges in obstetric clinical practice (Fajersztajn and Veras, 2017). Fetuses undergo many morphological and functional changes to compensate for reduced oxygenation, and many of the adverse effects of chronic fetal hypoxia are associated with failure of the fetus to achieve its full developmental potential (Hutter et al., 2010). In both the sheep and the human, haemoglobin and haematocrit levels increase to maintain oxygen delivery to vital organs (Browne et al., 1997). Moreover, hypoxia triggers a dramatic redistribution of blood flow away from non-essential tissues, such as the lower limbs and kidneys, and towards the brain, heart and upper body (Giussani, 2016). Despite the relative vasodilatation of the cerebral and coronary arteries, sustained severe hypoxia can lead to progressive cardiac ischaemia and dysfunction predisposing for heart failure, cognitive dysfunction, cerebral palsy and fetal or neonatal death (Fouron et al., 1991; Pollack and Divon, 1992). In addition to the immediate effects on fetal morbidity, hypoxia-induced FGR is
also highly implicated in the developmental programming of cardiovascular disease in later life (Barker et al., 1989; Barker and Clark, 1997; Barker et al., 2002; Giussani, 2006; Giussani and Davidge, 2013). Sustained fetal hypoxia occurs in ca. 10% of pregnancies at sea level as a result of a number of pregnancy complications, and is in most cases a direct consequence of maternal pre-placental or uteroplacental hypoxia leading to placental insufficiency (Hutter et al., 2010). Exposure of pregnant women to high altitude mimics the condition of placental insufficiency and its adverse effects on fetal development, and is associated with a range of pregnancy complications (Jensen and Moore, 1997; Hutter et al., 2010). High altitude also increases the risk of developing preeclampsia, which further contributes to the FGR observed in pregnancies at high altitude (Keyes et al., 2003).

Preeclampsia is a common complication of pregnancy and is classically diagnosed as new-onset hypertension and proteinuria after 20 weeks of gestation (Sibai et al., 2005; Steegers et al., 2010). While the pathogenesis of preeclampsia is controversial, it is widely recognised that preeclampsia is a disorder that involves significant placental dysfunction, presenting with reduced uteroplacental perfusion and placental hypoxia (Steegers et al., 2010; Young et al., 2010; Burton et al., 2019). Both placental hypoxia and oxidative stress can significantly disturb cellular protein homeostasis by inducing oxidative protein damage or impairing the formation of high energy disulphide bonds by protein disulphide isomerase (PDI; Figure 3.1; Berlett at Stadtman, 1997; Koumenis et al., 2007; Chipurupalli et al., 2019). This can promote protein unfolding, fragmentation and aggregation, resulting in ER stress and activation of the UPR, as occurs in preeclampsia and in pregnancies at high altitude (Schröder and Kaufman, 2005; Buchberger et al., 2010; Yung et al., 2012; Yung et al., 2014). UPR pathways exist in all cellular compartments involved in protein synthesis, including the UPR_Cyt,
Chronic hypoxia and the placental unfolded protein response

mitochondria UPR\textsuperscript{mt}, and UPR\textsuperscript{ER} (Yung et al., 2019). When the chaperone GRP78 is sequestered to bind unfolded or misfolded proteins in the ER, UPR signalling is initiated by the ER transmembrane signal activators PERK, ATF6 and IRE1 (Figure 3.1; Xu et al., 2005; Hetz, 2012). Cleavage of ATF6 produces an active transcription factor. IRE1 cleaves XBP1 mRNA to become a transcription factor and activates the MAPKs C-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), both of which regulate trophoblast survival and placental nutrient and oxygen exchange with the fetus (Iwawaki et al., 2009; Hetz, 2012; Darling and Cook, 2014). PERK phosphorylates eIF2α, which inhibits global protein translation while promoting expression of the transcription factor ATF4 (Hetz, 2012). Together, signalling downstream of the UPR\textsuperscript{ER} induces the expression of chaperone proteins, antioxidant enzymes and cellular protein degradation machinery (Xu et al., 2005; Hetz, 2012). Apart from increasing the protein folding capacity of the ER, ATF6 and XBP1 and can trigger morphological adaptations, including ER distension and expansion (Jones and Fox, 1980; Bommiasamy et al., 2009).

Another component of cellular UPR pathways is a group of phylogenetically conserved stress proteins termed heat shock proteins (HSPs), which are key to regulating cellular homeostasis by maintaining the integrity and function of other proteins (Hromadnikova et al., 2015). While only expressed at low levels under physiological conditions, they are significantly upregulated in response to stressors, such as oxidative stress, pH changes and radiation (Wagner et al., 1999). For example, as part of the UPR\textsuperscript{Cyt}, both HSP27 and HSP70 are able to modify the cellular response to proteotoxic stress by stabilising unfolded proteins in the cytosol, contributing to cellular antioxidant capacity and quality control and protecting the cell from UPR-induced apoptosis (Kumano et al., 2012; Terrab and Wipf, 2020). Downstream effects of the UPR pathways are
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manifold and the outcome depends on the degree, duration and location of their activation, ultimately determining cell fate, governing apoptosis, survival, proliferation and differentiation. ERK activation can promote cell survival, terminal differentiation or proliferation depending on the cell type, and has a vital role in trophoblast development (Hatano et al., 2003; Darling and Cook, 2014; Tang et al., 2014). Levels of ERK expression and activation have been found to be altered in compromised pregnancies, such as FGR and preeclampsia, but reports disagree on the direction of this change (Arroyo et al., 2010; Bahr et al., 2014; Tang et al., 2014; Dong and Shi, 2016; Jiang and Zhao, 2018; Wang, 2020). On the other hand, JNK activation has negative effects on cell viability in trophoblast cells following oxidative damage (Tang et al., 2014). Both JNK and CHOP activation downstream of ATF4 signalling have pro-apoptotic properties by increasing the levels of BH3-only proteins and decreasing BCL2 (Harding et al., 2000; McCullough et al., 2001; Ghosh et al., 2012; Darling and Cook, 2014). There is also evidence that hypoxia-reoxygenation can directly trigger mitochondrial release of cytochrome c, which induces cleavage of caspase 3 and apoptosis (Hung et al., 2002). In addition, cellular UPR signalling can affect trophoblast function by altering extracellular matrix composition and increasing the rate of collagen deposition (Maiers et al., 2017; Hisanaga et al., 2018; Klymenko et al., 2019). These pathways are shared in preeclamptic and hypoxic placentae, which show elevated production of type I collagen by fibroblasts (Chen et al., 2005; Ohmaru-Nakanishi et al., 2018; Xu et al., 2019). Villous fibrosis is a common histologic feature of preeclamptic and hypoxic placentae, and possibly a response to placental proteotoxic stress and inflammatory injury (Ducray et al., 2011; Devisme et al., 2013).
Figure 3.1. Endoplasmic reticulum and cytosolic unfolded protein response.

Under physiological conditions, the ER maintains protein homeostasis to aid protein folding, refolding and degradation. Under conditions of hypoxia and oxidative stress, GRP78 is sequestered into the lumen by increased levels of unfolded and misfolded proteins. This allows the UPR signal activators IRE1, PERK and ATF6 to relay information about the protein homeostasis of the ER to the cytosol and nucleus in order to improve cellular protein-folding capacity. ATF6 activation allows its translocation to the nucleus. IRE1 dimerisation activates MAPKs, including ERK and JNK. Activated PERK phosphorylates and inhibits eIF2α, which halts protein synthesis, while still allowing expression of the transcription factor ATF4. Proteotoxic stress located in the cytosol triggers the upregulation of the highly conserved family of heat shock proteins, including HSP27 and HSP70. Downstream effects of UPR activation include the upregulation of protein chaperones, expression of enzymes involved in protein quality control and the ERAD pathway, increase in antioxidant capacity, alteration of apoptotic and proliferative signalling and changes to extracellular matrix deposition. These transcriptional and translational changes are used to re-establish protein homeostasis and promote cellular survival. If the proteotoxic insult is too severe, single cells undergo apoptosis or autophagy to protect tissue viability.
These molecular and morphological changes in response to hypoxia and oxidative stress culminate in the development of placental dysfunction, with adverse consequences downstream on fetal development. For instance, hypoxia-induced placental dysfunction and oxidative stress is associated with a decrease in placental efficiency, exacerbating FGR, and may trigger protein and nucleic acid oxidation, protein synthesis inhibition and cell death in the fetus (Longini et al., 2007; Fowden et al., 2009; Yung et al., 2012; Perrone et al., 2016).

The objective of this chapter was to determine the effects of chronic hypoxia in the last third of gestation in sheep on markers of the unfolded protein response in the placenta, which are commonly seen in preeclampsia.
3.2 Methods

The expanded methodology has been described in the General Methods. Below is the summarised methodology used in this particular chapter, which has been cross-referenced to the General Methods for further detail.

3.2.1 Experimental procedures

In brief, pregnant Welsh Mountain ewes carrying singleton fetuses were weighed and randomly assigned to chronic normoxia or hypoxia at 103 dGA. Ewes assigned to chronic normoxia were housed in individual floor pens, while ewes assigned to chronic hypoxia were moved into one of four bespoke isobaric hypoxic chambers (section 2.2.1). Ewes were fed daily a bespoke maintenance diet consisting of concentrate and hay pellets, thereby facilitating the monitoring of food intake (section 2.1.2). From 105 dGA ewes were exposed to control normoxic conditions or gradually subjected to hypoxia, reaching 10 ± 1% inspired oxygen over 48 hours (Brain et al., 2015). This level of hypoxia was maintained for a month until 138 dGA. Ambient PO₂, PCO₂, humidity and temperature within each chamber were continuously monitored and recorded.

3.2.2 Post-mortem

At 138 dGA, normoxic and hypoxic ewes were weighed and moved to the post-mortem laboratory. Maternal weight gain was calculated as the difference between maternal weight at 103 dGA and at 138 dGA. Ewes in the hypoxic group remained hypoxic at 10 ± 1% inspired oxygen via a respiratory hood until euthanasia. Both normoxic and hypoxic ewes were killed humanely and a blood sample was taken from the umbilical vein for analysis of fetal blood (section 2.2.2). The fetus was delivered by Caesarean
section, weighed, and fetal CRL, AC, BPD and hind limb lengths were measured. Fetal BMI and PI were calculated, as described in section 2.2.4. Fetal organs were isolated and weighed. Relative organ weights were calculated by dividing absolute organ weights by fetal body weight at 138 dGA. Following hysterectomy, individual placentomes were dissected, characterised according to Vatnick et al., counted and weighed (Figure 2.4; Vatnick et al., 1991). 1 mm thick strips from two representative type A placentomes were placed in ice-cold 2 mM calcium chloride in 0.05M Sodium cacodylate buffer at pH 7.4 containing 2% glutaraldehyde and 2% formaldehyde for electron microscopy (section 2.4.6). Representative examples of type A and type D placentomes were then snap frozen in liquid nitrogen and stored at -80 °C or fixed in 4% paraformaldehyde (Sigma, UK), transferred to 70% ethanol after 24 hours and stored at 4 °C until further analysis.

3.2.3 Protein analysis

Flash frozen type A and type D placentomes were homogenised to tissue lysates using ice-cold cell lysis buffer (Cell Signaling Technology, UK) containing protease inhibitors (Roche Diagnostics, UK).

3.2.3.1 Measurement of protein carbonylation

To determine post-translational protein carbonylation as a result of oxidative damage, an OxyBlot™ analysis was performed, as previously described in section 2.3.1.3 (Millipore Ltd., UK). In short, protein lysates were treated to derivatise carbonyl groups to DNPH moieties, separated on a 12% SDS-PAGE agarose gel and transferred onto a nitrocellulose membrane (Hybond® ECL™, Sigma-Aldrich, UK). Non-specific binding was inhibited by blocking the membrane in 2.5% BSA in TBS-T for 1 hour at RT. The nitrocellulose membrane was incubated with a primary rabbit anti-DNPH
antibody (Chemicon Oxyblot™; diluted 1:200) for 1 hour at RT. Membranes were washed with TBS-T and incubated with a secondary antibody conjugated with horseradish peroxidase against Rabbit IgG (Thermo Fisher, UK; diluted 1:10,000) for 1 hour at RT. Following further washing with TBS-T, protein levels were visualised using an enhanced chemiluminescence kit (Pierce™ ECL, Thermo Fisher Scientific, UK). Protein band densities were quantified using ImageJ software (NIH, RRID:SCR_003070) and normalised against Ponceau S staining.

3.2.3.2 Western blotting

Western blotting was used to determine the relative levels of proteins of interest in protein lysates as described in section 2.3.1.2. Gel loading samples were resolved on 10-12% SDS-PAGE agarose gels and transferred onto nitrocellulose membranes (Hybond® ECL™, Sigma-Aldrich, UK). Non-specific binding was inhibited by blocking the membrane in 5% dry skim milk in TBS-T for 1 hour at RT. Following incubation with the primary antibody, membranes were washed with TBS-T and incubated with the relevant secondary antibodies against Rabbit IgG (Thermo Fisher, UK; diluted 1:10,000) or against Mouse IgG (Thermo Fisher, UK; diluted 1:10,000) for 1 hour at RT. Following further washing with TBS-T, protein levels were visualised using an enhanced chemiluminescence kit (Pierce™ ECL, Thermo Fisher Scientific, UK) on film (Amersham™ Hyperfilm™ ECL, GE Healthcare, UK). Protein band densities were quantified using ImageJ software (NIH; RRID:SCR_003070) and normalised against Ponceau S staining. A full list of primary antibodies, dilutions and incubation times can be found in Table 2.2.

3.2.4 Placental histology

For histological analysis, formalin-fixed paraffin-embedded type A placentomes were
sectioned to 7 µm thickness using a microtome (Leica Biosystems, UK), mounted onto Superfrost™ Plus microscope slides and incubated at 37 °C overnight, as described in detail in section 2.4.2.

### 3.2.4.1 Immunohistochemistry

Sections were rehydrated and incubated in 3% hydrogen peroxide (Fisher Scientific, UK) for 15 minutes to block endogenous peroxidase activity. After rinsing in distilled water, the sections were incubated in TBS-TT for 30 minutes. After rinsing in TBS, slides were blocked in 5% BSA in TBS for 1 hour and then incubated overnight in primary antibody in 5% BSA. The following day, sections were washed using TBS-TT and then incubated in biotinylated secondary antibody against Rabbit IgG (Vector Laboratories, Canada; diluted 1:200) in 5% BSA. After washing in TBS-TT, staining was visualised using the VECTASTAIN avidin-biotin complex method (Vector Laboratories, Canada) by adding metal DAB (Thermo Fisher Scientific, UK). Staining was stopped by rinsing in distilled water. Sections were dehydrated, and cover slips mounted using DPX Mountant (Sigma-Aldrich, UK). A full list of primary antibodies, dilutions and incubation times can be found in Table 2.5.

For sections immunostained with PCNA, systematic random sampling of the trophoblast area of whole placentomal cross sections was used to estimate the number of stained nuclei in each section, as described in section 2.4.3.1.

### 3.2.4.2 Picrosirius red stain

Sections were rehydrated, covered in 0.1% Direct Red 80 in saturated picric acid (both from Sigma-Aldrich, UK) for 1 hour and then washed in 0.5% acetic acid (Sigma-Aldrich, UK) twice. Sections were dehydrated and cover slips were mounted using DPX Mountant (Sigma-Aldrich, UK). The area fraction of collagen was determined.
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through systematic random sampling, as previously described in section 2.4.5.

3.2.4.3 Transmission electron microscopy

Tissue processing, embedding and sectioning was performed by the Cambridge Advanced Imaging Centre, as described in section 2.4.6. In brief, small pieces of type A placentome tissue were fixed immediately *post-mortem* by immersion and osmicated over the course of a week. The tissue was dehydrated and embedded in Quetol epoxy resin over the course of 11 days. 80 nm sections were cut on an Ultracut UCT (Leica, Germany) and mounted onto 400 mesh bare copper grids. Transmission electron microscopy was performed on an FEI Tecnai G2 transmission electron microscope and images were taken using an AMT camera.

3.2.5 Data and statistical analysis

The sample size is n=9 for the normoxic cohort and n=7 for the hypoxic cohort. All data are expressed as mean ± S.E.M. The effect of oxygenation was analysed using the Student’s *t*-test for unpaired data. The effects of oxygenation, time and interactions between oxygenation and time were compared by two-way repeated measures ANOVA. When a significant difference was detected (*p*<0.05), the Šidák *post hoc* test was used to control for multiple comparisons and isolate the differences. For all comparisons, values of *p*<0.05 were accepted as statistically significant. The software used was Graphpad Prism 7 (GraphPad, USA).
3.3 Results

3.3.1 Chamber oxygen status and maternal food intake

In ewes assigned to the chronic hypoxic cohort, the percentage of atmospheric oxygen in the hypoxic chambers was kept at 20.9 ± 0.1% during baseline (Figure 3.2). At 105 dGA, the percentage of inspired oxygen was gradually decreased to an average of 10.6 ± 0.03% between days 106 and 138 dGA.

![Chamber O₂ status](image)

**Figure 3.2. Chamber oxygen status.**

Values are mean ± S.E.M. for chamber atmospheric oxygen status. Groups are N (○, n=9) and H (●, n=7). Significant differences (p<0.05) are *N vs. H or †vs. baseline; two-way RM ANOVA.

There were no changes in maternal daily food and energy intake at the end of gestation compared to baseline in normoxic (1611 ± 121 g to 1220 ± 169 g and 15895 ± 1183 kJ to 11955 ± 1654 kJ, respectively) and in hypoxic ewes (1321 ± 121 g to 889 ± 158 g and 12845 ± 1183 kJ to 8715 ± 1557 kJ, respectively; Figure 3.3A). There were no significant differences in food and energy intake between the two cohorts. There were no changes in maternal weight with advancing gestation or in normoxic compared to hypoxic ewes (Figure 3.3B).
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3.3.2 Fetal blood and biometry

3.3.2.1 Fetal blood sample results

At 138 dGA, fetuses from the hypoxic cohort had increased haematocrit and increased haemoglobin concentration compared to normoxic fetuses at 138 dGA (Figure 3.4A and B). They also showed a tendency towards decreased $P_{50}$ ($p=0.05$; Figure 3.4C).
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Figure 3.4. Fetal oxygenation.
Values are mean ± S.E.M. for haematocrit (A), haemoglobin (B) and fetal P_{50} (C), at 138 dGA. Groups are N (○, n=9) and H (●, n=6). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data.

3.3.2.2 Fetal biometry
Fetuses from the hypoxic cohort compared to the normoxic cohort showed a decrease in body weight (3.72 ± 0.15 kg vs. 4.88 ± 0.17 kg), CRL, AC and hind limb lengths at 138 dGA (Figure 3.5). There was an increase in the ratio of CRL to body weight, the ratio of BPD to body weight and the ratio of AC to body weight at 138 dGA (Figure 3.5). There were no differences between fetuses from normoxic and hypoxic cohorts in BPD, the ratio of BPD to AC, the ratio of BPD to CRL, BMI and PI at 138 dGA (Figure 3.5). There were no differences between fetuses from normoxic and hypoxic cohorts in absolute and relative weights of the pituitary, adrenals, perirenal fat depots, the pancreas and thyroids (Figure 3.6). Fetuses from the hypoxic cohort had higher relative brain weight compared to fetuses from the normoxic cohort at 138 dGA, but there was no difference in absolute brain weight (Figure 3.6A). Fetuses from the hypoxic cohort had decreased absolute weights of the heart, lungs and kidneys compared to fetuses from the normoxic cohort at 138 dGA, but there was no difference in relative heart, lung and kidney weights (Figure 3.6). Both absolute and relative liver weights were decreased in fetuses from the hypoxic cohort compared to the normoxic cohort at 138 dGA (Figure 3.6E).
In hypoxic fetuses the ratio of brain to heart, brain to lung, brain to liver and brain to kidney weights was increased at 138 dGA (Figure 3.7). There was no difference in the ratio of pituitary to brain weight or the ratio of kidney to liver weight (Figure 3.7).

Figure 3.5. Fetal biometry.
Values are mean ± S.E.M. for fetal weight (A), CRL (B), BPD (C), AC (D), the ratio of BPD to AC (E), the ratio of BPD to CRL (F), the ratio of CRL to body weight (G), the ratio of CPD to body weight (H), the ratio of AC to body weight (I), BMI (J), PI (K) and hind limb lengths (L). Groups are N (○, n=9) and H (●, n=6). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data.
Figure 3.6. Fetal organ weights.

Values are mean ± S.E.M. for absolute and relative weight of fetal brain (A), pituitary (B), heart (C), lungs (D), liver (E), kidneys (F), adrenals (G), perirenal fat depots (H), pancreas (I) and thyroids (J) at 138 dGA. Groups are N (○, n=9) and H (●, n=6). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data.
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Figure 3.7. Fetal organ weight ratios.
Values are mean ± S.E.M. for the fetal ratio of pituitary to brain weight (A), the ratio of brain to heart weight (B), the ratio of brain to lung weight (C), the ratio of brain to liver weight (D), the ratio of brain to kidney weight (E) and the ratio of kidney to liver weight (F) at 138 dGA. Groups are N (○, n=9) and H (●, n=6). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data.

3.3.3 Placental biometry and the unfolded protein response

3.3.3.1 Placental biometry
There were no differences in absolute or relative distribution of the number and the weight of different placentome types (Figure 3.8A and B). There were no differences in the average weight of all placentomes or individual placentome types, or in the fetal to placental weight ratio between the normoxic and the hypoxic cohort at 138 dGA (Figure 3.8C and D).
3.3.3.2 Placental endoplasmic reticulum unfolded protein response

The levels of protein carbonylation were greater in hypoxic relative to normoxic placentae at 138 dGA (Figure 3.9A). The expression of the UPR signal activator ATF6 was increased in hypoxic relative to normoxic placentae (Figure 3.9B). Immunohistochemical analysis showed that ATF6 localises to the nucleus, where it mediates the transcriptional response to ER stress and activation of UPR target genes (Adams et al., 2019). The levels of the protein chaperone GRP78 and of the protein folding enzyme PDI were greater in hypoxic relative to normoxic placentae at 138 dGA (Figure 3.10). PDI staining was localised to the trophoblast rather than fetal or maternal tissues (Figure 3.10B). Transmission electron microscopy further revealed distended ER morphology in hypoxic placentomes compared with the ER in normoxic placentomes, which displayed a highly defined membrane structure (Figure 3.11).
**Figure 3.9. Placental oxidative protein damage and ATF6 levels.**

Values are mean ± S.E.M. for the relative ratio of placental levels of post-translational protein carbonylation (A) and ATF6 (B). Staining of the nuclei by ATF6 was confirmed through immunostaining. Two representative images taken at 40x magnification are shown. Scale bar = 50 µm. Groups are N (○, n=9) and H (●, n=7). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data.
Figure 3.10. Placental endoplasmic reticulum unfolded protein response.
Values are mean ± S.E.M. for the relative ratio of placental levels of GRP78 (A) and PDI (B).
Staining of the trophoblast by PDI was confirmed through immunostaining. Two representative images taken at 20x magnification are shown. Scale bar = 100 µm. Groups are N (○, n=9-10) and H (●, n=7). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data.
Figure 3.11. Placental endoplasmic reticulum morphology. Differences in placental ER structure at 138 dGA were examined by transmission electron microscopy. Two representative images taken at 5,000x magnification are shown. The arrows indicate the location of ER and the stars indicate the location of the nucleus. Scale bar = 500 nm.

3.3.3.3 Placental cytosolic unfolded protein response

As part of the UPR\textsuperscript{Cyt}, the levels of the cytosolic protein chaperones HSP27 and HSP70 were greater in hypoxic relative to normoxic placentae at 138 dGA (Figure 3.12). HSP70 shows more prominent localisation to the nuclei of the trophoblast in hypoxic compared to normoxic placentae (Figure 3.12B).

This was associated with an increase in the ratio of the phosphorylated form of the MAPKs ERK and JNK compared to total levels of these stress kinases in hypoxic relative to normoxic placentae at 138 dGA (Figure 3.13).

There was no difference in the relative number of nuclei stained by PCNA in hypoxic compared to normoxic placentomes (Figure 3.14).
Figure 3.12. Placental cytosolic unfolded protein response.

Values are mean ± S.E.M. for the relative ratio of placental levels of HSP27 (A) and HSP70 (B). Staining of the trophoblast by HSP70 and translocation to the nucleus under hypoxia was confirmed through immunostaining. Two representative images taken at 20x magnification are shown. Scale bar = 100 µm. Groups are N (○, n=10) and H (●, n=7). Significant differences (p<0.05) are *N vs. H; Student's t-test for unpaired data.
Figure 3.13. Placental stress kinase signalling.
Values are mean ± S.E.M. for the relative ratio of placental levels of phosphorylated compared to total ERK (A) and JNK (B). Groups are N (○, n=10) and H (●, n=7). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data.

Figure 3.14. Placental cellular proliferation.
Values are mean ± S.E.M. for the relative ratio of placental levels of nuclei stained with PCNA, as determined by immunostaining. Two representative images taken at 40x magnification are shown. Scale bar = 50 µm. Groups are N (○, n=5) and H (●, n=5).
3.3.3.4 Placental ATF4 signalling

There was no difference in placental levels of ATF4 in hypoxic relative to normoxic placentae (Figure 3.15A). There were no differences in placental levels of the apoptosis regulators CHOP, BCL2 and BAX in hypoxic compared to normoxic placentomes (Figure 3.15B-D). While lower total caspase 3 levels were detected in placentomes collected from the hypoxic cohort, cleaved caspase 3 could not be detected in either normoxic or hypoxic placentomes (Figure 3.15E).

There was no difference in the relative collagen content of hypoxic compared to normoxic placentomes (Figure 3.16).

3.3.3.5 Effect of placentome type on placental endoplasmic reticulum unfolded protein response

There were no significant differences in placental protein levels of post-translational protein carbonylation, the ER chaperone GRP78, the folding enzyme PDI and the cytoplasmic chaperones HSP27 and HSP70 between type A and type D placentomes in normoxic or hypoxic pregnancies (Figure 3.17). Levels of the UPRER transcription factor ATF6 were lower in type D placentomes from hypoxic pregnancies compared to both type A placentomes from hypoxic pregnancies and type D placentomes from normoxic pregnancies (Figure 3.17B).

There was no difference in the ratio of phosphorylated compared to total levels of the MAPKs ERK and JNK between type A and type D placentomes in normoxic or hypoxic pregnancies (Figure 3.17G and H). There were no significant differences in placental levels of ATF4, CHOP, BCL2, BAX or caspase 3 between type A and type D placentomes in normoxic or hypoxic pregnancies (Figure 3.17).
Figure 3.15. Placental ATF4 levels.
Values are mean ± S.E.M. for the relative ratio of placental levels of ATF4 (A), CHOP (B), BCL2 (C), BAX (D) and total levels of caspase 3 (E). Cleaved caspase 3 could not be detected. Groups are N (○, n=9-10) and H (●, n=7). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data.
Figure 3.16. Placental collagen content.
Values are mean ± S.E.M. for the placental percentage of picrosirius red staining. Two representative images of the whole section are shown. Scale bar = 5mm. Two representative images taken at 10x magnification are shown. Scale bar = 250 µm. Groups are N (○, n=7) and H (●, n=7).
Figure 3.17. Placental stress response in type A vs. type D placentomes.

Values are mean ± S.E.M. for the relative ratio of placental levels of protein carbonylation (A), ATF6 (B), GRP78 (C), PDI (D), HSP27 (E), HSP70 (F), phosphorylated compared to total ERK (G) and JNK (H), ATF4 (I), CHOP (J), BCL2 (K), BAX (L) and caspase 3 (M). Groups are NA (○, n=5), ND (□, n=5), HA (●, n=5) and HD (■, n=5). Significant differences (p<0.05) are for the main effect of hypoxia and placentome type; when there was an interaction, significant differences (p<0.05) are denoted by *; two-way RM ANOVA with Šidák’s post hoc test.
3.4 Discussion

3.4.1 Summary

The data in this chapter showed that the bespoke hypoxic chambers were able to maintain ewes in the chronic hypoxic cohort under stable hypoxic conditions of ca. 10.6% atmospheric oxygen for the last third of gestation from 105 until 138 dGA. This level of hypoxia did not lead to any changes to maternal food intake, energy intake and weight gain during the last third of gestation.

Gestational hypoxia in the last third of gestation increased fetal haematocrit and haemoglobin concentration at 138 dGA, which was associated with a tendency towards a decrease in fetal $P_{50}$. Hypoxic pregnancy was associated with a decrease in fetal body weight, CRL, AC and hind limb lengths at 138 dGA, while the relative CRL, BPD and AC compared to fetal body weight was increased. Fetuses from the hypoxic cohort showed an increase in relative brain weight compared to total body weight and compared to heart, lung, liver and kidney weights at 138 dGA. Fetuses from the hypoxic cohort also showed a decrease in relative liver weight and absolute heart, lung, kidney and liver weights compared to fetuses from the normoxic cohort at 138 dGA.

There were no differences in absolute or relative distribution of the number and the weight of the different placentomes types, or in the fetal to placental weight ratio between the normoxic and the hypoxic cohort at 138 dGA. Hypoxic pregnancy was associated with increased placental oxidative protein damage and increased levels of the UPR signal activator ATF6, which localizes to the nucleus. Placental levels of the ER chaperone GRP78, the protein folding enzyme PDI and the cytosolic chaperones HSP27 and HSP70 were higher in the hypoxic cohort, and both PDI and HSP70 were
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localized to the trophoblast. Hypoxic placentomes also showed increased activation of the MAPKs ERK and JNK compared to placentomes collected from the normoxic cohort. These molecular changes were associated with altered morphology of the ER in hypoxic placentomes, which showed evidence of dilation and expansion.

There were no considerable differences in activation of the UPR\textsuperscript{ER} pathways between type A and type D placentomes in normoxic or hypoxic pregnancies.

3.4.2 Chamber oxygen status and maternal food intake

Despite the severity of the hypoxic challenge in the present study, this degree of hypoxia did not affect maternal food or energy intake or maternal weight gain in the last third of gestation (Peacock, 1998). Thus, maternal, placental and fetal responses to gestational hypoxia in this model are independent from maternal undernutrition. In addition, previous studies in our own laboratory on the same ovine model of gestational hypoxia confirmed that ewes undergoing hypoxia in the last third of pregnancy showed no long-term alterations in maternal or fetal plasma concentrations of stress hormones, indicating that this level of hypoxia does not induce a significant stress response (Brain et al., 2015). In contrast to sheep, rodent species are less resilient to the effects of hypoxia. For example, this level of maternal hypoxia consistently induces significant reductions in maternal food intake of the order of 30-40% in rat pregnancy, making it more difficult to isolate the effects on outcomes of gestational hypoxia from maternal and thereby fetal nutrient restriction (de Grauw et al., 1986; Williams et al., 2005; Camm et al., 2011; Higgins et al., 2016; Nuzzo et al., 2018).
3.4.3 Fetal blood and biometry

An increase in fetal haemoglobin and haematocrit in response to chronic hypoxia is a well-documented adaptive response to increase oxygen carrying capacity in both humans and animal models (Gilbert et al., 1979; Alonso et al., 1989; Kitanaka et al., 1989; Kamitomo et al., 1993). It is thought that hypoxia-inducible factors orchestrate the increase in erythropoietin levels that mediate increased red blood cell production (Haase, 2013). In addition, there is evidence of adaptive changes to the oxygen-haemoglobin dissociation curve in response to hypoxia. For example, several studies have shown that humans experience a rightward shift of the oxygen-haemoglobin dissociation curve upon acclimatisation to high altitude, decreasing the affinity of haemoglobin to oxygen (Aste-Salazar and Hurtado, 1944; Lenfant et al., 1968). In contrast, animals adapted to high altitude, such as llamas, often show a leftward shift of their oxygen-haemoglobin dissociation curve (Banchero and Grover, 1972; Petschow et al., 1977; Moraga et al., 1996). There are also reports demonstrating that the affinity of haemoglobin for oxygen remains unchanged during adaptation to high altitude (Morpurgo et al., 1976; Mairbäurl et al., 1990; Tashi et al., 2014). In the ovine model of isobaric hypoxic pregnancy used in the present study, we observed no changes in maternal $P_{50}$, but hypoxic fetuses showed a tendency towards a leftward shift of the fetal oxygen-haemoglobin dissociation curve with a decrease in fetal $P_{50}$. This would in theory favour oxygen unloading from the maternal into the fetal circulation. However, the nature of the fetal blood samples, which were taken at post-mortem, requires that the data be interpreted with caution.

Gestational hypoxia in the last third of gestation in the present study had significant effects on fetal development, promoting fetal growth retardation. FGR has been described in detail in both high altitude pregnancies and also in other sheep models.
of chronic hypobaric hypoxia (Jacobs et al., 1988; Kamitomo et al., 1993; Giussani et al., 2001; Keyes et al., 2003). Alterations in fetal biometry in the present study indicate that asymmetric FGR is accompanied by significant fetal “brain sparing”, which increases relative brain weight (Wollmann, 1998; Cahill et al., 2014). This has been observed in several animal models of gestational hypoxia and occurs due to a shift in cardiac output towards essential tissues, such as the brain, while sparing “less essential” vascular beds, such as those perfusing the lower limbs, liver, lungs and kidneys (Jensen et al., 1991; Giussani et al., 1994; Ruijtenbeek et al., 2000; Giussani et al., 2007; Cahill et al., 2014). This is consistent with the biometry and the disparities in organ weights between normoxic and hypoxic fetuses in the current study, such as the lighter lungs, liver and kidneys in the hypoxic cohort. In addition, the resulting increase in fetal peripheral vascular resistance is likely to increase fetal cardiac afterload, affecting myocardial development in late gestation (Veille et al., 1993; Skilton et al., 2005; Salinas et al., 2010). Hypoxic fetuses in the present study also showed lower absolute heart weights at 138 dGA. Several studies of placental insufficiency in sheep that result in chronic fetal hypoxaemia and FGR have demonstrated that fetal myocardial development is altered in late gestation, including decreased heart weights and a reduction in cardiomyocyte number (Bubb et al., 2007; Louey et al., 2007; Morrison et al., 2007; Botting et al., 2014). In addition, both in utero and ex utero models of chronic fetal hypoxaemia have shown that these fetuses show impaired cardiac function, including decreased cardiac output, systolic and diastolic dysfunction and abnormalities in cardiomyocyte structure (Brain et al., 2015; Lawrence et al., 2018). These effects of prenatal hypoxia are likely to contribute to the increased risk of ischaemic heart disease and heart failure in adulthood (McMillen and Robinson, 2005; Derks et al., 2010; Giussani et al., 2012; Giussani and Davidge, 2013).
3.4.4 Placental biometry and the unfolded protein response

Despite significant FGR, there was no significant change in total placentome weight and placentome distribution, and there was no difference in the fetal to placental weight ratio, indicating that there was no change in placental efficiency (Fowden et al., 2009; Burton and Fowden, 2012). This is consistent with previous studies of both short-term and long-term hypoxia, as well as human pregnancies at high altitude, which is associated with a reduction in fetal weight, often independent of changes to placental weight, which may even be reduced at high altitude (Jackson et al., 1987; Jacobs et al., 1988; van Patot et al., 2009; Parraguez et al., 2011). While the data did not reach statistical significance due to high sample variability, mean placental weight in the hypoxic cohort was lower than in the control normoxic cohort. Low placental weight is also associated with an increased risk of developing preeclampsia and other pregnancy complications, though the true cause and effect relationship remains to be elucidated (Dahlstrøm et al., 2008; Wallace et al., 2012; McNamara et al., 2014; Dypvik et al., 2017). In the present study, we also did not observe a significant alteration in the distribution of placentome types, or any changes in their relative or absolute weight distribution. This suggests that placentome morphology is not a significant determinant of the placental response to chronic maternal hypoxia (Penninga and Longo, 1998; Zhang et al., 2016b). In reality, as the main interface between the developing fetus and its mother, the role of the placenta in pregnancy complications goes far beyond a simple relationship with placental weight and gross morphology, and more covert forms of placental dysfunction are likely to underlie the changes to transplacental exchange and endocrine activity. Many complications of pregnancy, including placental insufficiency, pregnancy at high altitude and preeclampsia, are associated with impaired oxygenation and increased uteroplacental
vascular resistance (Brosens et al., 1972; Zamudio et al., 1995; Browne et al., 2011; Krishna and Bhalerao, 2011). Reduced uteroplacental perfusion induces placental hypoxia and directly limits adequate oxygen and nutrient delivery to the fetus, causing FGR (Krishna and Bhalerao, 2011; Burton and Jauniaux, 2018). Further, the impaired oxygenation in hypoxic pregnancies and pregnancies complicated by preeclampsia have been consistently associated with the development of placental oxidative stress, exacerbating placental dysfunction (Zamudio et al., 2007b; Myatt, 2010; Richter et al., 2012; Schoots et al., 2018). Placental hypoxia and placental oxidative stress therefore trigger a vicious cycle that appears central to the pathogenesis of many types of adverse pregnancy, including high altitude, preeclampsia and FGR (Burton and Jauniaux, 2004; Zamudio et al., 2007b; Tissot van Patot et al., 2012; Schoots et al., 2018). Oxidative stress, beyond the level required for physiological signalling, can pose a major threat to cell viability by causing oxidative damage of lipids, proteins and nucleic acids (Valko et al., 2007; Zhang et al., 2016a). In the present study, we observed an increase in total protein carbonylation in hypoxic placentomes, a marker of oxidative protein damage, which is consistent with findings from in vitro models of experimental hypoxia and from animal models and women suffering from preeclampsia (Zusterzeel et al., 2001; Soleymanlou et al., 2005; Vanderlelie et al., 2005; Doridot et al., 2014; Burton et al., 2017a). Oxidative stress and oxidative protein damage are closely associated with disruption of ER homeostasis. As the main site of cellular protein synthesis, folding and post-translational modification, the ER is sensitive to a number of stressors, such as oxidative stress and hypoxia (Schröder and Kaufman, 2005; Ron and Walter, 2007; Wouters and Koritzinsky, 2008). The ER maintains strict quality control of proteins through activation of the UPR to maintain and restore ER function (Figure 3.1; Ron and Walter, 2007). Downstream
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consequences include rapid inhibition of nonessential protein synthesis and longer term upregulation of chaperone proteins and quality control enzymes. In addition, activation of ERK may induce metabolic adaptations through pyruvate kinase and alterations of glucose metabolism with advancing gestation in response to altered oxygen and energy supply (Bahr et al., 2014). The role of ERK in pregnancy is manifold, and while ERK is vital for healthy trophoblast proliferation and invasion in early pregnancy, ERK activity seems to be involved in cellular survival and energy metabolism towards the end of gestation. This may explain why ERK phosphorylation is often decreased in preeclamptic placentae in the first trimester, while the opposite is reported in the last trimester, as in the present study (Arroyo et al., 2010; Bahr et al., 2014; Tang et al., 2014; Dong and Shi, 2016; Jiang and Zhao, 2018; Wang, 2020).

If the cellular homeostatic response is unable to restore energy supply for protein synthesis with continued ER dysfunction and ER stress, ATF4 translocation, CHOP signalling and activation of JNK can trigger cell death in an attempt to maintain tissue homeostasis at the expense of the individual cell (Harding et al., 2000; McCullough et al., 2001; Ghosh et al., 2012; Darling and Cook, 2014).

Activation of these pathways has been documented in placentae from pregnancies complicated by FGR, preeclampsia and high altitude (Yung et al., 2007; Yung et al., 2008; Burton et al., 2009; Yung et al., 2012; Yung et al., 2014). For example, placentae from pregnant women at high altitude show increased activation of the UPRER, which can be viewed as a homeostatic response (Yung et al., 2012; Burton and Jauniaux, 2018). This may affect the nutrient transfer capacity of the placenta, providing a mechanistic explanation for the FGR observed at high altitude. More severe activation of the UPRER with expression of CHOP is observed in pregnancies complicated by FGR in combination with preeclampsia (Yung et al., 2008; Yung et al., 2012). This can
be recapitulated in vitro by treating trophoblast cells with increasing doses of the ER stress inducer tunicamycin. Interestingly, activation of the placental UPR is characteristic of early-onset preeclampsia with impaired spiral artery remodelling and significant maternal cardiovascular dysfunction, but less so of late-onset preeclampsia, which rarely presents with FGR (Yung et al., 2014; Mizuuchi et al., 2016; Du et al., 2017). Thus, the degree of activation of the placental UPR may have pathophysiological significance, as its severity will determine placental release of pro-inflammatory cytokines, trophoblast senescence and even apoptosis (Zhang and Kaufman, 2008; Hotamisligil, 2010; Burton and Jauniaux, 2018). This has led to the hypothesis that the level of placental UPR activation may provide the distinction between pregnancies complicated by combined FGR and preeclampsia, and pregnancies complicated by FGR alone (Burton and Jauniaux, 2018).

The placental molecular signature in this chapter closely resembles the activation of the placental UPR response of early-onset preeclampsia, including activation and nuclear translocation of ATF6, increased expression of the ER chaperones GRP78, activation of MAPKs and expression of cytosolic protein chaperones (Yung et al., 2014). The electron microscopy data presented in this study also showed that these functional changes to ER protein handling are associated with morphological distension and expansion in response to increased protein folding demands. Similar morphological dilation of the ER has been observed in the trophoblast during periods of ischaemia and in placentae from Nepalese women (Jones and Fox, 1980; Soma et al., 2005; Yung et al., 2007; Yung et al., 2008; Bommiasamy et al., 2009). Furthermore, we found increased levels of the cytosolic chaperones HSP27 and HSP70 in hypoxic placentomes, which are part of the URP_Cyt and considered the “second line of defence” to placental oxidative stress in chronic complications, such as in preeclampsia (Geisler
et al., 2004; Ekambaram, 2011; Hromadnikova et al., 2015). In contrast, we have found no evidence of activation of ATF4 signalling in placentomes from the hypoxic cohort, with no changes to levels of CHOP and cellular apoptosis markers BCL2 and BAX and the executioner caspase 3. We also did not observe any changes to placental collagen deposition or the rate of cellular proliferation. Thus our data are inconsistent with the notion that mild ER stress leads to inhibition of cellular proliferation while more severe ER stress is associated with an increase in apoptotic cell death in the placenta, as we observe activation of the UPRER in the absence of gross morphological changes to the placenta, such as proliferation, apoptosis or fibrosis. These findings are in keeping with the outcome in other studies on rats, which show that the placenta exhibits at least some degree of resilience in response to hypoxia once it is fully formed (Nuzzo et al., 2018). In combination with the lack of change to total placental weight and placentome distribution, this suggest that chronic hypoxia in the last third of gestation has no or only minor effects on macroscopic tissue composition of the established placenta, but it has rather profound effects on protein homeostasis and subcellular signalling. All proteins for secretion require translational and post-translational modifications in the ER, and any disturbance to ER homeostasis will have detrimental consequences for placental function, fetal development and maternal health. Interestingly, this study induced a late-onset hypoxic insult in the last third of pregnancy, but recapitulated the placental molecular signature of early-onset preeclampsia. While this may seem counterintuitive, it supports the notion that hypoxia and oxidative challenges are vital in inducing placental stress, and that they are key drivers in the pathogenesis of both early- and late-onset preeclampsia.
3.4.5 Conclusion

In this study, we have shown that hypoxic pregnancy in the last third of ovine gestation resulted in asymmetric FGR independent of changes in maternal food intake. While hypoxic pregnancy in the present study did not lead to changes to placental gross morphology, it was associated with significant ER stress, ER distension and activation of placental unfolded protein and stress responses. This is likely to have significant effects on placental metabolism, which will be addressed in the next chapter.
4. Chronic hypoxia and placental mitochondrial function

4.1 Introduction

Mitochondria and the mitochondrial ETC are one of the main sources of ROS, which can contribute to oxidative stress and alterations in mitochondrial structure and function (Hung et al., 2002; Colleoni et al., 2013). Thus, mitochondrial dysfunction has been heavily implicated in the pathoetiology of gestational disorders that share a mutual phenotype of restricted placental oxygen supply (Mailloux, 2015; Holland et al., 2017). As the sites of oxygen consumption-coupled ATP synthesis, mitochondria are known as the “powerhouses of the cell” (Rich, 2003; Holland et al., 2017). In brief, electrons supplied by reactions of the citric acid cycle are transferred to oxygen in a series of redox reactions through complexes I, II, III and IV, which pump protons into the mitochondrial intermembrane space (Figure 2.13). This establishes the mitochondrial membrane potential of a proton-motive gradient between the mitochondrial matrix and the intermembrane space, which drives ATP synthesis by an F$_1$F$_0$-type ATP synthase and releases ROS as a by-product (Holland et al., 2017). Glycolysis combined with OXPHOS can thus yield 30-38 molecules of ATP per molecule of glucose consumed, in contrast to only two molecules of ATP produced through glycolysis combined with anaerobic fermentation to lactate (Rich, 2003). In addition to cellular energy metabolism, mitochondria are also regulators of cell fate by mediating calcium release and caspase activation (Miller, 2013; Martinez et al., 2015).

Mitochondria generate ROS in the transfer of electrons from complexes I, III and, to a lesser extent, from complex II to oxygen, producing superoxide and hydrogen peroxide (Quinlan et al., 2013; Holland et al., 2017). Mitochondria are also susceptible to
damage by ROS and under conditions of hypoxia and oxidative stress the mitochondrial ETC can exacerbate oxidative stress by enhancing ROS production (Bindoli, 1988; Lushchak, 2014; Mailloux, 2015; Angelova and Abramov, 2016). Similar to the activation of the UPRER, mitochondria can activate the UPRmt in response to oxidative damage, designed to maintain mitochondrial function and to remove damaged mitochondria by autophagy (Figure 4.1; Hamanaka and Chandel 2010; Yung et al., 2019). Most mitochondrial proteins enter the mitochondrial matrix as nascent polypeptides, where they undergo folding and refolding assisted by mitochondrial chaperones HSP60 and GRP75 (Neupert and Herrmann, 2007; Chacinska et al., 2009). The co-chaperone TID1 activates the ATPase activity of GRP75 to assist in refolding of misfolded proteins (Voos et al., 2013). Should folding or refolding of proteins fail, mitochondria can activate their protein degradation machinery, mediated by the quality control proteases caseinolytic protease proteolytic subunit (ClpP) and paraplegin (Haynes et al., 2007; Voos et al., 2013). ClpP-mediated degradation of proteins produces short peptides that translocate to the cytosol, activating ATF5 (Haynes et al., 2007; Wu et al., 2018). ATF5 is the functional human orthologue to activating transcription factor associated with stress (ATFS-1) in C. elegans, which translocates to the nucleus to stimulate expression of mitochondrial chaperones and proteases (Zhao et al., 2002; Haynes and Ron, 2010; Fiorese et al., 2016). Mitochondrial proteostasis is complex and tightly linked to the activation of the UPRER, allowing noncanonical pathways to be able to trigger the UPRmt in the absence of ClpP activity (Quirós et al., 2017; Yung et al., 2019).

Additional placental mitochondrial adaptive responses that occur under conditions of hypoxia and oxidative stress, such as in preeclampsia, include remodelling of the ETC and alterations in citric acid cycle activity and β-oxidation, ultimately leading to
dyslipidaemia and decreased efficiency of ATP synthesis (Bartha et al., 2012; Shi et al., 2013; El Khouly et al., 2016). Alterations of complex-specific enzyme activity and abundance become more apparent with advancing gestation and prolonged hypoxia, and are associated with an increase in ROS generation and nitrotyrosine production, and impaired OXPHOS (Lenaz et al., 2010; Bleier and Dröse, 2013; Dröse, 2013; Myatt et al., 2014; Bleier et al., 2015; Vinogradov and Grivennikova, 2016; Fuhrmann and Brüne, 2017). These alterations in mitochondrial oxygen-dependent energy metabolism may be, at least partly, mediated by the downstream effects of HIF1α signalling and its target genes, which are dysregulated in placentae and maternal plasma during preeclampsia (Rajakumar et al., 2003; Akhilesh et al., 2013; Fuhrmann and Brüne, 2017). HIF1α upregulates expression of the hypoxamir miR-210, which is expressed threefold in placentae from preeclamptic compared to control pregnancies, and suppresses the essential iron sulphur cluster assembly enzyme (Pineles et al., 2007; Favaro et al., 2010; Devlin et al., 2011; Muralimanoharan et al., 2012; Gan et al., 2017; Li et al., 2019).

HIF1α itself also induces NADH dehydrogenase 1α subcomplex, which reduces complex I activity under hypoxic conditions in order to limit mitochondrial ROS production and to maintain mitochondrial membrane potential (Tello et al., 2011). Furthermore, HIF1α inhibits the citric acid cycle by inducing phosphoinositide-dependent kinase 1 (PDK1), driving the characteristic shift from OXPHOS to glycolysis through inhibitory phosphorylation of pyruvate dehydrogenase (PDH; Kim et al., 2006; Semenza, 2007, 2011). In combination with the hypoxia-mediated increase in lactate dehydrogenase (LDH) activity, this often leads to a relative increase in conversion of pyruvate to lactate compared to acetyl-CoA, altering the placental demand for glucose (Kim et al., 2006; De Saedeleer et al., 2012).
As the main substrate for placental energy production, glucose consumption and its transporter expression increase in the human placenta with advancing gestation to accommodate the growing fetus (Masahiro et al., 1995; Illsley, 2000; Stanirowski et al., 2020). Similarly, GLUT1 and GLUT3 expression increase in the ovine placenta towards the end of pregnancy, with an increase in the relative importance of GLUT3 towards term (Ehrhardt and Bella, 1995; Currie, 2001). In light of their importance for fetoplacental metabolism, the placental glucose transporter expression has been found to be altered in many pregnancy disorders, including gestational hypoxia and diabetes, FGR and preeclampsia. Glucose transport and consumption is upregulated in a number of \textit{in vitro} models of placental hypoxia (Esterman et al., 1997; Hayashi et al., 2004; Baumann et al., 2007). This is possibly mediated by HIF1α target genes insulin-like growth factor 1 and 2 (IGF1 and IGF2), which can upregulate GLUT expression via PDK1-dependent phosphorylation of protein kinase B (AKT; Figure 4.1; Alessi et al., 1997; Feldser et al., 1999; Pringle et al., 2009; Yu et al., 2012). While hypoxia induces increased activation of AKT in the trophoblast \textit{in vitro}, pregnancies complicated by gestational hypoxia, preeclampsia and FGR \textit{in vivo} can show changes in AKT activation and glucose transporter and IGF expression in both directions (Gratton et al., 2002; Díaz et al., 2005; Trollmann et al., 2007; Yung et al., 2008; Park et al., 2010; Zamudio et al., 2010; Yu et al., 2012; Dubova et al., 2013; Higgins et al., 2016; Matheson et al., 2016; Liao et al., 2017; Lüscher et al., 2017; Nuzzo et al., 2018; Stanirowski et al., 2020). These disparities may in part stem from the fact that full activation of AKT requires phosphorylation by PDK1 at its T308 residue as well as by the mammalian target of rapamycin complex 2 at its S473 residue. It has been proposed that these two sites present independent regulators of AKT signalling, and the ratio of T308 to S473 phosphorylation is altered in several models of gestation.
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complicated by undernutrition, hypoxia, endocrine manipulation, or a combination of those (Zhu et al., 2007; Yung et al., 2008; Zhu et al., 2009; Sferruzzi-Perri et al., 2011; Yung et al., 2012; Sferruzzi-Perri et al., 2013b; Vadlakonda et al., 2013). However, while it is acknowledged that the placental IGF2 axis is altered in placentae from complicated pregnancies, the individual contribution of each insult to site-specific phosphorylation has not yet been resolved.

While β-oxidation was initially regarded as negligible in the placenta, fat metabolism is now known to be an essential component of placental development and function, despite being poorly understood (Oey et al., 2003). The human placenta uses fatty acids as significant metabolic fuel, and placental mitochondrial β-oxidation disorders have been implicated in compromised pregnancies, including preeclampsia, HELLP syndrome and FGR (Shekhawat et al., 2003; Holdsworth-Carson et al., 2010; Shin et al., 2016). Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated transcription factor expressed in the trophoblast that sits at the cusp between placental vascular health and metabolic regulation (Parast et al., 2009; Matsuda et al., 2013; Garnier et al., 2015). As a regulator of cellular proliferation, inflammatory responses and metabolic processes, PPARγ is involved in the modulation of vascular reactivity by inhibiting ET-1 synthesis and stimulating NO production, but also regulates lipid distribution, insulin-sensitivity and secretion of adipocytokines, such as adiponectin (Figure 4.1; Delerive et al., 1999; Moller and Berger, 2003; Polikandriotis et al., 2005; McCarthy et al., 2011; Astapova and Leff, 2012). Adiponectin signals via two transmembrane adiponectin receptors 1 and 2 (AdipoR1 and AdipoR2) in the placenta (Caminos et al., 2005; Chen et al., 2006; Hastie and Lappas, 2014). In concert with a plethora of other cytokines, adiponectin is involved in the maternal metabolic adaptation to pregnancy, essential for healthy placental and fetal development.
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(Pajvani et al., 2003; Kasimanickam and Kasimanickam, 2011). The expression of both adiponectin and PPARγ is upregulated by activity of the other, and due to their overlapping roles in placental development, vascular health and lipid metabolism, their interaction is far more complex than sequential signalling (Barak et al., 1999; Walczak and Tontonoz, 2002; Yamauchi and Kadowaki, 2008). Adiponectin signalling has antioxidant effects through activation of eNOS and inhibition of iNOS induction to preserve bioactive NO, which has been shown to improve endothelial function in hyperlipidaemic rats by reducing nitrosative stress (Li et al., 2007). These mechanisms may, at least partly, explain the important anti-inflammatory functions of adiponectin in suppressing inflammatory cytokine signalling in the vascular endothelium, including TNFα, IL-1, IL-6 and IL-8 (Bruun et al., 2003; Tilg and Moschen, 2006). In addition, AMP-dependent kinase (AMPK) is activated downstream of adiponectin signalling, and some of its targets for phosphorylation include acetyl-CoA carboxylase (ACC) and eNOS (Heiker et al., 2010; Astapova and Leff, 2012). AMPK inhibits ACC, which leads to a decrease in cellular malonyl CoA concentration, triggering increased mitochondrial import of fatty acids for ß-oxidation by stimulating the activity of carnitine palmitoyl transferase 1 (CPT1; Figure 4.1; Tomas et al., 2002; Garcia and Shaw, 2017). In addition, ACC is directly regulated by PPARγ at the level of gene expression and they are often found co-induced or co-repressed (Piguet et al., 2009; Zhao et al., 2010). Thus, both PPARγ and adiponectin signalling are vital in mediating common shared pathways between metabolic homeostasis, vascular function and inflammation (Heiker et al., 2010). The activity of both is often altered in complicated pregnancies presenting with placental hypoxia, and preliminary investigations have been launched into the potential of PPARγ and AMPK agonists as methods of intervention against a variety of compromised pregnancies, including pregnancy at high altitude.
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(Holdsworth-Carson et al., 2010; Julian et al., 2014; Geach, 2015; Kumagai et al., 2018; Lane et al., 2019a). In contrast, adiponectin is more controversial, and conditions, such as diabetes and preeclampsia, often present with paradoxical elevations of adiponectin despite its anti-inflammatory and insulin-sensitising properties (Ramsay et al., 2003a; Koistinen et al., 2006; Nien et al., 2007; Semple et al., 2007; Abd-Alaleem et al., 2011; Woodward et al., 2017). These observations may be indications of a compensatory feedback mechanism secondary to cardiovascular dysfunction to improve fat utilisation and endothelial health, which has given adiponectin the reputation of a “rescue hormone” in more recent times (Ramsay et al., 2003a; Nien et al., 2007; Abd-Alaleem et al., 2011; Woodward et al., 2017).

In addition to functional changes in response to oxidative stress, mitochondrial networks are plastic, and mitochondrial content and dynamics are able to respond to a wide variety of stimuli, including hypoxic stress (Liesa et al., 2009; Holland et al., 2017). Compromised pregnancies that present with placental insufficiency are associated with changes in mitochondrial content (Qiu et al., 2012; Holland et al., 2017). While different laboratories use varying techniques to estimate mitochondrial content, including complex-specific protein levels, complex-specific respiratory activity and mitochondrial DNA transcript levels, citrate synthase activity is the most frequently used marker (Larsen et al., 2012). However, citrate synthase activity can be altered in response to endurance training or under conditions of glucose intolerance and lipotoxicity, which cannot be explained by a decrease in citrate synthase abundance, requiring reports on altered citrate synthase activity to be interpreted with caution (Siu et al., 2003; Kilikevicius et al., 2013; Holland et al., 2017; Alhindi et al., 2019). Differing findings may also be caused by subtle differences in the timing and severity of the insult, which may shift the delicate balance between compensatory mitochondrial
proliferation in response to disrupted cellular bioenergetics and ROS-induced mitophagy (Lee et al., 2000; Sitarz et al., 2012; Hastie and Lappas, 2014; Holland et al., 2017). ROS are powerful modulators of mitochondrial morphology, and mitochondrial dynamics are highly dependent on the cellular and mitochondrial redox state (Willems et al., 2015). One of the main functions of mitochondrial fusion and fission is likely the exchange and replacement of mitochondrial contents damaged by ROS, such as components of the ETC or mitochondrial DNA (Detmer and Chan, 2007). Placental mitochondria in pregnancy disorders associated with placental hypoxia and oxidative stress often show changes to mitochondrial swelling and loss of cristae structure, as well as changes to mitochondrial fusion and fission dynamics (Figure 4.1). For instance, mild forms of preeclampsia are more frequently associated with increases in mitochondrial fusion markers, while in severe forms of preeclampsia mitochondrial fission markers predominate (Wang and Walsh, 1998; Hung and Burton, 2006; Muralimanoharan et al., 2012; Vishnyakova et al., 2016; Holland et al., 2018; Fisher et al., 2020). These differences may reflect cellular attempts to rescue damaged mitochondria and to prevent trophoblast apoptosis in mild forms of preeclampsia, with a shift towards mitophagy and the limitation of mitochondrial respiration and free radical production in more severe forms of the disease (Ausman et al., 2018; Fisher et al., 2020).

Combined, previous studies provide strong evidence to support that placental mitochondrial function is central to the progression of healthy pregnancy, and mitochondrial dysfunction is invariably linked with complications of pregnancy. Therefore, the objective of this chapter was to determine the effects of chronic hypoxia in the last third of gestation leading to FGR in sheep on placental mitochondrial function and changes in placental energy metabolism, as in preeclampsia.
Figure 4.1. Effects of hypoxia on mitochondrial function and energy metabolism.
Mitochondrial respiration is one of the key determinants of placental oxidative stress and substrate metabolism. Oxidation of glucose and fatty acids supplies substrates to the citric acid cycle, which transfers electrons through the ETC in a series of redox reactions, establishing the mitochondrial membrane potential that drives oxidative phosphorylation of ATP. Mitochondria and their electron chains are susceptible to both internally and externally generated ROS and rely on the activation of the UPR\textsuperscript{mt} and the dynamic alteration of mitochondrial morphology to maintain mitochondrial function, ultimately determining cell fate. Hypoxia and oxidative stress also induce signalling cascades capable of altering the transport and metabolism of glucose and fatty acids, governing substrate supply and demand, for example driving glycogen deposition and anaerobic fermentation of pyruvate to lactate.
4.2 Methods

The expanded methodology has been described in the General Methods. Below is the summarised methodology used in this particular chapter, which has been cross-referenced to the General Methods for further detail.

4.2.1 Experimental procedures

Pregnant Welsh Mountain ewes carrying singleton fetuses were weighed and randomly assigned to chronic normoxia or chronic hypoxia at 103 dGA. Ewes assigned to chronic normoxia were housed in individual floor pens, while ewes assigned to chronic hypoxia were moved into one of four bespoke isobaric hypoxic chambers (section 2.2.1). From 105 dGA ewes were exposed to control normoxic conditions or gradually subjected to hypoxia, reaching 10 ± 1% inspired oxygen over 48 hours (Brain et al., 2015). This level of hypoxia was maintained for a month until 138 dGA.

4.2.2 Post-mortem

At 138 dGA, normoxic and hypoxic ewes were moved to the post-mortem laboratory. Ewes in the hypoxic group remained hypoxic at 10 ± 1% inspired oxygen via a respiratory hood until euthanasia. Both normoxic and hypoxic ewes were killed humanely by an overdose of sodium pentobarbitone (0.4 ml.kg⁻¹ i.v., Pentoject; Animal Ltd., UK). Following hysterectomy, individual placentomes were isolated, weighed, classified and counted (section 2.2.4). 1 mm thick strips of two representative type A and type D placentomes were placed in ice-cold 2 mM calcium chloride in 0.05M sodium cacodylate buffer at pH 7.4 containing 2% glutaraldehyde and 2% formaldehyde and in ice-cold BIOPS for electron microscopy and for high resolution oximetry, respectively. Representative examples of type A and type D placentomes
and pieces of the maternal left liver and biceps femoris were snap frozen in liquid nitrogen and stored at -80 °C or fixed in 4% paraformaldehyde (Sigma, UK), transferred to 70% ethanol after 24 hours and stored at 4 °C until further analysis.

4.2.3 Western blotting

Flash frozen type A and type D placentomes were homogenised to tissue lysates using ice-cold cell lysis buffer (Cell Signaling Technology, UK) containing protease inhibitors (Roche Diagnostics, UK). Western blotting was used to determine the relative levels of proteins of interest in protein lysates as described in section 2.3.1.2. Gel loading samples were resolved on 10-12% SDS-PAGE agarose gels and transferred onto nitrocellulose membranes (Hybond® ECL™, Sigma-Aldrich, UK). Non-specific binding was inhibited by blocking the membrane in 5% dry skim milk in TBS-T for 1 hour at RT. Following incubation with the primary antibody, membranes were washed with TBS-T and incubated with the relevant secondary antibodies against Rabbit IgG (Thermo Fisher, UK; diluted 1:10,000) or against Mouse IgG (Thermo Fisher, UK; diluted 1:10,000) for 1 hour at RT. Following further washing with TBS-T, protein levels were visualised using an enhanced chemiluminescence kit (Pierce™ ECL, Thermo Fisher Scientific, UK) on film (Amersham™ Hyperfilm™ ECL, GE Healthcare, UK). Protein band densities were quantified using ImageJ software (NIH; RRID:SCR_003070) and normalised against Ponceau S staining. A full list of primary antibodies, dilutions and incubation times can be found in Table 2.2.

4.2.4 Transcript analysis

RNA was extracted from flash frozen type A placentomes using QIAzol Lysis Reagent Solution and Qiagen miRNeasy purification columns (Qiagen, UK), as described in detail in section 2.3.2.1. RNA was reverse transcribed into cDNA using a RevertAid
First Strand cDNA Synthesis Kit (Thermo Fisher, UK) according to manufacturer’s specifications, as described in section 2.3.2.2. Quantitative real-time PCR was performed using the SYBR® Green system (Thermo Fisher, UK) according to manufacturer’s instructions in the 7500 Fast Real-Time PCR (Applied Biosystems, UK). mRNA transcript levels of unknown genes were determined by the threshold cycle ΔΔCt method and normalised to RPL19 and G6PDH expression, as described previously. All primer sequences can be found in Table 2.3.

4.2.5 Placental histology

4.2.5.1 Immunohistochemistry

Formalin-fixed paraffin-embedded type A placentomes were sectioned to 7 µm thickness using a microtome (Leica Biosystems, UK), mounted onto Superfrost™ Plus microscope slides and incubated at 37 ºC overnight, as described in detail in section 2.4.2. Sections were rehydrated and incubated in 3% hydrogen peroxide (Fisher Scientific, UK) for 15 minutes to block endogenous peroxidase activity. After rinsing in distilled water, the sections were incubated in TBS-TT for 30 minutes. After rinsing in TBS, slides were blocked in 5% BSA in TBS for 1 hour and then incubated overnight in primary antibody in 5% BSA. The following day, sections were washed using TBS-TT and then incubated in biotinylated secondary antibody against Rabbit IgG (Vector Laboratories, Canada; diluted 1:200) in 5% BSA. After washing in TBS-TT, staining was visualised using the VECTASTAIN avidin-biotin complex method (Vector Laboratories, Canada) by adding metal DAB (Thermo Fisher Scientific, UK). Staining was stopped by rinsing in distilled water. Sections were dehydrated, and cover slips mounted using DPX Mountant (Sigma-Aldrich, UK). A full list of primary antibodies, dilutions and incubation times can be found in Table 2.5.
4.2.5.2 Transmission electron microscopy

Tissue processing, embedding and sectioning was performed by the Cambridge Advanced Imaging Centre, as described in section 2.4.6. In brief, small pieces of type A placentome tissue were fixed immediately post-mortem by immersion and osmicated over the course of a week. The tissue was dehydrated and embedded in Quetol epoxy resin over the course of 11 days. 80 nm sections were cut on an Ultracut UCT (Leica, Germany) and mounted onto 400 mesh bare copper grids. Transmission electron microscopy was performed on an FEI Tecnai G2 transmission electron microscope and images were taken using an AMT camera.

4.2.6 High-resolution tissue oxygraphy

At post-mortem, small strips of type A and type D placentomes were placed in ice-cold BIOPS preservation solution (Table 2.6) and permeabilised using saponin, as described in section 2.5. 10 to 20 mg of placental tissue were analysed in duplicate in an Oxygraph-2K (Oroboros Instruments, Austria; Figure 2.13) at 37 °C. The substrate-uncoupler-inhibitor titration protocol (SUIT protocol; Figure 2.13C) allowed assessment of mitochondrial respiratory control in the three coupling control states LEAK, OXPHOS and ET in a sequence of pathway control states (Gnaiger, 2012; Figure 2.13B). Calculations made for unit-specific oxygen consumption, flux control ratios, substrate control ratios and coupling efficiencies are described in detail in section 2.5.

4.2.7 Enzyme activity analysis

For enzyme activity analysis, tissue lysates were prepared from homogenates of frozen type A placentomes using homogenisation buffer (300 µL per 10 mg of tissues; Table 2.4), as described in section 2.3.3. Citrate synthase, PDH, LDH and hexokinase
activities were measured in duplicate using the tissue lysate, assay buffers and saturating concentrations of substrates and cofactors by detecting absorbance changes at various wavelengths in an Evolution 220 spectrophotometer (Thermo Fisher Scientific, USA), as described in detail in section 2.3.3. Enzyme activities were calculated using the Beer-Lambert Law.

4.2.8 Measurement of tissue metabolites

For tissue metabolite analysis, tissue lysates were prepared from flash frozen type A placentomes, maternal liver and maternal skeletal muscle using ice-cold deionised water, as described in section 2.3.4.

Tissue contents of glucose and lactate were measured using a Yellow Springs 2700D Select Biochemistry Analyser (YSI Ltd., UK).

Tissue content of glycogen was measured by a commercially available glycogen assay kit (AB169558, Abcam, UK), as described in detail in section 2.3.4. Background control measurements from liver samples were subtracted from measurements of hydrolysed samples to control for endogenous glucose levels.

4.2.9 Data and statistical analysis

The sample size is n=9 for the normoxic cohort and n=7 for the hypoxic cohort. All data are expressed as mean ± S.E.M. The effect of oxygenation was analysed using the Student’s t-test for unpaired data. For all comparisons, values of \( p < 0.05 \) were accepted as statistically significant. The software used was Graphpad Prism 7 (GraphPad, USA).
4.3 Results

4.3.1 Placental mitochondrial stress

The expression of ATF5 was increased in hypoxic relative to normoxic placentae (Figure 4.2). Immunohistochemical analysis showed that ATF5 localises to the trophoblast, but limited translocation to the nucleus in either normoxic or hypoxic placentomes. This was associated with an increase in placental levels of the mitochondrial protein chaperone HSP60 in the hypoxic compared to the normoxic cohort (Figure 4.3A). HSP60 was localized to the mitochondria of trophoblast tissue. There was also an increase in the mitochondrial co-chaperone TID1 in hypoxic placentomes compared to normoxic placentomes (Figure 4.3B). There was a tendency towards a decrease in the mitochondrial endopeptidase ClpP in hypoxic placentomes, but this was not statistically significant ($p=0.08$; Figure 4.3C).

**Figure 4.2. Placental ATF5 levels.**

Values are mean ± S.E.M. for the relative ratio of placental levels of ATF5 at 138 dGA. Staining of the trophoblast was confirmed through immunostaining. Two representative images taken at 20x magnification are shown. Scale bar = 100 µm. Groups are N (○, n=9) and H (●, n=7). Significant differences ($p<0.05$) are *N vs. H; Student’s t-test for unpaired data.
Figure 4.3. Placental mitochondrial unfolded protein response.

(A) Values are mean ± S.E.M. for the relative ratio of placental levels of HSP60 at 138 dGA. Mitochondrial punctate staining was confirmed through immunostaining. Two representative images of the whole section and taken at 40x magnification are shown. Scale bars = 5mm and 50 µm, respectively. Values are mean ± S.E.M. for the relative ratio of placental levels of TID1 (B) and ClpP (B) at 138 dGA. Groups are N (○, n=9-10) and H (●, n=7). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data.
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Hypoxic placentomes showed decreased levels of the inner mitochondrial fusion protein OPA1 and the outer mitochondrial fusion protein MFN2 compared to normoxic placentomes (Figure 4.4A and B). There was a tendency towards an increase in the mitochondrial fission protein DRP1, but this was not statistically significant ($p=0.06$; Figure 4.4C).

**Figure 4.4. Placental mitochondrial fusion and fission markers.**

Values are mean ± S.E.M. for the relative ratio of placental levels of OPA1 (A), MFN2 (B) and DRP1 (C) at 138 dGA. Groups are N (○, n=9-10) and H (●, n=7). Significant differences ($p<0.05$) are *N vs. H; Student’s $t$-test for unpaired data.

These changes occurred in conjunction with a significant loss of the characteristic
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Mitochondrial cristae structure in hypoxic placentomes, which appear to be smaller and rounder, while normoxic placental mitochondria remained large and ovoid with highly defined cristae structures (Figure 4.5).

**Figure 4.5. Placental mitochondrial morphology.**
Differences in placental mitochondrial structure at 138 dGA were examined by transmission electron microscopy. Two representative images taken at 5,000x magnification are shown. The arrows indicate the location of mitochondria and the stars indicate the location of the nucleus. Scale bar = 500 nm.

### 4.3.2 Placental cellular respiration

There were no differences in mass or unit specific LEAK respiration (OctMₜ) between normoxic and hypoxic placentomes (Figure 4.6A and B). Hypoxic placentomes showed decreased mass and unit specific OXPHOS supported by the F-pathway (OctMₙ), the F-pathway together with the N-pathway supported by pyruvate (PMₙ; Figure 4.6A and B) and the F-pathway together with the N-pathway supported by both pyruvate and glutamate (GMₙ; Figure 4.6A and B). There were no differences in mass or unit specific maximum OXPHOS capacity (GMSₙ) between normoxic and hypoxic cohorts (Figure 4.6A and B). There were no mass specific differences in maximum ET capacity between the two cohorts (GMSₑ; Figure 4.6A).
Figure 4.6. Placental mitochondrial respiration.

Values are mean ± S.E.M. for placental mass specific oxygen consumption (A), unit specific oxygen consumption (B), FCRs of the F-, N- and S-pathways (C), SCR of products of β-oxidation vs. pyruvate and of pyruvate vs. glutamate (D), coupling efficiencies of mitochondrial OXPHOS and ET capacities (E), placental citrate synthase activity (F) and the relative ratio of placental levels of citrate synthase (G) at 138 dGA. Groups are N (○, n=5-9) and H (●, n=5-7). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data.
There were no differences in mass or unit specific ET capacity through the S-pathway (SE) or through respiratory complex IV (TmE; Figure 4.6A and B). Both the FCRs for the F-pathway (FCRF) and the N-pathway (FCRN) were decreased in hypoxic placentomes compared to normoxic placentomes, but there was no change in the FCR for the S-pathway (FCRS; Figure 4.6C). Both the SCRs of products of β-oxidation vs. pyruvate (SCRFA/P) and of pyruvate vs. glutamate (SCRPG) were decreased in hypoxic placentomes compared to normoxic placentomes (Figure 4.6D). There were no differences in coupling efficiencies for either ET or OXPHOS (Figure 4.6E). Hypoxic placentomes showed a decrease in citrate synthase enzyme activity, but there was no difference in citrate synthase abundance compared to normoxic placentomes (Figure 4.6F and G). Hypoxic placentomes also showed an increase in HIF1α levels at 138 dGA (Figure 4.7). Hypoxic pregnancy was associated with a tendency towards decreased complex I abundance in hypoxic compared to normoxic placentomes (p=0.07; Figure 4.8A). Both complexes II and IV were increased in the hypoxic cohort (Figure 4.8B and D). There was no difference in abundance of respiratory complex III between both cohorts (Figure 4.8C). There was a tendency towards an increase in complex V (ATP synthase) in hypoxic placentomes compared to normoxic placentomes (p=0.10; Figure 4.8E).

**Figure 4.7. Placental hypoxia-inducible factor 1α levels.**

Values are mean ± S.E.M. for the relative ratio of placental levels of HIF1α at 138 dGA. Groups are N (○, n=9) and H (●, n=7). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data.
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Figure 4.8. Placental expression of mitochondrial respiratory complexes.

Values are mean ± S.E.M. for the relative ratio of placental levels of complexes I (A), II (B), III (C), IV (D) and ATP synthase (E) at 138 dGA. Groups are N (○, n=9) and H (●, n=7). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data.
4.3.3 Placental glucose handling

While there were no differences in placental transcript levels of IGF1 between normoxic and hypoxic placentomes, there was an increase in IGF2 transcript levels in the hypoxic cohort (Figure 4.9A).

This was associated with an increase in the ratio of the phosphorylated form of PDK1 compared to total levels of PDK1 in hypoxic relative to normoxic placentae at 138 dGA (Figure 4.9B). AKT phosphorylation was also increased at both T308 and S473 residues in hypoxic placentomes, and there was no difference in the ratio between these two phosphorylation sites (Figure 4.9C, D and E).

While there was no difference in placental transcript levels of GLUT1, there was an increase in GLUT3 transcript levels in the hypoxic compared to the normoxic cohort (Figure 4.9F and G).

There were no statistically significant differences in enzyme activities of hexokinase, PDH and LDH between both cohorts, but hexokinase activity showed a tendency towards a decrease in hypoxic placentomes ($p=0.06$; Figure 4.9H, I and J).

Placentomes from hypoxic ewes showed a tendency towards increased glycogen storage compared to normoxic placentomes ($p=0.05$; Figure 4.10A). There were no differences between normoxic and hypoxic ewes in placental lactate content (Figure 4.10B).
Figure 4.9. Placental glucose handling.

(A) Values are mean ± S.E.M. for relative fold change of placental transcript levels of IGF2 at 138 dGA. Values are mean ± S.E.M. for the relative ratio of placental levels of phosphorylated compared to total PDK1 (B), of AKT phosphorylation at residue T308 (C) and at residue S473 (D) compared to total AKT, and of T308 compared to S473 phosphorylation of AKT (E) at 138 dGA. Values are mean ± S.E.M. for relative fold change of placental transcript levels of GLUT1 (F) and GLUT3 (G) at 138 dGA. Values are mean ± S.E.M. for placental activity of hexokinase (H), PDH (I) and LDH (J) at 138 dGA. Groups are N (○, n=9) and H (●, n=7). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data.
Figure 4.10. Placental glycogen and lactate contents.
Values are mean ± S.E.M. for placental glycogen (A) and lactate (B) at 138 dGA. Groups are N (○, n=9) and H (●, n=7). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data.

4.3.4 Placental adiponectin signalling

Hypoxic placentomes showed a relative increase in adiponectin transcript levels compared to normoxic placentomes, but there were no differences in AdipoR1 and AdipoR2 expression (Figure 4.11A and B). Transcript levels of PPARγ were decreased in hypoxic placentomes at 138 dGA (Figure 4.11C). This was associated with a decrease in the ratio of the phosphorylated form of AMPK compared to total levels of AMPK in hypoxic relative to normoxic placentae (Figure 4.11D). Placental expression of IL-1β, IL-6 and IL-8 was not different between both cohorts, but there was an increase in relative transcript levels of TNFα in hypoxic placentomes (Figure 4.11E to H). While there were no differences in the ratio of the phosphorylated form of ACC compared to total levels of the fatty acid synthesis enzyme, the total levels of ACC were decreased in hypoxic placentomes (Figure 4.11I). Hypoxic placentomes also showed increased expression of CPT1 compared to normoxic placentomes at 138 dGA (Figure 4.11J).
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**Figure 4.11. Placental adiponectin signalling.**

Values are mean ± S.E.M. for the relative fold change of placental transcript levels of adiponectin (A), AdipoR1 and AdipoR2 (B) and PPARγ (C) at 138 dGA. (E) Values are mean ± S.E.M. for the relative ratio of placental levels of phosphorylated compared to total AMPK at 138 dGA. Values are mean ± S.E.M. for the relative fold change of placental transcript levels of TNFα (E), IL-1β (F), IL-6 (G) and IL-8 (H) at 138 dGA. (I) Values are mean ± S.E.M. for the relative ratio of placental levels of phosphorylated compared to total ACC and of total ACC at 138 dGA. (J) Values are mean ± S.E.M. for the relative fold change of placental transcript levels of CPT1 at 138 dGA. Groups are N (○, n=9) and H (●, n=7). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data.
There were no changes in maternal hepatic glucose, glycogen and lactate content or in skeletal muscle glycogen and lactate content (Figure 4.12) at 138 dGA.

**Figure 4.12. Maternal tissue glucose, glycogen and lactate contents.**
Values are mean ± S.E.M. for maternal hepatic glucose (A), glycogen (B) and lactate content (C) and for maternal skeletal muscle glycogen (D) and lactate content (E) at 138 dGA. Groups are N (○, n=9) and H (●, n=7).

### 4.3.5 Effect of placentome type on placental mitochondrial function

There were no significant differences in placental protein levels of citrate synthase, the UPR\textsuperscript{mt} signal activator ATF5, the mitochondrial chaperone HSP60, the mitochondrial co-chaperone TID1, the mitochondrial protease ClpP and the mitochondrial fusion protein OPA1 between type A and type D placentomes of normoxic and hypoxic pregnancies at 138 dGA (Figure 4.13A-F). Levels of the mitochondrial fission protein DRP1 were higher in type D compared to type A placentomes in normoxic pregnancies, but lower in type D compared to type A placentomes in hypoxic pregnancies at 138 dGA (Figure 4.13G).
There were no differences in mass or unit specific OctM$_L$, OctM$_P$, PM$_P$, GM$_P$, GMS$_P$, GMS$_E$, S$_E$ and Tm$_E$ between type A and type D placentomes of normoxic and hypoxic pregnancies at 138 dGA (Figure 4.14 and Figure 4.15). There were no changes to FCR$_F$, FCR$_N$, FCR$_S$, SCR$_{FA/P}$, SCR$_{P/G}$, the OXPHOS coupling efficiency or the ET coupling efficiency in type A vs. type D placentomes of normoxic and hypoxic pregnancies at 138 dGA (Figure 4.16).

**Figure 4.13. Mitochondrial stress response in type A vs. type D placentomes.**

Values are mean ± S.E.M. for the relative ratio of placental levels of citrate synthase (A), ATF5 (B), HSP60 (C), TID1 (D), ClpP (E), OPA1 (F) and DRP1 (G). Groups are NA (○, n=5), ND (□, n=5), HA (●, n=5) and HD (■, n=5). Significant differences (p<0.05) are for the main effect of hypoxia and placentome type; when there was an interaction, significant differences (p<0.05) are denoted by *; two-way RM ANOVA with Šidák’s post hoc test.
Figure 4.14. Mitochondrial mass specific respiration in type A vs. type D placentomes. (A) Values are mean ± S.E.M. for placental mass specific oxygen consumption during OctM_L. (B) Values are mean ± S.E.M. for placental mass specific oxygen consumption during OctM_P, PM_P, GM_P and GMS_P. (C) Values are mean ± S.E.M. for placental mass specific oxygen consumption during GMS_E, S_E and Tm_E. Groups are NA (○, n=5), ND (□, n=5), HA (●, n=5) and HD (■, n=5). Significant differences (p<0.05) are for the main effect of hypoxia; two-way RM ANOVA with Šidák’s post hoc test.
Figure 4.15. Mitochondrial unit specific respiration in type A vs. type D placentomes. (A) Values are mean ± S.E.M. for placental unit specific oxygen consumption during OctM_L. (B) Values are mean ± S.E.M. for placental unit specific oxygen consumption during OctM_P, PM_P, GM_P and GMS_P. (C) Values are mean ± S.E.M. for placental unit specific oxygen consumption during GMS_E, S_E and Tm_E. Groups are NA (○, n=5), ND (□, n=5), HA (●, n=5) and HD (■, n=5). Significant differences (p<0.05) are for the main effect of hypoxia; two-way RM ANOVA with Šidák's post hoc test.
Figure 4.16. Mitochondrial respiratory ratios in type A vs. type D placentomes. (A) Values are mean ± S.E.M. for placental FCRs of the F-, N- and S-pathways. (B) Values are mean ± S.E.M. for placental SCRs of products of β-oxidation vs. pyruvate and of pyruvate vs. glutamate. (C) Values are mean ± S.E.M. for placental coupling efficiencies of mitochondrial OXPHOS and ET capacities. Groups are NA (○, n=5), ND (□, n=5), HA (●, n=5) and HD (■, n=5). Significant differences (p<0.05) are for the main effect of hypoxia; two-way RM ANOVA with Šídák’s post hoc test.
4.4 Discussion

4.4.1 Summary

The data in this chapter show that chronic hypoxia in sheep in the last third of pregnancy promoted significant changes in mitochondrial metabolism and structure in the placenta. There was an increase in the UPR\textsuperscript{mt} signal transducer ATF5 in hypoxic placentomes, as well as increased levels of the mitochondrial chaperone HSP60 and the mitochondrial co-chaperone TID1. In contrast, hypoxic placentomes showed decreased levels of the mitochondrial fusion proteins OPA1 and MFN2, which was associated with a smaller mitochondrial size and loss of mitochondrial cristae structure.

There were no changes to total placental OXPHOS or ET capacity, however hypoxic placentomes showed significant reductions in fatty-acid-dependent mitochondrial respiration, as well as decreased respiratory capacity through the N-pathway via complex I. This occurred with decreased relative flux via the F- and N-pathways, and decreased substrate preference for substrates of β-oxidation vs. pyruvate and for pyruvate vs. glutamate in hypoxic placentomes. While hypoxic placentomes showed a decrease in citrate synthase enzyme activity, there were no differences in citrate synthase protein levels compared to normoxic placentomes. These changes in mitochondrial respiratory activity were accompanied by an increase in HIF1α levels in hypoxic placentomes, which was associated with a tendency towards a fall in respiratory complex I abundance and an increase in respiratory complexes II and IV. There was no change in complex III abundance, while ATP synthase showed a tendency towards increased abundance in hypoxic placentomes.

Increased placental transcript levels of IGF2 in hypoxic placentomes occurred in association with increased activating phosphorylation of PDK1 and AKT. While there
was no difference in placental transcript levels of GLUT1, there was an increase in GLUT3 expression in the hypoxic compared to the normoxic cohort. There were no differences in PDH and LDH activities between hypoxic and normoxic placentomes, but hypoxic placentomes showed a tendency towards decreased activity of the glycolytic enzyme hexokinase.

Decreased expression of PPARγ in hypoxic placentomes was associated with decreased activation of the nutrient sensing kinase AMPK. Paradoxically, hypoxic placentomes showed an increase in expression of adiponectin, but not of adiponectin receptors. TNFα was expressed at higher levels in hypoxic placentomes. Placentomes collected from the hypoxic cohort also showed decreased total levels of the fatty acid synthesis enzyme ACC and increased expression of the mitochondrial enzyme CPT1.

There were no considerable differences in activation of the UPR\textsuperscript{mt} pathways and mitochondrial respiratory activity between type A and type D placentomes in normoxic or hypoxic pregnancies at 138 dGA.

### 4.4.2 Placental mitochondrial stress

Even though mitochondrial dysfunction has been widely reported in placentae of complicated pregnancies, pathways involved in the UPR\textsuperscript{mt} are poorly understood (Holland et al., 2017). While canonical signalling within the UPR\textsuperscript{mt} is centred around the quality control protease ClpP, which produces short peptides that drive activation of the UPR\textsuperscript{mt} signal activator ATF5, recently a noncanonical pathway has been described. In that instance, UPR\textsuperscript{mt} was activated in placentae from early-onset preeclampsia following downregulation of ClpP (Yung et al., 2019). This was associated with a decrease in nuclear translocation of ATF5 and occurred through translational inhibition mediated by the UPR\textsuperscript{ER}, but independent of activation of the
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$\text{UPR}^{\text{ER}}$ marker ATF4. The conserved mitochondrial chaperone systems that include HSP60/HSP10 and GRP75/TID1 were elevated and mitochondrial dynamics were shifted towards a fission phenotype. In addition, while knockdown of ClpP stimulated ER stress, knockdown of TID1 suppressed the development of ER stress induced by tunicamycin in placental explants, suggesting that a positive feedback loop may exist between the $\text{UPR}^{\text{ER}}$ and $\text{UPR}^{\text{mt}}$ pathways (Yung et al., 2019). Interestingly, these changes to $\text{UPR}^{\text{mt}}$ signalling were not observed in placentae collected from women suffering from late-onset preeclampsia (Yung et al., 2019). In the present study, we observed similar activation of the $\text{UPR}^{\text{mt}}$, with an increase in the mitochondrial chaperone HSP60 and co-chaperone TID1, despite a tendency towards a decrease of ClpP and limited nuclear translocation of ATF5, and despite late-onset hypoxia in our model. Comparable changes were also found in a rat model of early-onset gestational hypoxia, which showed an increase in the mitochondrial ATPase GRP75 and TID1 in the labyrinth zone (Nuzzo et al., 2018). While this study also observed increased activation of the $\text{UPR}^{\text{ER}}$ in hypoxic placentae, these two pathways do not colocalise in the rodent placenta due to the functional and anatomical separation of the junctional and the labyrinth zone. This prevents the positive feedback between the $\text{UPR}^{\text{mt}}$ and the $\text{UPR}^{\text{ER}}$ and may explain some of the differences between the canonical and the noncanonical signalling pathways discovered in different models of complicated pregnancy.

Activation of the $\text{UPR}^{\text{mt}}$ in the present study was associated with decreased fusion signalling via OPA1 and MFN2, and potentially increased mitochondrial fission signalling via DRP1, which was confirmed by electron microscopy. This is likely a consequence of altered placental redox status in hypoxic placentomes and is consistent with other studies showing similar activation of the $\text{UPR}^{\text{mt}}$ (Vishnyakova et
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al., 2016; Zhou et al., 2017; Yung et al., 2019). However, the current literature is divided about the direction of these changes, possibly due to variations in the degree and duration of placental stress in each study. For example, low grade stress with mild UPR\textsuperscript{mt} activation may favour compensatory fusion to salvage damaged mitochondria and to combine functional components of the ETC, while severe activation of the UPR\textsuperscript{mt} may lead to mitochondrial fission for removal by mitophagy, or even mitochondria-mediated cell death (Houtkooper et al., 2013; Mouchiroud et al., 2013; van der Bliek et al., 2013). While we have not assessed functional markers of mitophagy in the present study, we have not found any changes to apoptotic markers, as discussed in detail in section 3.4.4. While it has been proposed that increased mitochondrial fission may be an adaptive mechanism to limit mitochondrial respiration under conditions of hypoxia to limit generation of mitochondrial ROS, in the present study, there was no change in total OXPHOS capacity. However, increased fission in the present study may allow the ease with which individual mitochondria can alter the specific composition of their ETC (Twig et al., 2008). This facilitates more subtle modulation of mitochondrial respiration and free radical production, while still maintaining total respiratory capacity (Twig et al., 2008).

4.4.3 Placental cellular respiration

As a highly active organ involved in nutrient transfer to the fetus, gas exchange and hormone secretion, the placenta uses almost 40% of the uteroplacental oxygen uptake in healthy pregnancies (Cetin and Antonazzo, 2009). Thus, it seems likely that a scarcity of oxygen supply may cause a potential energy crisis with significant effects on placental metabolic function (Yung et al., 2012). However, it has been reported that fetal oxygen delivery is constant at different altitudes and between different ethnicities.
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when normalised to fetal weight, such that placentae may be able to reduce oxygen consumption to maintain oxygen delivery to the fetus (Siems et al., 1992; Postigo et al., 2009). Like many other tissues, placentae are able to alter their metabolic functions to varying levels of ATP supply and demand (Tissot van Patot et al., 2010). For instance, protein translation and folding is one of the most energy demanding processes, and mild ER stress observed at high altitude may be a homeostatic mechanism to slow protein synthesis and cell proliferation (Yung et al., 2012). More recently, noncanonical activation of the UPR\textsuperscript{mt} involving the downregulation of ClpP and the upregulation of TID1 has been found to suppress mitochondrial respiration (Yung et al., 2019). This is attributed to a decrease in complex II and total OXPHOS respiration, as occurs following knockdown of ClpP (Cole et al., 2015; Yung et al., 2019). Loss of ClpP has been shown to decrease the formation of mitoribosomes, which affects mitochondrial protein synthesis, with consequences on the composition of respiratory complexes (Szczepanowska et al., 2016). In addition, TID1 is found to directly interact with complex I, suppressing its activity (Colleoni et al., 2013; Ng et al., 2014). In the present study, while we also observed decreased complex I activity and a tendency towards decreased complex I abundance under chronic hypoxia, complex-specific respiration of the other parts of the ETC was not altered between normoxic and hypoxic placentomes. These changes are in keeping with the specific activation of UPR\textsuperscript{mt} observed in hypoxic placentomes, which showed a significant increase in TID1 levels, but no significant changes to ClpP. As a major source of mitochondrial ROS, lower complex I activity in hypoxic placentomes may serve to limit excessive mitochondrial ROS production, thereby attenuating damage to mitochondrial proteins and DNA, and maintaining mitochondrial viability (Murphy, 2009). Despite significant reduction of OXPHOS capacity dependent on complex I, total OXPHOS capacity
remained unchanged, suggesting compensation downstream within the mitochondrial ETC. The relative increase in complex II and complex IV abundance, and the tendency towards an increase in ATP synthase levels, may act to maintain mitochondrial respiration and ATP synthesis under conditions of hypoxia, and has also been observed in mouse models of gestational hypoxia towards the end of term (Sferruzzi-Perri et al., 2019). These discoveries are important as they argue against placental respiratory downregulation to maintain fetal oxygen delivery in the face of pregnancy complicated by chronic hypoxia. On the one hand it has been demonstrated that both the human fetus at high altitude and the sheep fetus under experimental hypoxia of ca. 12 to 13% are able to maintain fetal oxygen supply to almost normal sea levels values, and that the restriction of fetal growth is not directly related to reduced fetal oxygen delivery (Kitanaka et al., 1989; Zamudio et al., 2007a; Postigo et al., 2009). However, other groups have argued the opposite, proposing that transplacental oxygen delivery remains a major determinant of fetal growth under conditions of hypoxia in human pregnancies, for instance by inducing compensatory metabolic alterations in the fetus (Moore et al., 2004; Wilson et al., 2007; Cetin et al., 2020). We know from previous studies in our own laboratory that maternal hypoxia at the same degree as in the present study leads to a sustained decrease in fetal $P_aO_2$ to ca. 12 to 13 mmHg. In conjunction with the maintenance of total placental oxygen consumption and significant alterations in fetal growth with evidence of fetal brain sparing, this indicates that placental metabolic alterations in response to 10% chronic hypoxia in the last third of gestation in sheep are not adaptive, and thereby unable to maintain fetal oxygen delivery.

The data in the present study confirm that the ovine placenta can use $\beta$-oxidation to support mitochondrial respiration, and that OXPHOS capacity supported by the F-
pathway amounts to almost 40% of total OXPHOS capacity in placentomes collected from normoxic controls. Thus, defects in placental β-oxidation in complicated pregnancies, such as preeclampsia, may contribute to placental dysfunction observed in these diseases (Bartha et al., 2012). The decreased SCR for octanoyl carnitine vs. pyruvate indicates that OXPHOS using products of β-oxidation is relatively more susceptible to hypoxia than OXPHOS using products of glycolysis. As ATP synthesis from β-oxidation requires ca. 8-11% more oxygen compared to glycolysis, shifting OXPHOS away from the F-pathway may be an adaptive mechanism to maintain ATP synthesis (Murray, 2012). A similar phenomenon has been observed in the placental junctional zone from hypoxic mice at the end of term (Sferruzzi-Perri et al., 2019). In addition, reduced β-oxidation has been observed in trophoblast cells subjected to hypoxia, which occurred in the absence of changes in lipid transport and synthesis (Bildirici et al., 2018). This was correlated with an increase in lipid retention by the trophoblast and the formation of lipid droplets, which may further contribute to the placental dysfunction and reduce lipid trafficking to the fetus.

Many studies have focused on changes in placental mitochondrial content as an indicator of altered mitochondrial function. However, while mitochondrial content can provide some insight into mitochondrial dynamics and biogenesis, it does not reflect changes to mitochondrial respiratory activity or energy metabolism. Secondly, assessing true mitochondrial content is difficult without performing full ultrastructural analyses, and other putative markers of mitochondrial content are not fully validated, delivering inconsistent results (Larsen et al., 2012). This has led to the contradictory preconceptions that reductions in oxidative respiration in hypoxic tissues is associated with decreased mitochondrial biogenesis, but also that hypoxia and high altitude can stimulate mitochondrial biogenesis (Soma et al., 2005; Gutsaeva et al., 2008; Colleoni
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et al., 2013; Matheson et al., 2016). Thus, while we have measured both citrate synthase enzyme activity and abundance, we have not normalised the respirometry data against these markers. The respiratory activity shown in this chapter is presented in two ways: normalised to wet tissue mass and normalised to maximum ET capacity, which provides an estimate for the number mitochondrial units present. The data showed no differences using these two normalisation methods. It is also important to note that any measurements of oxygen consumption using respirometry can only assess maximum capacity under saturating concentrations of substrates, providing merely an estimate of true oxygen consumption *in vivo*. However, the more recent functional studies on tissue respiratory activity, including the present study, deliver more accurate insight into the behaviour of mitochondria *in situ* compared to studies that have attempted to investigate placental mitochondrial dysfunction using isolated primary trophoblast cells or mitochondria isolated from preeclamptic placentae (Colleoni et al., 2013; Vishnyakova et al., 2016; Holland et al., 2018; Yung et al., 2019).

### 4.4.4 Placental glucose handling

Previous studies have shown that the placenta can respond to environmental stressors by adapting both morphologically and functionally to optimise fetal growth (Fowden et al., 2009; Vaughan and Walsh, 2012; Sferruzzi-Perri et al., 2013a). This is also a phenomenon observed at high altitude and under gestational hypoxia in both humans and animal models, including sheep and rodents, in which placentae show changes in placental vascularisation, thinning of villous membranes to reduce barrier thickness and increased oxygen diffusion capacity (Ali et al., 1996; Mayhew, 1998; Tissot van Patot et al., 2003; Zamudio, 2003; Parraguez et al., 2006; Hvizdošová-Kleščová et al., 2013; Zhou et al., 2013; Cuffe et al., 2014). While these morphological
adaptations are widely reported, less is known about the functional adaptations of the placental phenotype and changes to nutrient transport and nutrient metabolism within the placenta. In order to fulfil its functions in nutrient transfer and as an endocrine organ, the trophoblast itself is a major consumer of energy during pregnancy (Bax and Bloxam, 1997). Many complications of pregnancy are associated with abnormal placental carbohydrate metabolism and altered resource allocation, which becomes increasingly critical towards term (Bloxam et al., 1987; Bax and Bloxam, 1997; Magnusson et al., 2004). This is possibly the result of adaptive attempts to match placental metabolism to altered oxygen supply and energy demand following activation of HIF1α, including a switch to anaerobic glycolysis to maintain ATP synthesis (Rajakumar et al., 2003; Jeyabalan et al., 2008; Tissot van Patot et al., 2010; Bahr et al., 2014). While it has been reported that glucose delivery is not always altered in placentae from pregnancies complicated by FGR, this suggests that the reduction in uteroplacental blood flow should be compensated for by mechanisms to increase placental glucose uptake or to reduce placental glucose consumption, such as the use of alternative glycolytic pathways to balance the glucose demands of placenta and the growing fetus (Bloxam et al., 1987; Magnusson et al., 2004; Bahr et al., 2014). Under hypoxic conditions, the extent to which this is possible depends on both the maternal nutritional status and the severity and duration of the hypoxic insult. In a study by Higgins et al., 13% hypoxia in the last third of gestation in mice increased both glucose uptake and clearance in the placenta with minimal FGR, while 10% hypoxia for the same duration reduced glucose clearance at the end of gestation, associated with FGR of ca. 20%. Thus, the authors suggested that mild hypoxia triggers beneficial placental functional and morphological adaptation, while 10% hypoxia had detrimental consequences for materno-fetal resource allocation,
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indicating that the threshold, at which placental adaptation is no longer possible, lies
somewhere between 13% and 10% maternal inspired oxygen (Higgins et al., 2016).
However, that study in mice was confounded by significant maternal hypophagia in
the hypoxic cohort. Even though some of the changes were not observed in a pair-fed
equivalent normoxic cohort, the effects of maternal hypoxia and maternal
undernutrition are not easy to disentangle due to possible interaction between both
gestational insults. One of the benefits of using the sheep model in the present study
included the maintenance of normal feeding patterns in hypoxic ewes despite similar
severity of the hypoxic insult. This permits isolation of the direct effects of hypoxia,
independent from the effects of reductions in maternal food intake. In the present study,
alterations in placental glucose handling were associated with placental changes in
the expression of the HIF1α target gene IGF2 in the placenta, which may be one of
the mechanisms by which maternal hypoxia can modify placental metabolic function
(Feldser et al., 1999; Higgins et al., 2016). For instance, deletion of the IGF2 gene in
the placenta impairs nutrient transport in the mouse placenta and prevents the
beneficial morphological adaptations in response to maternal undernutrition (Coan et
al., 2010; Sferruzzi-Perri et al., 2011; King et al., 2013; Díaz et al., 2014). In the present
study, we found increased placental expression of IGF2, as well as increased
activation of the downstream signalling kinases PDK1 and AKT. A plethora of literature
exists regarding changes in AKT activation in rodent placentae under conditions of
hypoxia, which shows conflicting findings (Yung et al., 2008; Park et al., 2010; Higgins
et al., 2016; Matheson et al., 2016; Nuzzo et al., 2018; Wang et al., 2019). This is, at
least in part, due to the antagonising effects of hypoxia and nutrient restriction on S473
phosphorylation, as well as gestational age-dependent and species-specific effects
(Higgins et al., 2016). Increased placental AKT activation has been observed in other
sheep models of adverse pregnancy, including hyperthermia and nutrient restriction (Zhu et al., 2007; Arroyo et al., 2010).

In the present study, activation of the IGF2 signalling pathway occurred alongside an increase in GLUT3 expression. It has been proposed that increased transplacental supply of glucose at high altitude may promote glycolytic metabolism to spare oxygen for delivery to the fetus and to maintain ATP levels for active nutrient transport (Illsley et al., 2010; Higgins et al., 2016). The same is observed in placentae from fetal growth restricted fetuses, and may, at least partly, occur due to an increase in HIF1α signalling in the chronically hypoxic trophoblast (Janzen et al., 2013). This also matches the shift in substrate preference away from products of ß-oxidation towards products of glycolysis measured in the present study. However, we observed no significant changes in activity of the glycolytic enzyme hexokinase, and the data even showed a tendency towards decreased activity. We also observed no changes in LDH activity and placental lactate content. While there were no significant changes to PDH activity \textit{in vitro}, it is important to note that PDH activity \textit{in vivo} is inhibited by phosphorylation of all four isoforms of PDK (Rardin et al., 2009). Due to the increase in PDK1 phosphorylation, measurement of PDH activity \textit{in vitro} in the present study, under conditions where PDH phosphorylation may be lost during tissue processing, may not truly reflect PDH activity. Taken together, data showing a reduction in substrate preference for pyruvate compared to glutamate, the tendency towards a decrease in hexokinase activity and the increase in placental glycogen content suggest that placentomes from hypoxic pregnancies may be downregulating aerobic glycolysis while maintaining anaerobic glycolysis to produce lactate. Interestingly, placental glycogen content is increased in several complications of pregnancy, including preeclampsia and FGR (Arkwright et al., 1993; Shenoy et al., 2010; Salgado and...
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Salgado, 2011; Akison et al., 2017). A mouse model with a PIGF knockout also showed increased accumulation of glycogen in the junctional zone, which was associated with decreased placental efficiency and a preeclamptic phenotype (Parchem et al., 2018). The function of glycogen in the placenta is still a matter of debate, but is mostly seen as an energy reservoir for both the placenta and the fetus, which can maintain placental metabolism under conditions of oxygen deprivation through increased activity of glycogen phosphorylase and glycogen synthase (Longo et al., 1973; Arkwright et al., 1993; Tsoi et al., 2003; Akison et al., 2017). Data in the present study indicate that the hypoxic placenta is not preserving the fetal resource allocation of oxygen. Instead, the increased placental glucose transport capacity serves to build placental glycogen stores, thereby maintaining resource allocation of glucose long-term at the expense of relative maternal hypoglycaemia. In humans and sheep at high altitude and in chronically hypoxic FGR fetuses, glucose levels in both the umbilical artery and vein are decreased (Mann, 1970; Economides and Nicolaides, 1989; Lueder et al., 1995; Zamudio et al., 2010; Cetin et al., 2020). Zamudio and colleagues interpreted the increase in maternal arteriovenous glucose difference in combination with a reduction in fetal glucose delivery as an indication of increased placental anaerobic glucose consumption (Zamudio et al., 2010). However, we extend these findings by showing that placental metabolic adaptations in response to chronic hypoxia may involve increased glucose storage as glycogen rather than glucose catabolism. This could act as a protective mechanism to ensure longer term, energy reserves, but the data may also indicate a placental imbalance between glycolysis and glycogenesis, impairing transplacental transport of glucose to the fetus and contributing to FGR. These ideas warrant further investigation into placental glucose and oxygen delivery and consumption in this ovine model through four-way
catheterisation of the umbilical vein and artery, and the uterine artery and vein (Ward et al., 2004).

4.4.5 Placental adiponectin signalling

One of the most striking findings in placentomes collected from hypoxic ewes in the present study was an almost sevenfold increase in the expression of the insulin-sensitising and anti-inflammatory adipocytokine adiponectin. This upregulation occurred in the absence of increased expression and activation of its co-regulators PPARγ and AMPK, both of which were significantly downregulated in hypoxic placentomes. Accumulating clinical evidence suggests that adiponectin may play a role in the physiological adaptations to pregnancy by mirroring changes in maternal insulin-sensitivity throughout the course of pregnancy (Aye et al., 2013). For instance, early pregnancy is associated with increased maternal accumulation of glycogen and fat, while advancing gestation is marked by a state of insulin-resistance to limit glucose uptake into maternal tissues to support gluconeogenesis and lipolysis for transplacental delivery of glucose and fatty acids to the growing fetus. These changes are mirrored by an increase in adiponectin in early gestation, possibly to aid in maternal accretion of energy and nutrient stores, with a subsequent decrease in maternal adiponectin levels in the second half of gestation to promote resource allocation to the fetus (Catalano et al., 2006; Mazaki-Tovi et al., 2007; Aye et al., 2013). Similarly, maternal serum adiponectin levels are inversely correlated with fetal growth across the entire range of birth weights (Lowe et al., 2010). However, despite the marked increase in placental adiponectin expression, we observed no other evidence of increased maternal adiponectin signalling, or of changes in maternal insulin sensitivity in hypoxic compared to normoxic control pregnancies. There were no
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differences in hepatic glucose content or in hepatic and skeletal muscle glycogen storage. In addition, there were no changes to hepatic or skeletal muscle lactate content. This suggests that the relative decrease in maternal venous glucose concentration observed in hypoxic ewes (data not shown) in the present study is not due to an increase in insulin-sensitivity and maternal peripheral glucose uptake, but rather the result of increased glucose uptake by the placenta. Furthermore, adiponectin is thought to have anti-inflammatory properties capable of alleviating endothelial function, for example by stimulating NO-dependent vasodilatation (Cheng et al., 2007; Plant et al., 2008; Ebrahimi-Mamaeghani et al., 2015). Again, we observed none of these protective effects on maternal cardiovascular function, which will be discussed in detail in the next chapter. As occurs in preeclampsia, it is possible that increased placental adiponectin expression may be a secondary adaptive response to counteract increased vascular inflammation and endothelial dysfunction in complicated pregnancy (Redman et al., 1999; Ramsay et al., 2003a; Chen et al., 2006; Aloush et al., 2018). Several cytokines, including TNFα, can modulate placental adiponectin gene expression, which was also upregulated in placentae collected from hypoxic ewes in the present study (Greer et al., 1994; Ramsay et al., 2003b). Similar observations of elevations in adiponectin have been made in patients with diabetes and insulin resistance, possibly in an attempt to enhance fat utilisation and reduce lipotoxic stress, which adds weight to the hypothesis that adiponectin may serve as a “rescue hormone” to attenuate systemic vascular dysfunction (Koistinen et al., 2006; Semple et al., 2007; Woodward et al., 2017).

Adiponectin signalling through AdipoR1 and AdipoR2 is proposed to modulate trophoblast metabolism and may work to alleviate placental inflammatory and oxidative stress in adverse pregnancy (McDonald and Wolfe, 2009; Heiker et al., 2010;
Aye et al., 2013). Preeclampsia often presents with impaired placental β-oxidation, which can increase circulating triglycerides leading to lipid accumulation in maternal liver and muscle (Ramsay et al., 2003b). This may, at least in part, occur due to HIF1α-mediated suppression of lipid catabolism and increased lipid transport and storage, and has been observed in several cell types under hypoxia (Gordon et al., 1977; Laurenti et al., 2011). To support this theory, we have also demonstrated reduced utilisation of products of β-oxidation during mitochondrial OXPHOS in placentomes collected from hypoxic ewes. One of the downstream effects of adiponectin is to sensitize tissues to insulin while promoting β-oxidation of fatty acids to reduce lipotoxic stress (Yamauchi and Kadowaki, 2008). Thus, an adaptive increase in adiponectin could act to attenuate fat accumulation in tissues from preeclamptic women (Ramsay et al., 2003a). Consistent with placental adaptations to promote β-oxidation in the face of reduced fatty acid utilisation, we measured a decrease in levels of the catalytic enzyme ACC. This reduces levels of malonyl CoA, relieving its inhibition on CPT1, which performs the rate-limiting step during oxidation of long-chain fatty acids and is required for fatty acid transport into mitochondria for β-oxidation (Wakil and Abu-Elheiga, 2009). Interestingly, the canonical signalling pathway of adiponectin leads to increased phosphorylation of AMPK, which in turn phosphorylates ACC to inhibit its activity (Achari and Jain, 2017). However, in the present study, AMPK phosphorylation was decreased and the potential decrease in ACC activity was attributed to a decrease in total protein levels rather than increased phosphorylation. This suggests that placentomes from the hypoxic cohort were unlikely to be suffering from an acute energy crisis, possibly through an increase in cellular glucose storage as glycogen (Viollet et al., 2010). Adiponectin itself increases insulin sensitivity through interaction of their respective receptors, such that activation of the PI3K/AKT pathway through
combined signalling of IGF2 and adiponectin may be sufficient to increase placental cellular energy levels that renders AMPK activation redundant (Achari and Jain, 2017).

4.4.6 Conclusion

In summary, we have shown that hypoxic pregnancy independent of changes in maternal nutrition in ewes in the last third of gestation is associated with an increase in placental mitochondrial stress and the activation of a noncanonical UPR<sup>mt</sup> pathway. This related to an increase in mitochondrial fission and a decrease in mitochondrial fusion dynamics, which was confirmed by transmission electron microscopy. Hypoxic placentae showed loss of their characteristic cristae structure, and they were rounder and smaller compared to the traditionally elongated mitochondria in normoxic placentomes. These morphological changes occurred together with placental functional changes to both ETC composition and respiratory activity. Decreased complex I oxygen consumption was buffered by increased abundance of respiratory complex II and IV to maintain total placental capacity for OXPHOS. OXPHOS showed a shift away from products of ß-oxidation towards products of glycolysis, which was associated with increased IGF2 signalling to increase placental glucose uptake and reduced PPARγ and AMPK signalling. There was an increase in the placental expression of the adipocytokine adiponectin, which may be a secondary adaptive response to improve placental lipid homeostasis and inflammation. Combined, therefore, the data show that the placenta undergoes significant morphological and functional changes in response to hypoxia-mediated activation of mitochondrial stress pathways. These alterations are designed to maintain cellular respiration and energy supply, similar to changes measured in placentae from preeclamptic women.
5. **Chronic hypoxia and maternal cardiovascular function**

5.1 **Introduction**

During healthy pregnancy, there are maternal haemodynamic adaptations that are important both for appropriate fetal growth and development, as well as the cardiovascular well-being of the mother. These adaptations include the development of a low resistance uteroplacental circulation with advancing gestation, which favours an increase in uteroplacental blood flow to support the growing fetus (Osol and Mandala, 2009; Townsley, 2013). The fall in utero-placental vascular resistance decreases the maternal systemic vascular resistance and thereby the maternal cardiac afterload, reducing her arterial blood pressure with advancing gestation (Osol and Mandala, 2009). There is also an increase in maternal blood volume, which contributes to an increase in maternal cardiac output, buffering the fall in maternal arterial blood pressure and ensuring appropriate perfusion of the uteroplacental vascular beds (Hennessy et al., 1996; Thilaganathan and Kalafat, 2019). Left ventricular remodelling with changes to systolic and diastolic function occurs as an adaptive response to the increase in cardiac output and reduction in systemic vascular resistance in pregnancy to yield a high volume-low resistance circulation (Savu et al., 2012; Melchiorre et al., 2016). Because the maternal plasma volume expansion exceeds the increase in red blood cell production during advancing gestation, pregnancy leads to haemodilution and a state of physiological anaemia (Townsley, 2013).

Like many other complications of pregnancy, such as placental insufficiency and pregnancy at high altitude, preeclampsia is associated with increased uteroplacental
vascular resistance and reduced uteroplacental perfusion (Brosens et al., 1972; Zamudio et al., 1995; Browne et al., 2011; Krishna and Bhalerao, 2011). This directly limits adequate oxygen and nutrient delivery to the fetus, causing intrauterine FGR (Poston, 1997; Lang et al., 2003). In addition, the altered placental vascularisation prevents the development of a high capacitance-low resistance utero-placental bed, raising maternal total peripheral vascular resistance and thus maternal arterial blood pressure (VanWijk et al., 2000; Thilaganathan and Kalafat, 2019). Placental hypoxia and placental oxidative stress also trigger the release of pro-inflammatory cytokines, syncytiotrophoblast debris and anti-angiogenic factors into the maternal bloodstream, contributing to maternal cardiovascular dysfunction by disrupting the complex interactions between several vasoactive factors (Benyo et al., 1997; Benyo et al., 2001; Maynard et al., 2003; Burton and Jauniaux, 2004; Hung et al., 2004; Levine et al., 2004; Zamudio et al., 2007b; Redman and Sargent, 2008; Maynard and Karumanchi, 2011; Tissot van Patot et al., 2012; Malti et al., 2014; Schoots et al., 2018; Tong and Giussani, 2019). Any disruption of the intricate balance of agents that simultaneously regulate uteroplacental and systemic vascular function, as occurs in gestational hypoxia, can create a vicious cycle that appears central to the pathogenesis of many placental disorders, including preeclampsia (Gilbert et al., 2008; Tissot van Patot et al., 2012; Shah and Khalil, 2015).

The occurrence of vascular dysfunction in preeclampsia is paralleled by alterations in maternal cardiac function that go beyond the physiological and reversible mild eccentric maternal cardiac hypertrophy that results from normal pregnancy. Clinical investigations have shown that patients suffering from preeclampsia undergo pathological changes to cardiac structure and function both before and at the clinical onset of symptoms that persist after termination of pregnancy (Wang et al., 2014). For
instance, the increase in vascular resistance compared to healthy pregnancy is 
associated with a reduction in cardiac output, left ventricular hypertrophy and 
remodelling, and impaired cardiac relaxation and contractility (Valensise et al., 2008; 
Melchiorre et al., 2013; De Haas et al., 2017; Thilaganathan and Kalafat, 2019). While 
the long-term added risks of preeclampsia for maternal morbidity and mortality are 
difficult to decipher, women with a history of preeclampsia show increased 
susceptibility to cardiovascular dysfunction in later life, including cardiac ischaemic 
injury and arrhythmia (Smith et al., 2001; Ramsay et al., 2003b; Thilaganathan and 
Kalafat, 2019). Related to these risk factors, preeclamptic women also have increased 
risk of developing chronic kidney disease post-partum (Kristensen et al., 2019). This 
indicates that the renal dysfunction associated with preeclampsia, including increased 
afferent arteriolar resistance and reduced glomerular ultrafiltration coefficients, can 
induce irreversible pathological changes that persist after termination of pregnancy 
(Jeyabalan and Conrad, 2007). Clinically, during the progression of preeclampsia, 
these alterations become evident in increased maternal plasma creatinine 
concentrations, as well as in proteinuria and albuminuria (Kappers et al., 2011; Manaj 
et al., 2011; Cunningham et al., 2016). The histological nephropathy characteristic of 
preeclampsia is termed “renal endotheliosis” and is associated with glomerular 
endothelial swelling, loss of endothelial fenestrae, occlusion of capillary lumens, 
glomerulosclerosis, glomerular hypertrophy and collagen deposition in the mesangium 
(Foidart et al., 1983; Gaber et al., 1994; Nochy et al., 1994). Interestingly, these 
symptoms are very similar to the high-altitude renal syndrome that includes reduced 
renal blood flow, polycythaemia, hyperuricaemia, systemic hypertension, 
microalbuminuria and glomerular hypertension, which are thought to be a result of the 
hypoxia-mediated shift in vascular angiogenic balance (Luks et al., 2008; Hurtado et
Chronic hypoxia and maternal cardiovascular function

al., 2012). This supports the growing evidence that preeclamptic nephropathy is mediated by the plethora of anti-angiogenic and vasoactive factors secreted from the stressed hypoxic placenta, contributing to renal endotheliosis, proteinuria and hypertension during the course of disease progression.

Of the factors released by the trophoblast, sFlt-1 is one of the key drivers of maternal vascular inflammation and endothelial dysfunction in preeclampsia due to its actions as a soluble decoy receptor and antagonist to VEGF and PIgf (Shibuya, 2001; Tanbe and Khalil, 2010). The ratio of sFlt-1 to both provides a representation of maternal and placental angiogenic balance (Torry et al., 1999; Zhou et al., 2002; Charnock-Jones et al., 2004; Li et al., 2005). Gestational hypoxia, placental oxidative stress and preeclampsia are associated with a shift towards anti-angiogenic signalling in both the placenta and the maternal circulation (Maynard et al., 2003; Taylor et al., 2003; Levine et al., 2004; Li et al., 2005; Maynard and Karumanchi, 2011; Tam et al., 2011; Nevo et al., 2013). An increased ratio of sFlt-1 to PIgf and VEGF is often evident even before the onset of symptoms (Maynard et al., 2003; Karumanchi and Bdolah, 2004). Another anti-angiogenic soluble decoy co-receptor often associated with elevated sFlt-1 levels in preeclampsia is sEng. sEng inhibits TGF-β signalling, which leads to dysregulation of placental vascular development, trophoblast invasion and placental endocrine homeostasis (Jones et al., 2006; Xuan et al., 2007; Shao et al., 2009). In the systemic circulation sEng prevents TGF-β-dependent vasodilatation via eNOS and NO (Venkatesha et al., 2006). sEng is increased in women suffering from preeclampsia, and likely acts in concert with sFlt-1 to amplify the symptoms in severe preeclampsia leading to development of HELLP syndrome (Levine et al., 2006; Venkatesha et al., 2006).
HELLP syndrome is a serious complication that occurs in 10-20% of cases of preeclampsia (Haram et al., 2009). Healthy endothelial cells maintain vascular integrity and prevent platelet adhesion through a complex balance between vasodilator autacoids, such as the prostacyclin prostaglandin I\(_2\) (PGI\(_2\)) and NO, and vasoconstrictors, such as angiotensin-II, TXA\(_2\), 5-HT and ET-1 (Vane et al., 1990; Morris et al., 1996; Bolte et al., 2001). In healthy pregnancy, the uterine artery shows a diminished contractile response to administration of TXA\(_2\) analogues (Weiner et al., 1992). In contrast, in preeclampsia, an increased ratio of the pro-aggregatory TXA\(_2\) compared to anti-aggregatory PGI\(_2\) may underlie platelet activation, which is associated with spiral artery thrombosis, reduced uteroplacental perfusion and microangiopathy haemolysis (Friedman, 1988; Zeeman and Dekker, 1992). Activation and aggregation of platelets leads to the release of both 5-HT and TXA\(_2\), which is involved in the onset of hypertension during preeclampsia, and has been mimicked in several animal models (O'Brien et al., 1986; Woods, 1989; Ballegeer et al., 1992; Kriston et al., 1999; Bolte et al., 2001; Perneby et al., 2011). It has been proposed that in preeclampsia and hypertension there is an increase in RhoA and Rho-associated protein kinase (ROCK) pathway signalling that leads to an increase in vascular tone via TXA\(_2\)-mediated vasoconstriction in the uterine vasculature (Goulopoulou et al., 2012; Gu et al., 2017). This stands in contrast to vascular homeostasis in healthy pregnancy, during which the contribution of Rho kinase in TXA\(_2\)-mediated vasoconstriction is reduced. At least part of the vasodilator response to NO in the vasculature is thought to occur through the inhibition of the Rho kinase pathway, while Rho kinase itself can negatively regulate the phosphorylation and activation of eNOS, adding a further level of complexity to the crosstalk between TXA\(_2\) and NO in pregnancy (Ming et al., 2002; Takemoto et al., 2002; Lee et al., 2004).
Within this extensive network of vasoactive agents, the three gasotransmitters NO, H2S and CO are important regulators of vascular function and are involved in several physiological adaptive responses to pregnancy (Rengarajan et al., 2020). All three gasotransmitters are implicated in the pregnancy-associated decrease in uterine artery vascular resistance via endothelial- and smooth muscle-dependent mechanisms that allow increased uteroplacental perfusion with advancing gestation (Kublickienė et al., 1997; Xiao et al., 1999; Magness et al., 2001; Bird et al., 2003; Thakor et al., 2010a; Herrera et al., 2012; Venditti et al., 2013; Aljunaidy et al., 2016; Sheibani et al., 2017; Lechuga et al., 2019b).

NO is an important modulator of vascular tone and a key vasodilator in the uteroplacental and umbilical circulation, in addition to its vasoprotective and anti-atherosclerotic properties (Magness et al., 2001; Cindrova-Davies, 2014). Under conditions of hypoxia or oxidative stress, vascular NO bioavailability can be reduced due to a decrease in eNOS activity and free radical scavenging by superoxide to form peroxynitrite (Kossenjans et al., 2000; Thompson and Dong, 2005; Farrow et al., 2008; Fish et al., 2010; Matsubara et al., 2015). Gestational hypoxia, preeclampsia and gestational diabetes are associated with reduced NO-dependent vasodilatation, both in the uteroplacental and the maternal systemic vasculature (Mateev et al., 2003; Sandrim et al., 2008; Matsubara et al., 2015). NO shares a close relationship with ET-1, which is a powerful antagonist of NO- and endothelium-dependent vasodilatation (Yanagisawa et al., 1988; Khimji and Rockey, 2010; Bourque et al., 2011). Expression of ET-1 and its receptors is increased in gestational hypoxia and is implicated in the increase in uteroplacental vascular resistance and the impairment of uteroplacental perfusion in hypoxia-induced FGR, in both human and sheep pregnancies (Nova et al., 1991; Greenberg et al., 1997; Yamashita et al., 2001; Thaete et al., 2004; Baksu
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et al., 2005; Julian et al., 2008). ET-1 is also elevated in the plasma of women suffering from preeclampsia and in animal models of placental hypoxia, and is a key mediator of hypertension due to its potent systemic vasoactive effects on the maternal endothelium (George and Granger, 2011; Jain, 2012).

ET-1 is also elevated in the plasma of women suffering from preeclampsia and in animal models of placental hypoxia, and is a key mediator of hypertension due to its potent systemic vasoactive effects on the maternal endothelium (George and Granger, 2011; Jain, 2012). Both human and sheep pregnancies are associated with upregulation of endothelial H$_2$S production via CBS and CSE, as well as increased K$_{ATP}$ activation downstream of H$_2$S signalling, which contributes to the maternal vascular adaptation to pregnancy (Zhu et al., 2013; Sheibani et al., 2017; Lechuga et al., 2019a; Lechuga et al., 2019b). While H$_2$S is generally thought to mediate smooth muscle relaxation by increasing K$_{ATP}$ channel activity, there is preliminary evidence that it is also involved in the regulation of other potassium channels, including the BK$_{Ca}$ channel (Jackson-Weaver et al., 2013; Huang et al., 2014). In addition, H$_2$S is involved in modulating the renin-angiotensin system and can interfere with anti-angiogenic factors, such as sFlt-1 (Bryan et al., 2011; Lu et al., 2017; Dongó et al., 2018). CSE expression and activity, as well as circulating H$_2$S levels are reduced in pregnancies complicated by placental insufficiency, preeclampsia and FGR, possibly through miR-21-mediated downregulation of CSE, which is associated with mitochondrial depolarisation, cytotrophoblast apoptosis and villous remodelling (Torry et al., 1999; Zhou et al., 2002; Tsatsaris et al., 2003; Yang et al., 2012; Cindrova-Davies et al., 2013; Wang et al., 2013; Hu et al., 2015; van Goor et al., 2016; Lu et al., 2017). Reduced H$_2$S bioavailability, which can be induced in animal models through treatment with PAG or in CSE knockout mice, is associated with increased levels of sFlt-1 and sEng, impaired uteroplacental blood flow and placental oxidative stress,
linking adverse downstream consequences, such as FGR with adverse upstream consequences, such as maternal hypertension and renal damage (Torry et al., 1999; Zhou et al., 2002; Tsatsaris et al., 2003; Yang et al., 2012; Wang et al., 2013; Holwerda et al., 2014; Lu et al., 2017). One of the ways, in which H₂S improves vascular function in pregnant women with pre-eclampsia is thought to occur through upregulation of miR-133b, which in turn downregulates sFlt-1 release, increasing the bioavailability of PIGF and VEGF (Hu et al., 2017a).

In this chapter, adopting an integrative approach combining experiments in vivo with those in vitro at the cellular and molecular levels, the objective was to investigate whether hypoxic pregnancy leading to FGR in sheep also promotes maternal cardiovascular and renal dysfunction, as in preeclampsia.
5.2 Methods

The expanded methodology has been described in the General Methods. Below is the summarised methodology used in this particular chapter, which has been cross-referenced to the General Methods for further detail.

5.2.1 Experimental procedures

Pregnant Welsh Mountain ewes carrying singleton fetuses were weighed and randomly assigned to chronic normoxia or hypoxia at 103 dGA. Ewes assigned to chronic normoxia were housed in individual floor pens, while ewes assigned to chronic hypoxia were moved into one of four bespoke isobaric hypoxic chambers (section 2.2.1). From 105 dGA ewes were exposed to control normoxic conditions or gradually subjected to hypoxia, reaching 10 ± 1% inspired oxygen over 48 hours (Brain et al., 2015). This level of hypoxia was maintained for a month until 138 dGA. Venous samples were taken from the jugular vein at 103, 105 and 138 dGA, and plasma was stored for further analysis, as described previously. At 138 dGA, normoxic and hypoxic ewes were moved to a nearby ultrasound room and underwent all ultrasound procedures while maintaining the hypoxic exposure in hypoxic ewes using a respiratory hood. Ultrasonography was performed on uterine, femoral and carotid arteries using a Toshiba Powervision 7000 System with a convex 3.75 MHz Toshiba PVK-357AT transducer, as described in section 2.2.3.

5.2.2 Post-mortem

At 138 dGA, normoxic and hypoxic ewes were moved to the post-mortem laboratory. Ewes in the hypoxic group remained hypoxic at 10 ± 1% inspired oxygen via a respiratory hood until euthanasia. Both normoxic and hypoxic ewes were killed
humanely and the maternal heart was immediately placed in ice-cold Krebs-Henseleit bicarbonate buffer for Langendorff heart perfusion experiments, while the left kidney was immediately taken for perfusion fixing. Other maternal organs were isolated and weighed. Relative organ weights were calculated by dividing absolute organ weights by maternal body weights at 138 dGA. A 3rd order branch of the femoral artery was dissected into ice-cold Krebs solution for in vitro wire myography. Following hysterectomy, a 3rd order branch of the uterine artery was dissected into ice-cold Krebs solution for in vitro wire myography. Representative examples of type A and type D placentomes were snap frozen in liquid nitrogen and stored at -80 °C or fixed in 4% paraformaldehyde (Sigma, UK), transferred to 70% ethanol after 24 hours and stored at 4 °C until further analysis.

5.2.3 Ex vivo experiments

5.2.3.1 Wire myography experiments

Third order branches of the maternal uterine or femoral arteries were dissected immediately post-mortem into ice-cold Krebs buffer (Table 2.7). 2 mm sections of the vessels were mounted onto individual channels of a small vessel wire myograph (Muti-wire Myograph System 610M and 620M, DMT, UK; Figure 2.14). Vessels were allowed to warm up to 37 °C and their resting diameter was determined, as previously described. To assess vasoconstrictor function, uterine arteries were exposed to increasing concentrations of 5-HT and U46619, and femoral arteries were exposed to increasing concentrations of PE and ET-1 (section 2.6.1). To assess basal vascular tone dependent on Rho kinase activity, uterine arteries were exposed to increasing concentrations of the Rho kinase inhibitor Y27632 (section 2.6.1). To assess vasodilator function, uterine and femoral arteries were pre-constricted and then
exposed to increasing concentrations of ACh, SNP and NaHS (section 2.6.1). Vascular tension was recorded throughout experiments on Labchart 7 Pro (Version 7.2.4, AD Instruments, Australia).

5.2.3.2 Langendorff heart perfusion experiments
The maternal heart was mounted onto a Langendorff apparatus (Figure 2.17) immediately post-mortem, as previously described (Niu et al., 2013). Mounted hearts were perfused at 70 mmHg with a recirculating Krebs-Henseleit bicarbonate buffer solution (Table 2.8), and a small water-filled balloon was inserted into the left ventricle, mimicking physiological LVEDP. Basal heart rate, LVSP, LVEDP, systolic duration and diastolic duration were recorded using a pressure transducer (Argon Medical Devices, USA) connected to the balloon (section 2.6.2). $\Delta P/\Delta t_{\text{max}}$, $\Delta P/\Delta t_{\text{min}}$, CFR, LVDP, contractility index and $\tau_{\text{au}}$ were calculated as previously described in section 2.6.2.

5.2.4 Western blotting
Flash frozen type A and type D placentomes were homogenised to tissue lysates using ice-cold cell lysis buffer (Cell Signaling Technology, UK) containing protease inhibitors (Roche Diagnostics, UK). Western blotting was used to determine the relative levels of proteins of interest in protein lysates as described in section 2.3.1.2. Gel loading samples were resolved on 10-12% SDS-PAGE agarose gels and transferred onto nitrocellulose membranes (Hybond® ECL™, Sigma-Aldrich, UK). Non-specific binding was inhibited by blocking the membrane in 5% dry skim milk in TBS-T for 1 hour at RT. Following incubation with the primary antibody, membranes were washed with TBS-T and incubated with the relevant secondary antibodies against Rabbit IgG (Thermo Fisher, UK; diluted 1:10,000) or against Mouse IgG (Thermo Fisher, UK; diluted 1:10,000) for 1 hour at RT. Following further washing with TBS-T, protein levels
were visualised using an enhanced chemiluminescence kit (Pierce™ ECL, Thermo Fisher Scientific, UK) on film (Amersham™ Hyperfilm™ ECL, GE Healthcare, UK). Protein band densities were quantified using ImageJ software (NIH; RRID:SCR_003070) and normalised against Ponceau S staining. A full list of primary antibodies, dilutions and incubation times can be found in Table 2.2.

5.2.5 Transcript analysis

RNA and miRNA was extracted from flash frozen type A placentomes using QIAzol Lysis Reagent Solution and Qiagen miRNeasy purification columns (Qiagen, UK), as described in detail in section 2.3.2.1.

5.2.5.1 RNA

RNA was reverse transcribed into cDNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, UK) according to manufacturer’s specifications, as described in section 2.3.2.2. Quantitative real-time PCR was performed using the SYBR® Green system (Thermo Fisher, UK) according to manufacturer’s instructions in the 7500 Fast Real-Time PCR (Applied Biosystems, UK). mRNA transcript levels of unknown genes were determined by the threshold cycle ΔΔCt method and normalised to RPL19 and G6PDH expression, as described previously. All primer sequences can be found in Table 2.3.

5.2.5.2 miRNA

miRNA was reverse transcribed into cDNA using a miRCURY® LNA® RT Kit (Qiagen, UK) according to manufacturer's specifications, as described in section 0. Quantitative real-time PCR was performed using the SYBR® Green system (Thermo Fisher, UK) according to manufacturer's instructions in the 7500 Fast Real-Time PCR (Applied
miRNA transcript levels of unknown genes were determined by the threshold cycle ΔΔCt method and normalised to miR-26b and let-7f-5p expression, as described previously.

5.2.6 Plasma and urine analysis

5.2.6.1 Angiogenic factors

Plasma sFlt-1, sEng, VEGF and PIGF were measured by commercially available ELISA kits (MyBioSource, USA) according to manufacturer’s instructions, as described in detail in section 2.3.5.1. Measurements from plasma samples taken on 103 and 105 dGA before the onset of chronic normoxia or hypoxia were averaged as baseline measurements.

5.2.6.2 Creatinine and total protein

Plasma and urine concentrations of creatinine (Abcam, UK) and total protein (Thermo Fisher, UK) were measured using commercially available colourimetric kits according to manufacturer’s instructions, as described in detail in sections 2.3.5.2 and 2.3.5.3, respectively. Glomerular filtration rate was estimated from plasma creatinine concentrations, as previously described.

5.2.7 Placental and renal histology

Type A placentomes were immersion fixed in 4% paraformaldehyde, while the maternal left kidney was perfusion fixed immediately post-mortem, as described in detail in section 2.4.1. Formalin-fixed paraffin-embedded tissues were sectioned to 7 µm thickness using a microtome (Leica Biosystems, UK), mounted onto Superfrost™ Plus microscope slides and incubated at 37 °C overnight, as described in detail in section 2.4.2.
5.2.7.1 Immunohistochemistry

Sections were rehydrated and incubated in 3% hydrogen peroxide (Fisher Scientific, UK) for 15 minutes to block endogenous peroxidase activity. After rinsing in distilled water, the sections were incubated in TBS-TT for 30 minutes. After rinsing in TBS, slides were blocked in 5% BSA in TBS for 1 hour and then incubated overnight in primary antibody in 5% BSA. The following day, sections were washed using TBS-TT and then incubated in biotinylated secondary antibody against Rabbit IgG (Vector Laboratories, Canada; diluted 1:200) in 5% BSA. After washing in TBS-TT, staining was visualised using the VECTASTAIN avidin-biotin complex method (Vector Laboratories, Canada) by adding metal DAB (Thermo Fisher Scientific, UK). Staining was stopped by rinsing in distilled water. Sections were dehydrated, and cover slips mounted using DPX Mountant (Sigma-Aldrich, UK). A full list of primary antibodies, dilutions and incubation times can be found in Table 2.5.

5.2.7.2 Periodic acid-Schiff stain

Kidney sections were rehydrated, covered in 0.5% periodic acid (Sigma-Aldrich, UK) for 5 minutes, rinsed in distilled water and then covered in Schiff’s reagent (Sigma-Aldrich, UK) for 15 minutes. Sections were dehydrated and cover slips were mounted using DPX Mountant (Sigma-Aldrich, UK). The cortical region of each section was analysed for renal corpuscle area, glomerular area, total proximal tubule diameter, proximal tubule lumen diameter and glomerulosclerosis index through systematic random sampling, as described in section 2.4.4. Urinary space and proximal tubule wall thickness were calculated, as previously described.

5.2.7.3 Picosirius red stain

Kidney sections were rehydrated, covered in 0.1% Direct Red 80 in saturated picric
acid (both from Sigma-Aldrich, UK) for 1 hour and then washed in 0.5% acetic acid (Sigma-Aldrich, UK) twice. Sections were dehydrated and cover slips were mounted using DPX Mountant (Sigma-Aldrich, UK). The area fraction of collagen was determined through systematic random sampling, as previously described in section 2.4.5.

5.2.8 Longitudinal cardiovascular measurements

A second cohort of pregnant ewes were surgically prepared with catheters and flow probes to permit continuous monitoring of arterial blood pressure and uterine blood flow in normoxic and hypoxic groups via the CamDAS™ wireless data acquisition system (Allison et al., 2016; Shaw et al., 2018).

5.2.8.1 Surgery and CamDAS™

The second cohort of pregnant Welsh mountain ewes carrying singleton fetuses underwent laparotomy at 116 ± 1 dGA for instrumentation with the wireless data acquisition system under general anaesthesia, as previously described in section 2.7.2 (Allison et al., 2016). In brief, animals were induced and then maintained under general anaesthesia using 1.5 to 2.0% isofluorane (IsoFlo; Abbott Laboratories Ltd., UK) in 60:40 oxygen:nitrogen. A midline abdominal incision was made, as previously described (Fletcher et al., 2000; Allison et al., 2016; Shaw et al., 2018). A Transonic flow probe (MC2RS-JSF-WC120-CS12-GCP, Transonics, UK) was positioned around the maternal uterine artery, as before, and then exteriorised through a keyhole incision in the ewe’s right flank (Jellyman et al., 2004; Allison et al., 2016; Shaw et al., 2018). Following closure of the abdominal cavity, catheters were inserted via the maternal femoral vein into the maternal inferior vena cava, and via the maternal femoral artery into the maternal descending aorta. Catheters were exteriorised through a keyhole
incision in the maternal left flank. While under general anaesthesia, the ewe was fitted with a bespoke jacket housing the wireless data acquisition system. The CamDAS™ (Maastricht Instruments, the Netherlands) has been previously described in detail (Allison et al., 2016). In brief, the pressure box containing pressure transducers (COBE; Argon Division, Maccim Medical, USA) was connected to catheters, and a miniaturised flow module was connected to the uterine Transonic flow probe. The system is powered by Lithium batteries housed within the same jacket, allowing continuous wireless transmission and recording of maternal uterine blood flow and maternal arterial blood pressure beat-by-beat onto a laptop computer via Bluetooth technology.

5.2.8.2 Experimental procedures

From 120 dGA ewes were fed the daily maintenance diet (5g hay.kg⁻¹ and 40g sheep nuts.kg⁻¹; Manor Farm Feeds Ltd., Oakham, UK) and pregnancies were randomly assigned to chronic normoxia or chronic hypoxia cohorts, as before. Ewes assigned to the hypoxic cohort were transferred to the hypoxic chambers, while ewes assigned to chronic normoxia were housed in individual floor pens occupying the same floor area. At 125 dGA, ewes in the hypoxic cohort were gradually subjected to hypoxia, reaching 10 ± 1 % inspired oxygen over 48 hours (section 2.7.5). Exposure to chronic hypoxia in these ewes lasted 10 days, until 135 dGA. Continuous CamDAS™ recording of maternal arterial blood pressure and uterine blood flow were converted into minute averages off-line and uterine vascular resistance was calculated, as described previously in section 2.7.5. Arterial blood samples were taken daily to measure maternal blood gases, ABE and metabolic status, as previously described (Allison et al., 2016). At the end of in vivo experiments, normoxic and hypoxic ewes were transported to the post-mortem laboratory and humanely killed as before. The positions of the uterine Transonic flow probe and maternal catheter tips were verified.
5.2.9 Data and statistical analysis

The sample size for the uninstrumented cohort is $n=9$ for the normoxic cohort and $n=7$ for the hypoxic cohort. The sample size for the CamDAS™ cohort is $n=5$ for the normoxic cohort and $n=5$ for the hypoxic cohort. All data are expressed as mean ± S.E.M. The effect of oxygenation was analysed using the Student’s $t$-test for unpaired data. The effects of oxygenation, time and interactions between oxygenation and time were compared by two-way repeated measures ANOVA. When a significant difference was detected ($p<0.05$), the Šidák post hoc test was used to control for multiple comparisons and isolate the differences. For all comparisons, values of $p<0.05$ were accepted as statistically significant. The software used was Graphpad Prism 7 (GraphPad, USA).
5.3 Results

5.3.1 Maternal biometry

There were no differences between normoxic and hypoxic ewes in absolute and relative maternal lung weights, liver weight, adrenal weights, perirenal fat depot weights, pancreas weights and thyroid weights at 138 dGA (Table 5.1). There was no difference in absolute heart weight between normoxic and hypoxic ewes, but relative heart weight was higher in hypoxic ewes at 138 dGA (Figure 5.1A). There was also a tendency towards a decrease in absolute kidney weights, which failed to reach statistical significance (Figure 5.1B). However, there was no difference in relative kidney weights between the normoxic and the hypoxic cohort (Figure 5.1B).

<table>
<thead>
<tr>
<th></th>
<th>Normoxic</th>
<th>Hypoxic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>absolute (g)</td>
<td>relative (g.kg⁻¹)</td>
</tr>
<tr>
<td>Left lung</td>
<td>185.8 ± 14.2</td>
<td>3.33 ± 0.17</td>
</tr>
<tr>
<td>Right lung</td>
<td>248.6 ± 20.6</td>
<td>4.45 ± 0.25</td>
</tr>
<tr>
<td>Liver</td>
<td>883.0 ± 38.5</td>
<td>15.9 ± 0.5</td>
</tr>
<tr>
<td>Adrenal</td>
<td>2.28 ± 0.23</td>
<td>0.042 ± 0.005</td>
</tr>
<tr>
<td>Perirenal fat depot</td>
<td>169.1 ± 12.9</td>
<td>3.06 ± 0.24</td>
</tr>
<tr>
<td>Pancreas</td>
<td>50.6 ± 2.9</td>
<td>0.92 ± 0.07</td>
</tr>
<tr>
<td>Thyroid</td>
<td>1.89 ± 0.23</td>
<td>0.034 ± 0.004</td>
</tr>
</tbody>
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Table 5.1. Maternal organ weights. 
Values are mean ± S.E.M. for absolute and relative weight of maternal left lung, right lung, liver, adrenals, perirenal fat depots, pancreas and thyroids at 138 dGA. Groups are N (n=9) and H (n=7).
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5.3.2 Maternal uteroplacental vascular function

5.3.2.1 In vivo measurements of uteroplacental vascular resistance

At 138 dGA, the measurements for uterine artery pulsatility index were greater in hypoxic relative to normoxic ewes (Figure 5.2A).

In the second cohort of instrumented ewes, maternal uterine artery vascular resistance was calculated via continuous wireless CamDAS™ recording of maternal cardiovascular parameters. This showed a fall in uterine artery vascular resistance with advancing gestation in normoxic ewes, but not in hypoxic ewes (Figure 5.2B). Between 122 and 124 dGA, average values for uterine vascular resistance were not different between normoxic and hypoxic ewes (0.29 ± 0.05 mmHg.(ml.min⁻¹)⁻¹ vs. 0.20 ± 0.03 mmHg.(ml.min⁻¹)⁻¹). In normoxic pregnancies, values for uterine vascular resistance were significantly lower at 135 dGA compared to baseline (0.20 ± 0.06 mmHg.(ml.min⁻¹)⁻¹ vs. 0.29 ± 0.05 mmHg.(ml.min⁻¹)⁻¹). In contrast, in hypoxic pregnancies, values for uterine vascular resistance were similar at 135 dGA compared to baseline (0.19 ± 0.04 mmHg.(ml.min⁻¹)⁻¹ vs. 0.20 ± 0.03 mmHg.(ml.min⁻¹)⁻¹).

**Figure 5.1. Maternal heart and kidney weights.**

Values are mean ± S.E.M. for absolute and relative weights of maternal heart and kidneys at 138 dGA. Groups are N (○, n=9) and H (●, n=7). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data.
Daily changes in maternal arterial blood gas, acid-base and metabolic status during baseline and exposure to chronic normoxia or chronic hypoxia of ca. 10% inspired oxygen in the second cohort of animals have been previously reported (Allison et al., 2016). In brief, these data confirm a significant reduction in maternal arterial PO$_2$ from 105.7 ± 3.7 mmHg to 42.0 ± 1.2 mmHg and in arterial oxygen saturation from 103.5 ± 0.5 % to 78.6 ± 5.7 % (Table 5.2).

Ewes exposed to chronic hypoxia had significantly elevated haematocrit and haemoglobin concentration by the end of exposure relative to baseline values and relative to normoxic ewes (Table 5.2). There was no significant change between groups in maternal arterial PCO$_2$, P$_{50}$, O$_2$ content, pH, acid-base excess, blood bicarbonate, glucose or lactate concentrations (Table 5.2).
Chronic hypoxia and maternal cardiovascular function

<table>
<thead>
<tr>
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<th>Normoxic</th>
<th>Hypoxic</th>
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<tr>
<td></td>
<td>baseline</td>
<td>135 dGA</td>
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<tr>
<td>( P_aO_2 ) (mmHg)</td>
<td>104.2 ± 1.9</td>
<td>98.0 ± 5.0</td>
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<tr>
<td>( P_aCO_2 ) (mmHg)</td>
<td>32.3 ±1.3</td>
<td>30.5 ± 2.5</td>
</tr>
<tr>
<td>Sat Hb (%)</td>
<td>102.6 ± 1.3</td>
<td>101.6 ± 3.5</td>
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<tr>
<td>( P_{50} ) (mmHg)</td>
<td>27.0 ± 4.0</td>
<td>23.7 ± 7.3</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>28.6 ± 1.1</td>
<td>28.5 ± 0.9</td>
</tr>
<tr>
<td>Haemoglobin (g.dL(^{-1}))</td>
<td>9.41 ± 0.42</td>
<td>9.20 ± 0.30</td>
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<tr>
<td>( O_2 ) content (mL.dL(^{-1}))</td>
<td>13.2 ± 0.6</td>
<td>12.8 ± 0.1</td>
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<tr>
<td>pH</td>
<td>7.49 ± 0.03</td>
<td>7.46 ± 0.06</td>
</tr>
<tr>
<td>ABE (mmol.L(^{-1}))</td>
<td>4.19 ± 1.06</td>
<td>4.33 ± 2.33</td>
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<tr>
<td>HCO(_3)- (mmol.L(^{-1}))</td>
<td>26.2 ± 1.1</td>
<td>26.7 ± 2.4</td>
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<tr>
<td>Glucose (mmol.L(^{-1}))</td>
<td>2.60 ± 0.14</td>
<td>2.21 ± 0.03</td>
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<tr>
<td>Lactate (mmol.L(^{-1}))</td>
<td>0.56 ± 0.11</td>
<td>0.51 ± 0.06</td>
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Table 5.2. Maternal arterial blood gas, acid-base and metabolic status.
These data were provided by Dr. Beth Allison. Values are mean ± SEM for maternal arterial blood gas, acid-base and metabolic status at baseline and at 135 dGA. Groups are N (○, n=5) and H (●, n=5). Significant differences (\( p < 0.05 \)) are *N vs. H, or † vs baseline; two-way RM ANOVA.

5.3.2.2 Uterine artery in vitro wire myography

Uterine arteries from the hypoxic cohort showed a tendency towards a higher maximal response to 125 mM potassium compared to uterine arteries isolated from the normoxic cohort (\( p = 0.06 \); Figure 5.3A). Uterine arteries isolated from hypoxic ewes showed increased response to 5-HT, with increased tension generated at high 5-HT concentrations, an increase in the area under the curve of the 5-HT response and an increase in the Rho-dependent response to 5-HT (Figure 5.3B, C and D).
There was no difference in the Rho-independent response to 5-HT between uterine arteries from the normoxic and the hypoxic cohort (Figure 5.3D). Uterine arteries from the hypoxic cohort showed increased loss of vascular tone following dose-dependent inhibition of Rho kinase by Y27632, with an increase in the area under the curve of the Y27632 response (Figure 5.3E and F).

There was no difference in the dose-dependent response to the thromboxane analogue U46619 and there was no difference in the area under the curve of the U46619 response between uterine arteries isolated from the normoxic and the hypoxic cohort (Figure 5.3D and H).

There was no difference in the dose-dependent relaxation in response to ACh and there was also no difference in the area under the curve of the ACh response between uterine arteries isolated from the normoxic and the hypoxic cohort (Figure 5.4A and B). There were no differences in the dose-dependent responses to SNP and NaHS and there were no differences in the area under the curve of the responses to SNP and NaHS between uterine arteries isolated from the normoxic and the hypoxic cohort (Figure 5.4).

There were also no differences in the NO-dependent, NO-independent, potassium channel-dependent and potassium channel-independent components of the NaHS-induced vasodilatation (Figure 5.4G and H).

There were no differences in relative phosphorylation of eNOS in placentomes from the normoxic and the hypoxic cohort (Figure 5.5A). There was a tendency towards an increase in placental transcription of eNOS ($p=0.07$) and a significant increase in transcript levels of iNOS in hypoxic placentomes (Figure 5.5B and C).
Figure 5.3. Uterine artery vasoconstrictor properties.

Values are mean ± S.E.M. for the tension generated in response to 125 mM KCl (A), dose-dependent tension generated in response to 5-HT (B), area under the curve of the response to 5-HT (C), area under the curve of the Rho-dependent and -independent response to 5-HT (D), dose-dependent relative relaxation of Y27632 (E), area under the curve of the response to Y27632 (F), dose-dependent tension generated in response to U46619 (G) and area under the curve of the response to U46619 (H). Groups are N (○, n=6) and H (●, n=3). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data or two-way RM ANOVA, where appropriate.
Figure 5.4. Uterine artery vasodilator properties.
Values are mean ± S.E.M. for the dose-dependent relative relaxation of ACh (A), area under the curve of the response to ACh (B), dose-dependent relative relaxation of SNP (C), area under the curve of the response to SNP (D), dose-dependent relative relaxation of NaHS (E), area under the curve of the response to NaHS (F), area under the curve of the NO-dependent and NO-independent response to NaHS (G), area under the curve of the BK$_{Ca}$-dependent and BK$_{Ca}$-independent response to NaHS (H). Groups are N (○, n=3-6) and H (●, n=2-3).
**Chronic hypoxia and maternal cardiovascular function**

**Figure 5.5. Placental NO biology.**
Values are mean ± S.E.M. for the relative ratio of placental levels of phosphorylated compared to total eNOS (A) and for the relative fold change of placental transcript levels of eNOS (B) and iNOS (C) at 138 dGA. Groups are N (○, n=5-9) and H (●, n=5-7). Significant differences (*p<0.05) are *N vs H; Student’s t-test for unpaired data.

### 5.3.3 Maternal angiogenic balance

The placental transcription of the anti-angiogenic factors sFlt-1 and sEng was increased in hypoxic relative to normoxic placentae at 138 dGA (Figure 5.6A and B). There were no differences in VEGF and PIGF expression between the two cohorts (Figure 5.6C and D).

In normoxic ewes, the concentration of sFlt-1 in plasma and the ratio of sFlt-1 to VEGF and to PIGF in plasma did not change in samples taken at baseline and those taken at 138 dGA (Figure 5.7A, E and F). In hypoxic ewes, the concentrations of sFlt-1 and the ratio of sFlt-1 to VEGF and to PIGF were significantly higher at 138 dGA relative to baseline and when compared to values in normoxic ewes at 138 dGA.
Chronic hypoxia and maternal cardiovascular function

There was no change in VEGF concentration in normoxic ewes with advancing gestation, but in hypoxic ewes VEGF levels decreased from baseline to 138 dGA (Figure 5.7C). Neither normoxic nor hypoxic ewes showed changes in sEng or PlGF plasma concentrations with advancing gestation (Figure 5.7B and D). There were no differences in sEng, VEGF and PlGF plasma concentrations between normoxic and hypoxic ewes, neither at baseline nor at 138 dGA.

While there was no difference in placental expression and protein abundance of the H₂S producing enzyme cystathionine-β-synthase (CBS), there was a decrease in placental expression and abundance of CSE in hypoxic compared to normoxic placentomes at 138 dGA (Figure 5.8A-D). CSE was localised mostly to the endothelium and smooth muscle cells of placental villi (Figure 5.8E). In contrast, CBS was localised more broadly to the placental vasculature, the trophoblast and the uterine stroma and epithelium (Figure 5.8F).

![Figure 5.6. Placental transcript levels of angiogenic factors.](image)

Values are mean ± S.E.M. for the relative fold change of placental transcript levels of sFlt-1 (A), sEng (B), VEGF (C) and PlGF (D) at 138 dGA. Groups are N (○, n=9) and H (●, n=7). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data.
Figure 5.7. Maternal circulating angiogenic balance.
Values are mean ± S.E.M. for the maternal plasma levels of sFlt-1 (A), sEng (B), VEGF (C), PIGF (D), of the ratio of sFlt-1 compared to VEGF (E) and of the ratio of sFlt-1 compared to PIGF (F) at baseline and at 138 dGA. Groups are N (○, n=7) and H (●, n=7). Significant differences (p<0.05) are *N vs. H or † vs. baseline; two-way RM ANOVA.
Figure 5.8. Placental hydrogen sulphide synthesis enzymes.
Values are mean ± S.E.M. for the relative fold change of placental transcript levels of CSE (A) and CBS (C) at 138 dGA. Values are mean ± S.E.M. for the relative ratio of placental levels of CSE (B) and CBS (D) at 138 dGA. The representative localisation of CSE (E) and CBS (F) in type A placentomes at 138 dGA is shown at 5x magnification. Scale bar = 500 µm. Groups are N (○, n=9-10) and H (●, n=7). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data.

Hypoxic placentomes showed a decrease in H₂S production compared to normoxic placentomes at 138 dGA (Figure 5.9A). There was no difference in the relative fold change of the CSE-regulating miRNA miR-21 between hypoxic and normoxic placentomes at the end of gestation (Figure 5.9B). However, hypoxic placentomes showed decreased expression of the endogenous H₂S regulating miRNA miR-133b at
138 dGA (Figure 5.9C). Relative fold change of miR-133b transcript levels correlated positively and significantly with placental H$_2$S production at 138 dGA ($R^2$=0.3531; Figure 5.9D).

Figure 5.9. Placental hydrogen sulphide biology.
Values are mean ± S.E.M. for placental H$_2$S production at 138 days gestation (A) and for relative fold change of placental transcript levels of miR-21 (B) and miR-133b (C) at 138 dGA. The correlation of placental H$_2$S production with relative levels of placental miR-133b transcripts (D) was measured by calculating the Pearson product-moment coefficient. Groups are N (○, n=9-10) and H (●, n=7). Significant differences ($p<0.05$) are *N vs. H; Student’s $t$-test for unpaired data or Pearson’s correlation, where appropriate.

5.3.4 Maternal systemic vascular function

5.3.4.1 In vivo measurements of peripheral vascular resistance

At 138 dGA, there were no differences in femoral or carotid artery pulsatility indices between the two cohorts (Figure 5.10A).

Measurement of maternal arterial blood pressure via wireless CamDAS™ recording
showed a fall with advancing gestation in normoxic ewes but not in hypoxic ewes (Figure 5.10B). Between 122 and 124 dGA, average values for maternal arterial blood pressure were not different between normoxic and hypoxic ewes (80.4 ± 1.2 mmHg vs. 81.2 ± 3.2 mmHg). In normoxic pregnancies, values for maternal arterial blood pressure were significantly lower at 135 dGA compared to baseline (73.4 ± 1.1 mmHg vs. 80.4 ± 1.2 mmHg; p<0.05). In contrast, in hypoxic pregnancies, values for maternal arterial blood pressure were similar at 135 dGA compared to baseline (79.6 ± 4.8 mmHg vs. 81.2 ± 3.2 mmHg; p>0.05).

![Figure 5.10. In vivo measures of maternal systemic cardiovascular function.](image)

Values are mean ± S.E.M. for femoral and carotid artery pulsatility indices (A) and the change from baseline in maternal arterial blood pressure (B). Groups are N (○, n=5-9) and H (●, n=5-7). Significant differences (p<0.05) are *N vs. H or † vs. baseline; Student’s t-test for unpaired data or two-way RM ANOVA, where appropriate.

5.3.4.2 Femoral artery *in vitro* wire myography

There was no difference in potassium-induced constriction of femoral arteries between the normoxic and the hypoxic cohort, neither in their relative constriction nor in their area-under-the-curve response (Figure 5.11A and B). The femoral artery contractile response to PE and ET-1 was significantly increased in the hypoxic cohort, both in
relative constriction (Figure 5.11C and E) and in the area-under-the-curve response (Figure 5.11D and F).

There was no difference in ACh-induced relaxation of femoral arteries between the normoxic and the hypoxic cohort, neither in their relative constriction nor in their area-under-the-curve response (Figure 5.12A and B). However, hypoxic pregnancy was associated with a significant impairment of the NO-dependent component of the ACh-induced relaxation (Figure 5.12C). There were no differences in NO-independent, CSE-dependent and CSE-independent components of the ACh-induced relaxation between the two cohorts (Figure 5.12C and D). There were no significant differences between the two cohorts in femoral artery relaxation in response to SNP and NaHS, neither in relative relaxation (Figure 5.12E and G) nor in the area-under-the-curve response (Figure 5.12F and H). Interestingly, against our predictions, NaHS led to femoral artery constriction rather than relaxation (Figure 5.12G).

5.3.4.3 Langendorff heart perfusion

Hypoxic hearts showed a decrease in basal LVDP and this was associated with a tendency towards a decrease in the contractility index of the left ventricle ($p=0.10$; Figure 5.13A and C). There was no difference in $\Delta P/\Delta t_{max}$ between normoxic and hypoxic hearts (Figure 5.13B). Basal LVEDP was increased in hypoxic hearts compared to normoxic hearts (Figure 5.13D). There were no significant differences in $\Delta P/\Delta t_{min}$ and in $\tau$ between normoxic and hypoxic hearts (Figure 5.13E and F).

There were no significant differences in heart rate, the total length of the cardiac cycle, diastolic duration and the ratio of systolic compared to diastolic duration between the two cohorts (Figure 5.13G, H and I). There was a tendency towards a decrease in systolic duration in hypoxic hearts, however this did not reach statistical significance.
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\((p=0.08; \text{Figure 5.13H})\). There were no differences in absolute and relative CFR between normoxic and hypoxic hearts (Figure 5.13J and K).

**Figure 5.11. Maternal femoral artery vasoconstrictor properties.**

Values are mean ± S.E.M. for the dose-dependent tension generated in response to KCl (A), area under the curve of the response to KCl (B), dose-dependent relative tension generated compared to the maximal response to KCl of PE (C), area under the curve of the response to PE (D), dose-dependent relative tension generated compared to the maximal response to KCl of ET-1 (E) and area under the curve of the response to ET-1 (F). Groups are N (○, \(n=6\)) and H (●, \(n=4\)). Significant differences \((p<0.05)\) are *N vs. H; Student’s \(t\)-test for unpaired data or two-way RM ANOVA, where appropriate.
Figure 5.12. Maternal femoral artery vasodilator properties.
Values are mean ± S.E.M. for relative relaxation in response to ACh (A), area under the curve of the response to ACh (B), area under the curve of the NO-dependent and -independent response to ACh (C), area under the curve of the CSE-dependent and CSE-independent response to ACh (D), relative relaxation in response to SNP (E), area under the curve of the response to SNP (F), relative relaxation in response to NaHS (G), area under the curve of the response to NaHS (H). Groups are N (○, n=6) and H (●, n=6). Significant differences (p<0.05) are *N vs. H; Student’s t-test for unpaired data.
Figure 5.13. Maternal basal cardiac function.

Values are mean ± S.E.M. for measures of cardiac contractility LVDP (A), $\Delta P/\Delta t_{\text{max}}$ (B) and contractility index (C), for measures of cardiac relaxability LVEDP (D), $\Delta P/\Delta t_{\text{min}}$ (E) and $\tau$ (F), for heart rate (G), diastolic and systolic duration (H), the ratio of systolic to diastolic duration (I), absolute CFR (J) and relative CFR normalised to heart weight (K). Groups are N (○, n=4-7) and H (●, n=4). Significant differences ($p<0.05$) are *N vs. H; Student’s $t$-test for unpaired data.
5.3.5 Maternal kidney function

Plasma creatinine concentrations were higher in hypoxic relative to normoxic ewes at the end of gestation (Figure 5.14A). Hypoxic ewes had significantly lower estimated glomerular filtration rate at 138 dGA compared to normoxic ewes (Figure 5.14B). However, there was no difference in the ratio of protein to creatinine in urine between groups at 138 dGA (Figure 5.14 C).

![Figure 5.14. Maternal renal function.](image.png)

Values are mean ± S.E.M. for the maternal plasma levels of creatinine (A), for estimated glomerular filtration rate (B) and for the ratio of total protein to creatinine in maternal urine (C) at 138 dGA. Groups are N (○, n=7) and H (●, n=7). Significant differences (p<0.05) are *N vs. H or † vs. baseline; two-way RM ANOVA.

At 138 dGA, renal glomeruli showed no morphological differences in glomerular or Bowman’s space between normoxic and hypoxic ewes (Figure 5.15A and B). There was also no difference in glomerulosclerosis index between normoxic and hypoxic kidneys (Figure 5.15C).
Figure 5.15. Maternal renal glomerular health.
Values are mean ± S.E.M. for the cross-sectional area of glomerular space (A) and Bowman’s space (B), and for the renal glomerulosclerosis index (C). Two representative images taken at 20x magnification are shown. Scale bar = 100 µm. Groups are N (○, n=7) and H (●, n=7).

There were no morphological differences in proximal tubule lumen diameter, proximal tubule wall thickness and the proximal tubule wall to lumen ratio between the two groups at 138 dGA (Figure 5.16).

At 138 dGA, there were no differences in renal glomerular (Figure 5.17), cortical interstitial (Figure 5.18) and medullary collagen content (Figure 5.19) between hypoxic and normoxic cohorts.
Figure 5.16. Maternal renal proximal tubules.
Values are mean ± S.E.M. for proximal tubule lumen diameter (A), wall diameter (B) and wall to lumen ratio (C). Two representative images taken at 40x magnification are shown. Scale bar = 50 µm. Groups are N (○, n=7) and H (●, n=7).

Figure 5.17. Maternal renal glomerular collagen content.
Values are mean ± S.E.M. for the percentage of picrosirius red staining in renal glomeruli. Two representative images taken at 20x magnification are shown. Scale bar = 100 µm. Groups are N (○, n=6) and H (●, n=6).
Figure 5.18. Maternal renal cortical interstitial collagen content.
Values are mean ± S.E.M. for the percentage of picrosirius red staining in the renal cortical interstitial space. Two representative images taken at 20x magnification are shown. Scale bar = 100 µm. Groups are N (○, n=6) and H (●, n=6).

Figure 5.19. Maternal renal medullary collagen content.
Values are mean ± S.E.M. for the percentage of picrosirius red staining in the renal medulla. Two representative images taken at 20x magnification are shown. Scale bar = 100 µm. Groups are N (○, n=6) and H (●, n=6).
5.4 Discussion

5.4.1 Summary

The data in this chapter show that chronic hypoxia for the last third of gestation did not change maternal organ weights at 138 dGA, except for an increase in relative heart weight in hypoxic ewes.

The data also show that the same level of hypoxia for 10 days in the chronically instrumented cohort of animals was sufficient to decrease maternal arterial PO$_2$ and oxygen saturation, while increasing maternal haemoglobin and haematocrit by 135 dGA.

Ewes undergoing hypoxic pregnancy had higher uteroplacental vascular resistance by the end of gestation, both when the uterine artery pulsatility index was calculated non-invasively using Doppler ultrasonography and when uterine artery blood flow was measured invasively using a Transonic flow probe. Hypoxic ewes did not show the characteristic decrease in uterine artery vascular resistance that was observed in normoxic ewes towards the end of gestation. Uterine arteries from the hypoxic cohort had an increased contractile response to 5-HT, which is, at least partly, attributed to an increase in the Rho-dependent component of the contraction. This is consistent with the increased loss of vascular tone in hypoxic uterine arteries following inhibition of Rho kinase by Y27632. There was no difference in uterine artery vasodilatation in response to SNP and no change in relative phosphorylation of eNOS in hypoxic compared to normoxic placentomes. However, there was a tendency towards increased expression of eNOS at the transcript level, and the expression of iNOS was significantly increased in hypoxic placentomes.
Chronic hypoxia and maternal cardiovascular function

Placental expression of sFlt-1 and sEng was increased in the hypoxic cohort. This was associated with an increase in plasma concentrations of sFlt-1 and of the ratio of sFlt-1 compared to VEGF and to PIGF in hypoxic ewes at the end of gestation. Hypoxic placentomes also showed decreased expression and protein abundance of CSE. This occurred in conjunction with a fall in the placental production of H₂S in hypoxic placentomes, which positively correlated with a decrease in the placental expression of the H₂S-regulating miR-133b.

Normoxic ewes showed a decrease in mean arterial blood pressure towards the end of gestation, which was not observed in the hypoxic cohort. The maternal femoral artery contractile responses to PE and ET-1 were significantly increased in the hypoxic cohort. While there were no significant differences between the two cohorts in the femoral artery relaxation in response to ACh or SNP, femoral arteries isolated from the hypoxic ewes showed a significant reduction in the NO-dependent component of the ACh-mediated relaxation. Hearts isolated for Langendorff perfusion from the hypoxic ewes also showed signs of dysfunction, including a decrease in basal LVDP, an index of impaired systolic function and a an increase in LVEDP, a measure of impaired relaxability.

Plasma creatinine concentrations were higher in hypoxic relative to normoxic ewes at the end of gestation, which resulted in reduced estimated glomerular filtration rate in hypoxic ewes. However, there was no biochemical evidence of proteinuria or histological evidence of renal pathology in hypoxic compared to normoxic ewes.

5.4.2 Maternal blood and biometry

The only observed changes to maternal biometry were an increase in relative heart weight and a tendency towards decreased absolute kidney weights, which did not reach statistical significance. Right ventricular hypertrophy is often observed in
response to long-term exposure to hypoxia and in residents at high altitude due to mild pulmonary hypertension (Hultgren and Miller, 1967; Bärsch and Gibbs, 2007; Sakai et al., 2010). In contrast, cardiac adaptations in preeclampsia often include irreversible left ventricular hypertrophy in response to an increase in peripheral vascular resistance and cardiac afterload (Eghbali et al., 2005; Chung and Leinwand, 2014). In the present study, the relative increase in maternal heart weight is likely to be due to a combination of both factors, and is discussed in detail in section 5.4.5. Similarly, renal blood flow is reduced in both residents of high altitude as well as in women suffering from preeclampsia, which may underlie a possible decrease in kidney weights in the hypoxic cohort (Luks et al., 2008; Hurtado et al., 2012). In contrast, in healthy pregnancy, the physiological increase in glomerular filtration rate by 40 to 50% can cause kidney volume to increase by up to 30% (Rasmussen and Nielsen, 1988; Müller-Deile and Schiffer, 2014). The effects of gestational hypoxia in the last third of gestation on kidney function and morphology in this present study are discussed in detail in section 5.4.6.

Pregnancy is associated with a plethora of maternal physiological adaptations, including a rise in cardiac output and plasma volume and an increase in maternal ventilation, which is associated with changes to maternal blood gases and acid-base status (Lucius et al., 1970; Templeton and Kelman, 1976). These challenges to the maternal cardiovascular system are further exacerbated by exposure to high altitude, which leads to a reduction in \( P_aO_2 \), lowering arterial haemoglobin saturation and triggering compensatory hyperventilation and acute respiratory alkalosis (Zamudio, 2007). Metabolic compensation counteracts the respiratory alkalosis, resulting in a normal or slightly elevated blood pH at high altitude. In the present study, we found that 10% hypoxia for only 10 days in late gestation was sufficient to reduce maternal
arterial PO$_2$ and oxygen saturation without affecting maternal acid-base status. Despite decreased oxygen saturation at high altitude, an increase in haemoglobin and haematocrit allows the maintenance of, or even an increase in, oxygen content compared to pregnancy at sea level, which is consistent with the results obtained from ewes exposed to chronic hypoxia in the present study (Zamudio, 2007; Moore, 2010). While this could, in theory, maintain oxygen delivery to the placenta and the fetus, high levels of maternal haemoglobin and haematocrit are associated with pregnancy complications, predictive of FGR and the development of preeclampsia (Murphy et al., 1986; Lu et al., 1991; Zamudio et al., 1993b; Scanlon et al., 2000; Scholl, 2005; Gonzales et al., 2009). During healthy pregnancy, plasma volume expansion triggers a state of physiological haemodilution that plays an important role in maintaining the placental microcirculation, increasing uteroplacental blood flow and fetal growth (Kaibara et al., 1984; Bollini et al., 2005). Failure of blood volume expansion during high altitude and preeclamptic pregnancies antagonises the physiological decrease in blood viscosity, and a lack of reduction of haematocrit levels in the second half of gestation is associated with poor pregnancy outcome (Zamudio et al., 1993b; Kametas et al., 2004; Golboni et al., 2011; Khoigani et al., 2012). Thus, the increase in haematocrit associated with gestational hypoxia and with preeclampsia may contribute to compromised uteroplacental perfusion, exacerbating fetoplacental hypoxia and FGR (Heilmann, 1993; Kametas et al., 2004).

5.4.3 Maternal uteroplacental vascular function

Healthy pregnancy is associated with an increase in uteroplacental blood flow to sustain the demands of the growing fetus (Trudinger et al., 1985; Meschia, 2011). Fetal weight is closely associated with uteroplacental perfusion, highlighting the key
role of the placenta in maintaining appropriate materno-fetal exchange of oxygen and nutrients (Ferrell, 1989). In addition, the establishment of a high capacitance-low resistance uteroplacental vascular bed is crucial in maintaining low maternal peripheral vascular resistance, allowing the pregnancy-associated decrease in arterial blood pressure despite an increase in cardiac output (Osol and Mandala, 2009). The pathophysiology of abnormal uteroplacental perfusion and its impact on placental function, fetal development and maternal health has been studied for several decades (Burton and Jauniaux, 2018). Ultrasound imaging and colour Doppler ultrasonography have been indispensable in the study of the umbilicoplacental and the uteroplacental circulations, as well as in the screening and diagnosis of pregnancy disorders, such as preeclampsia and FGR (Fleischer et al., 1986; Jauniaux et al., 1991; Velauthar et al., 2014; Alfirevic et al., 2017). In the present study, we observed a significant increase in the uterine artery pulsatility index in hypoxic ewes compared to normoxic controls at the end of gestation. This is consistent with invasive measurements using a uterine artery flow probe in the chronically instrumented CamDAS™ cohort, which showed that chronic hypoxia prevents the pregnancy-associated fall in uterine artery vascular resistance with advancing gestation. Alterations in placental structure and function in response to chronically hypoxic pregnancy have been characterised in several animal models, showing evidence of increased uteroplacental vascular resistance and hypoxia (Mateev et al., 2003; Chang et al., 2009; Aljunaidy et al., 2016; Turan et al., 2017). For example, extensive studies by Zhang and colleagues have demonstrated in both sheep and rats that gestational hypoxia affects uterine artery vascular tone, possibly through epigenetic mechanisms or by modulating the expression and function of BKCa channels (Hu et al., 2012; Hu et al., 2017b; Ducsay et al., 2018; Hu et al., 2018). This maladaptive uterine haemodynamic profile opposes
the physiological increase in uterine blood flow, especially towards the end of term, promoting oxidative stress and a vicious cycle that becomes evident in preeclampsia-like symptoms (Mateev et al., 2003; Thaete et al., 2004; Tomlinson et al., 2010; Herrera et al., 2014; Matheson et al., 2016; Turan et al., 2017). Interestingly, a longitudinal study on several thousand women using uterine artery Doppler ultrasonography showed that a third of all women showed a de novo increase in uterine artery pulsatility index in late gestation, which was associated with a higher prevalence of preeclampsia (Binder et al., 2017). These findings support the idea that preeclampsia does not necessarily originate in the trophoblast, and that changes in uteroplacental perfusion can reflect maternal systemic vascular changes in the first instance (Burton and Nelson, 2011; Leslie and Thilaganathan, 2012; Thilaganathan and Kalafat, 2019; Thilaganathan, 2020). Thus, impaired placental perfusion may trigger secondary trophoblast dysfunction, and maternal cardiovascular dysfunction is likely hold a more critical role in the pathophysiology of preeclampsia than initially assumed (Ridder et al., 2019; Thilaganathan, 2020). In addition, uteroplacental vascular function is the common denominator that connects a number of pregnancy complications, including gestational hypoxia, preeclampsia and gestational diabetes.

It has been difficult to tease out single players in the pathogenesis of these disorders, because pregnancy itself strongly shifts the balance between vasoactive agents, such that it is difficult to disentangle which changes occur as part of normal physiological adaptations to pregnancy, and which changes are pathological. For example, during healthy pregnancy, uterine arteries are less responsive to the vasoconstrictor eicosanoid TXA$_2$ (Weiner et al., 1992). In contrast, there is evidence that trophoblast cells from preeclamptic pregnancies increase their TXA$_2$ production, evident in the accumulation of thromboxane metabolites in the placenta (Wang et al., 1992; Weiner
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et al., 1992; Zhao et al., 2008). Interestingly, at least part of the reason why smoking during pregnancy is associated with reduced risk of developing preeclampsia may be due to the inhibition of TXA2 by nicotine (Marcoux et al., 1989; Zhang et al., 1999). In addition, exposure of villous explants to hypoxia for longer than two days leads to a similar shift in prostanoid profile as in preeclampsia (Blumenstein et al., 2001; Vangrieken et al., 2018). This has led to the hypothesis that decreased production of vasodilator autacoids, such as PGI2 compared to TXA2 in the hypoxic placenta may contribute to vascular dysfunction in preeclampsia (Friedman, 1988; Zeeman and Dekker, 1992; Pai et al., 2016). While this may be the case in the systemic vasculature, as has been demonstrated in several animal models, our results show that uterine arteries from hypoxic pregnancies are unaltered in their dose-dependent response to the TXA2 analogue U46619 (Woods, 1989; Losonczy et al., 1995; Kriston et al., 1999; Perneby et al., 2011). This is consistent with observations from other laboratories demonstrating that, during pregnancy, inhibition of TXA2-associated signal transduction is only seen in the uteroplacental vascular bed, but not in the carotid or the mesenteric arteries (Weiner et al., 1992; Goulopoulou et al., 2012). This suggests that local factors in the placenta may be unique in being able to offset changes to TXA2-mediated signalling. Others have hypothesised that the modulation of TXA2 signalling in the placenta stems from a reduction in Rho kinase-mediated vascular signalling, which may be deregulated in placental endothelial cells in preeclampsia (Ark et al., 2005; Friel et al., 2008; Goulopoulou et al., 2012; Gu et al., 2017). Administration of the Rho kinase inhibitor fasudil in rodent models of preeclampsia improved vascular endothelial cell viability, reduced circulating levels of sFlt-1 and attenuated hypertension, proteinuria and FGR (Butruille et al., 2012; Gu et al., 2017). In the present study, the increase in uterine artery contractility in response to 5-HT is,
at least partly, mediated by an increase in the Rho-kinase-mediated component of this response. Using Y27632 to inhibit the Rho kinase demonstrated that Rho kinase contributes more to uterine artery vascular tone in vessels isolated from hypoxic pregnancies compared to normoxic pregnancies. Stimulation of Rho kinase activity under conditions of hypoxia has been previously observed in smooth muscle cells of the pulmonary vasculature (Wang et al., 2001; McMurtry et al., 2003). Interestingly, observations on the coronary and pulmonary artery contractile responses in lambs born to high altitude hypoxia show strikingly similar findings to the maternal uterine artery contractile responses isolated from chronically hypoxic ewes, presenting with increased contractility in response to potassium and 5-HT and an increase in the Rho-dependent component of 5-HT-mediated vasoconstriction, but no changes in the dose-dependent response to U46619 (Maruko et al., 2009; Blood et al., 2013). To our knowledge, this is the first attempt at characterising a Rho-dependent constrictor phenotype of the maternal uterine vascular reactivity in chronically hypoxic pregnancy. It is not clear how exactly hypoxia mediates an increase in Rho kinase activity, but it is possible that reduced NO bioavailability under conditions of hypoxia and oxidative stress relieves its inhibition on Rho kinase signalling (Sauzeau et al., 2000; Chitaley and Webb, 2002). This may also explain the reduction in the contribution of Rho kinase signalling pathways during normal pregnancy, as NO becomes increasingly important as a vasodilator in the uteroplacental circulation with advancing gestation. The deregulation of NO signalling in the uteroplacental vascular bed has been identified in humans and in animal models of compromised pregnancies (Mateev et al., 2003; Kusinski et al., 2012; Kulandavelu et al., 2013; Matsubara et al., 2015; Aljunaidy et al., 2016; Lorca et al., 2019). Interestingly, at least part of the AMPK-mediated vasodilator effects in the uteroplacental vascular bed are mediated by opposing PE-induced
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vasoconstriction and increasing endothelial NO production (Skeffington et al., 2016; Kumagai et al., 2018; Lorca et al., 2020). The decrease in placental AMPK activation in the present study may, at least partly, underlie the increase in uteroplacental vascular resistance observed in the present study, and its manipulation may have potential as a future intervention in pregnancies presenting with reduced uteroplacental perfusion (Kumagai et al., 2018; Lane et al., 2019b; Lorca et al., 2020).

We found no changes in the uterine artery response to the NO donor SNP, and we also observed no changes in protein abundance or activation of eNOS in the placenta. However, eNOS transcript levels showed a tendency towards an increase, and placental iNOS expression was significantly upregulated in hypoxic ewes. This may be a compensatory attempt to sustain placental development and fetal growth, which fails at the level of protein translation. For example, several models of FGR are associated with increased expression of eNOS and increased NO production, which is even more pronounced in pregnancies presenting with chronic fetal hypoxia (Galan et al., 2001; Hagen et al., 2005; Ziebell et al., 2007; Tikvica et al., 2008; Blomberg et al., 2010; Krause et al., 2011). In addition, while the level of NO production by eNOS during healthy pregnancy is crucial for uteroplacental vascular homeostasis, high levels of NO synthesis by iNOS may be detrimental to vascular regulation due to its interaction with ROS to form peroxynitrite (Kissner et al., 1997; Matsubara et al., 2015).

Upregulation of iNOS can occur in response to pro-inflammatory cytokine signalling and has been reported in both experimental models and clinical hypertension (Hong et al., 2000; Smith et al., 2011; Amaral et al., 2012; Oliveira-Paula et al., 2014). The same has been observed in the systemic vasculature of a rat model of experimental preeclampsia based on reduction of uteroplacental perfusion pressure (RUPP; Amaral et al., 2013). That study showed that treatment of these “preeclamptic” rats with an
iNOS inhibitor decreased vascular oxidative and nitrosative stress and attenuated hypertension. However, another investigation into the effects of iNOS inhibition in normal pregnant rats showed that this disrupted renal haemodynamics, resulting in hypertension (Alexander et al., 2002). Thus, it is likely that iNOS has diverging roles in the systemic, renal and uteroplacental vasculature, which remain to be clarified in healthy and complicated pregnancy.

5.4.4 Maternal angiogenic balance

Maternal vascular function relies on a complex homeostasis between several vasoactive agents, including cytokines and angiogenic, constrictor, dilator and growth promoting agents (Gilbert et al., 2008; Tissot van Patot et al., 2012; Shah and Khalil, 2015). Any disruption of this intricate balance, especially in the presence of pregnancy as an underlying stressor, may cause widespread endothelial dysfunction and inflammation. Hypoxia during pregnancy leads to vascular alterations both locally in the utero-placental unit and systemically in the form of general vascular dysfunction (Gilbert et al., 2008; Tissot van Patot et al., 2012; Zhou et al., 2013; Shah and Khalil, 2015; Aljunaidy et al., 2016). Increased placental production and secretion of sFlt-1 is thought to be a major player in the development of preeclampsia, contributing to hypertension and proteinuria through the sequestration and antagonism of the angiogenic factors VEGF and PlGF (Maynard et al., 2003; Gu et al., 2008). Our measurements of ovine plasma concentrations of sFlt-1 in the last third of gestation in the present study are similar to human values in healthy control pregnancies (Maynard et al., 2003; Noori et al., 2010; Saxena et al., 2013). In addition, we observed an increase in sFlt-1 concentrations in hypoxic ewes, which mirrors the plasma concentration in women suffering from term preeclampsia and gestational
hypertension (Maynard et al., 2003; Noori et al., 2010). These levels are lower than sFlt-1 concentrations in women, who develop severe preterm preeclampsia (Noori et al., 2010). VEGF plasma concentrations in healthy pregnancy increase drastically in the first trimester and decline thereafter, but remain elevated compared to non-pregnant states (Lygnos et al., 2006; Ren et al., 2014; Tandon et al., 2017). We measured similar plasma concentrations of VEGF in pregnant ewes during the last third of gestation, with a significant decrease throughout this period in hypoxic ewes. Both reductions and elevations in VEGF concentrations at term have been reported in cases of gestational hypertension, preeclampsia and FGR (Hunter et al., 2000; Polliotti et al., 2003; Levine et al., 2004; Lygnos et al., 2006; Ren et al., 2014; Tang et al., 2019). These disparities may have a number of causes, such as the heterogeneity within cases of compromised pregnancy, as well as the use of different antibodies during ELISA tests that measure free vs. bound versions of the antigen. Thus, rather than the measurement of single markers, determining the ratio of sFlt-1 to VEGF or to PIGF longitudinally provides a more accurate measurement, and these calculations have shown better performance in the prediction of preeclampsia (Verlohren et al., 2010; Rana et al., 2012; Chaiworapongs et al., 2013; Chappell et al., 2013; Zeisler et al., 2016; Caillon et al., 2018; Nikuei et al., 2020). Therefore, the increased ratio of sFlt-1 to PIGF and of sFlt-1 to VEGF in hypoxic ewes in the present study may, at least partly, underlie alterations in both uteroplacental and systemic vascular reactivity.

We also demonstrated that hypoxia in the last third of gestation leads to increased placental expression of both sFlt-1 and sEng, which is likely to be the source of increased circulating maternal sFlt-1 levels in hypoxic ewes (Palmer et al., 2016). Hypoxia has previously been shown to be the main driver of this shift towards anti-angiogenic signalling, both in vitro and in vivo (Gerber et al., 1997; Gilbert et al., 2007;
Makris et al., 2007; Appel et al., 2015). More recently, the close interrelationship between gestational hypoxia and altered H2S biology in the placenta has been implicated in the deregulation of angiogenic balance. Evidence suggests that hypoxia suppresses placental levels of CSE via miR-21-mediated mechanisms, which is associated with FGR, impaired uteroplacental perfusion, placental hypoxia and maternal vascular dysfunction (Yang et al., 2012; Cindrova-Davies et al., 2013; Lu et al., 2017). Both women with hypertension and pre-eclampsia present with lower circulating levels of H2S, indicating that H2S has important anti-hypertensive properties (Wang et al., 2013; van Goor et al., 2016). In addition, several studies have reported decreased expression of both CSE and CBS in human preeclamptic placentae (Cindrova-Davies et al., 2013; Holwerda et al., 2014; Hu et al., 2016). Decreased levels of placental CSE expression and activity in hypoxic pregnancy may have widespread adverse effects, such as direct consequences on systemic and utero-placental vascular tone through loss of function in the endothelium or smooth muscle cells, indirect impairment of vascular function by altering the balance of angiogenic factors, or by exacerbating oxidative stress due to loss of its antioxidant properties (Osmond and Kanagy, 2014; Dongó et al., 2018). While the importance of H2S for healthy pregnancy has been known for more than a decade, the potential link between placental H2S biology and sFlt-1 signalling has only recently come under focus. Various studies in vitro and in vivo have now confirmed that inhibition of CSE using PAG or siRNAs induces imbalance of angiogenic factors and symptoms of preeclampsia, which can be reversed by treatment with H2S donors or L-cysteine (Wang et al., 2013; Hu et al., 2017a). The stability of sFlt-1 mRNA is inversely related to the expression of miR-133b, which is downregulated in preeclamptic placentae and in trophoblast cells treated with siRNAs against CBS and CSE (Hu et al., 2015; Hu et
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Our data from hypoxic placentomes are strikingly similar to these observations. Notably, we have shown for the first time that the ovine placenta expresses the \( H_2S \) synthesising enzymes CSE and CBS, and that CSE is localised mostly to the smooth muscle and endothelial cell layers of the placental vasculature, while CBS is more widely present in placental villi, trophoblast and the maternal uterine stroma and epithelium. While there were no changes to CBS at the transcriptional or the translational levels, we measured a decrease in placental CSE abundance by more than half. The important reduction in placental levels of CSE may account for the decrease in placental \( H_2S \) production at term. Our findings support the theory that \( H_2S \) enhances the expression of miR-133b, which targets sFlt-1 mRNA, likely through imperfect base-pairing (Wu and Brewer, 2012; Hu et al., 2017a). The molecular basis of \( H_2S \)-mediated modulation of miRNAs remains unknown, but may rely on S-sulfhydration of cysteine residues of RNA binding proteins, such as thymidylate synthase and RNA methyltransferase (King and Redman, 2002; Lin et al., 2003; Xie et al., 2014b; Bibli et al., 2019). In our study, we have demonstrated that the ovine placenta is susceptible to hypoxia-mediated dysregulation of \( H_2S \) biology via a reduction in CSE abundance, which may contribute to the elevated sFlt-1 levels in hypoxic ewes, consistent with studies performed on preeclamptic patients and in animal models of preeclampsia.

Alongside measurement of sFlt-1, the TGF-\( \beta \) antagonist sEng is now a commonly assessed anti-angiogenic marker, as it shows similar patterns of upregulation in women with preeclampsia. In healthy pregnancy, serum concentrations of sEng are stable throughout gestation, with a slight increase in the last trimester (Levine et al., 2006). In preeclampsia, placental sEng expression and release is increased, such that circulating concentrations of sEng are elevated both before and after the onset of
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clinical symptoms (Jeyabalan et al., 2008; Sachan et al., 2016). However, a recent clinical study observed lower serum levels of sEng in preeclamptic patients compared to healthy control pregnancies in the third trimester (Rădulescu et al., 2016). In the present study, our measurements of sEng concentration in plasma are slightly lower than in women at term, and we found no differences in sEng levels with advancing gestation, or between normoxic and hypoxic ewes. In contrast, we did observe an increase in placental gene expression of sEng, which is consistent with previous findings showing that 3% and 8% hypoxia induces the release of sEng in villous explants (Yinon et al., 2008; Barsoum et al., 2011). However, other reports have found that sEng expression is not modulated by severe hypoxia of 1% in the villous trophoblast (Munaut et al., 2008). It is possible that in vitro measurements of trophoblast behaviour under conditions of different degrees of hypoxia are different compared to in vivo experiments, and that sEng release into the culture medium of villous explants may not translate to increased secretion of sEng by the placenta into the maternal circulation, despite increased placental gene expression.

5.4.5 Maternal systemic vascular function

In non-pregnant animals and humans, exposure to hypoxia initially triggers a chemoreflex, which increases sympathetic output, leading to a transient increase in blood pressure after the onset of hypoxia (Sugimura et al., 2008; Cowburn et al., 2017). Exposure to hypoxia also triggers the release of local agents in the vasculature that promote vasodilatation, maintaining perfusion and oxygen delivery to tissues (Marshall, 1999; Marshall, 2015; Dinenno, 2016). Hence, in the absence of pregnancy, the effect on arterial blood pressure of chronic hypoxia depends on the interaction between these opposing influences on total peripheral vascular resistance. Interestingly, after
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Lowland residents ascend to high altitude, arterial blood pressure returns to baseline after a few days under the influence of hypobaric hypoxia, which is attributable to a sustained blunting of the systemic response to sympathetic stimulation and increased sensitivity to NO-mediated vasodilatation, termed “hypoxic sympatholysis” (Doyle and Walker, 1991; Marshall, 2001; Bartlett and Marshall, 2003). However, it has also been reported that long-term residence at high altitude can increase the risk of hypertension due to a compensatory increase in sympathetic nervous output and increased noradrenaline spillover (Calbet, 2003; Hansen and Sander, 2003; Calbet et al., 2014). Previous studies using the same model of gestational hypoxia in sheep have shown that maternal concentrations of catecholamines during pregnancy remain unchanged during the entire period of chronic hypoxia (Brain et al., 2015). In addition, past studies from other laboratories have reported that exposure of non-pregnant animals to chronic hypoxia can have no effect or even reduce their basal arterial blood pressure (Vilar et al., 2008; Zhou et al., 2013). Combined, therefore, past and present evidence support that diverging influences of chronic hypoxia on arterial blood pressure between pregnant and non-pregnant animals stem from an effect of hypoxia at the uteroplacental level. In non-pregnant animals, the weight of the evidence suggests that chronic hypoxia has no long-term effect on arterial blood pressure, or it may even reduce it. Conversely, in pregnancy, chronic hypoxia favours a maternal hypertensive phenotype, associated with the effects of increased uteroplacental vascular resistance acting upstream on the maternal cardiac afterload.

In the present study, we demonstrated that hypoxic pregnancy is sufficient to induce an increase in uteroplacental vascular resistance, which is associated with altered placental expression of angiogenic factors and a shift in maternal angiogenic balance. The systemic vascular endothelium is essential in regulating arterial compliance,
vascular resistance and blood pressure, and changes in circulating angiogenic factors during preeclampsia may predispose the maternal vasculature to constriction, leukocyte adherence, oxidative stress and inflammation (Lamarca, 2012). It has been suggested that inhibition of VEGF signalling by sFlt-1 reduces NO bioavailability, which is, at least partly, responsible for the development of maternal hypertension by allowing the vasoactive effects of ET-1 to predominate (Kappers et al., 2009). Inhibition of VEGF signalling in both humans and rats using the receptor tyrosine kinase inhibitor sunitinib led to an almost threefold increase in circulating ET-1 levels, inducing a rise in arterial blood pressure, renal dysfunction and proteinuria (Kappers et al., 2010). Preeclamptic women show increased expression of pre-pro-endothelin and circulating ET-1, and several animal models of preeclampsia have shown that dysregulation of ET-1 signalling may link endothelial dysfunction with hypertension in pregnancy (Nova et al., 1991; LaMarca et al., 2005; Carbillon, 2006; Roberts et al., 2006; Roberts and Von Versen-Hoeynck, 2007). As ET-1 is produced locally in the vasculature with autocrine and paracrine functions, its contribution towards systemic endothelial dysfunction is not to be discounted, even if its levels are not necessarily increased in mild cases of preeclampsia (Taylor et al., 1990). Chronic immune activation of the vascular endothelium as a result of placental ischaemia may also contribute to increased vasoreactivity to ET-1 (Granger et al., 2001). For example, in RUPP models, CD4+ T helper lymphocytes are key players in the development of endothelial dysfunction, which is associated with increased plasma sFlt-1 and TNFα concentrations, hypertension and reduced glomerular filtration rate (Wallace et al., 2011; Novotny et al., 2012). TNFα- and sFlt-1-mediated hypertension can be attenuated by inhibiting ET-1 signalling using an endothelin receptor A blocker (Alexander et al., 2001; LaMarca et al., 2005; LaMarca et al., 2008a). These
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observations indicate that ET-1 is important in mediating the maternal vascular dysfunction in response to angiogenic imbalance and vascular inflammation. In the present study, we measured an increase in placental expression of TNFα and significant shifts in maternal angiogenic balance in response to hypoxic exposure. Further, hypoxic ewes did not show the fall in maternal arterial blood pressure observed in normoxic ewes with advancing gestation, and had greater constrictor reactivity in their femoral arteries to both PE and ET-1, which is likely the result of the decrease in the NO-dependent component of vasorelaxation in response to ACh.

In addition to adverse changes to the reactivity of the maternal peripheral circulation, in the present study we found that chronic hypoxia in late gestation induced indices of cardiac dysfunction. Pregnancy itself is already considered a burden on the maternal circulation by inducing an increase in maternal blood volume and cardiac output (Valdés and Corthorn, 2011). In addition, by the third trimester, the gravid uterus pushes the diaphragm upwards, which leads to a displacement of the heart and the cardiac vessels (Wang et al., 2014). Thus, even normal uncomplicated pregnancies are, to some extent, associated with reversible left ventricular hypertrophy and diastolic dysfunction, as determined by echocardiography (Savu et al., 2012; Melchiorre et al., 2016). These strains on maternal cardiac function are further aggravated by complications during pregnancy. For instance, preeclampsia is associated with more pronounced left ventricular hypertrophy and dysfunction by midgestation, which persists even post-partum (Melchiorre et al., 2011; Melchiorre et al., 2013; Castleman et al., 2016; De Haas et al., 2017; Ambia et al., 2018). These changes are most prominent in early-onset and severe cases of preeclampsia, which subsequently require preterm delivery, and correlate with the degree of adverse maternal and fetal outcomes (Melchiorre et al., 2012; Verlohren et al., 2017; Borges
et al., 2018; Ferrazzi et al., 2018; Vaught et al., 2018; Thilaganathan and Kalafat, 2019). Similarly, in a rat model of preeclampsia, an increase in left ventricular weight and hypertrophy of myocardial cells was observed by 16 weeks post-partum, which was associated with myocardial and coronary artery fibrosis and reduced tolerance to periods of ischaemia (Wang et al., 2014). In contrast to fetal cardiovascular function, little is known about the effects of hypoxia on cardiac function in the mother. There is evidence that even mild hypoxia can lead to changes in cardiomyocyte morphology via HIF1α signalling, and that this pathway is also involved in the physiological cardiac adaptation to pregnancy (Dorn, 2007; Chu et al., 2012; Chung et al., 2012; Soñanez-Organis et al., 2016; Kumar et al., 2018). In our study, we show that chronic hypoxia in late gestation resulted in an increase in the relative heart weight in chronically hypoxic ewes, as well as indices of impaired left ventricular relaxation and contraction, akin to dysfunction measured in women suffering from preeclampsia (Valensise et al., 2008; Melchiorre et al., 2013).

5.4.6 Maternal kidney function

Alongside the haemodynamic changes that occur in the mother as part of the physiological adaptation to pregnancy, renal function is also significantly altered during pregnancy. Hypervolaemia-induced haemodilution and increased renal perfusion lead to glomerular hyperfiltration in healthy pregnant women compared to non-pregnant levels (De Alvarez, 1958; Chapman et al., 1998; Hladunewich et al., 2007). The serum marker most commonly used in clinical practice to assess renal function is creatinine (Traynor et al., 2006). Creatinine concentration in the maternal serum of healthy pregnancies decreases to between 35 and 70 μmol.L⁻¹ from a normal non-pregnant reference range of 45 to 90 μmol.L⁻¹ (Fischer, 2007; Maynard and Thadhani, 2009).
Preeclamptic pregnancy is associated with plasma creatinine levels close to non-pregnant levels due to reduced renal blood flow and glomerular filtration rate (Manaj et al., 2011). The renal pathology of preeclampsia is associated with accumulation of extracellular fluid and oedema, promoting a vicious cycle between renal and systemic endothelial dysfunction (Roberts et al., 2003; Roberts and Gammill, 2005; Thadhani et al., 2005). Pregnancy toxaemia in sheep is regarded as the ovine equivalent of preeclampsia, presenting with haemodynamic and renal abnormalities similar to those seen in preeclamptic women (Parry and Taylor, 1955, 1956; Ferris et al., 1969). Our measurements of plasma creatinine levels at the end of gestation in normoxic and hypoxic ewes fall within normal reference ranges for human pregnancies. However, hypoxic ewes showed significantly higher plasma creatinine concentrations compared to normoxic controls, at the high end of the reference range. Under normal circumstances, creatinine is freely filtered and excreted solely by the kidneys, and is neither reabsorbed nor secreted by the kidneys (Baum et al., 1975). With caution, plasma creatinine concentrations can be used as an index of renal glomerular filtration rate by estimating creatinine clearance. While this lacks the sensitivity to detect subtle changes, such that glomerular filtration rate must decrease by more than half before creatinine rises above its reference range, it does provide a first measure of renal function when timed urine collection is not feasible (Baum et al., 1975; Maynard and Thadhani, 2009). In our study, controlling for maternal body weight using the Cockcroft-Gault equation, this calculation did reflect that hypoxic ewes had lower estimated glomerular filtration rate compared to their normoxic equivalents at the end of term. Similarly, during long-term residence at high altitude, renal blood flow and glomerular filtration rate are decreased, at least partly, due to secondary polycythaemia (Lozano and Monge, 1965; Ou et al., 1998). There is some evidence
that exposure to high altitude hypoxia can increase progression of chronic kidney disease to end-stage renal disease (Luks et al., 2008). There is also evidence that exposure of rats to severe hypobaric hypoxia equivalent to over 7,000 metres above sea level can directly induce renal damage with histological and molecular markers of renal injury and increased plasma creatinine and hypertension (Chhabra et al., 2018).

In order to assess kidney damage in clinical practice, the measurement of the urine protein to creatinine ratio in spot urine tests has become the preferred method for quantification of proteinuria in suspected preeclamptic women compared to timed 24 hour urine collection (Baum et al., 1975). In pregnancy, a urine protein to creatinine ratio of more than 30 mg.mmol\(^{-1}\) indicates significant proteinuria. However, as this measure is subject to circadian rhythms and day-to-day variations, it is more useful as a “rule-in” test for detecting severe proteinuria, but is less useful in ruling out cases of mild preeclampsia (Aggarwal et al., 2008; Côté et al., 2008; Naresh et al., 2013; Müller-Deile and Schiffer, 2014). Thus, while our results indicate that ewes undergoing chronic hypoxic pregnancy did not suffer from significant proteinuria, these data do not rule out more subtle forms of renal dysfunction. In addition, recently, the American College of Obstetricians and Gynaecologists removed proteinuria as a diagnostic requirement for preeclampsia, because it correlates poorly with maternal and fetal outcomes, and absence of proteinuria in acutely ill preeclamptic women can delay life-saving intervention (American College of Obstetricians and Gynecologists, 2013). From more recent studies, it is possible that up to 10% of women with preeclampsia show no signs of proteinuria (Thornton et al., 2010).

Histologically, the renal alterations in response to pregnancy toxaemia in sheep are very similar to renal endotheliosis in human preeclampsia, including swelling of
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glomerular tufts, thickening of the glomerular basement membrane with evidence of protein deposits and fusion of epithelial cell foot processes (Ferris et al., 1969). In the present study, we observed no changes in renal glomerular and proximal tubule morphology, and kidneys collected from hypoxic ewes showed no evidence of renal fibrosis. While we did observe a tendency towards a decrease in kidney weight, which may reflect a reduction in renal perfusion in hypoxic ewes, these data indicate that any insult to renal endothelial function is not severe is enough or has not progressed for long enough to induce histological alterations in renal morphology.

5.4.7 Conclusion

The objective of this chapter was to determine the effects of hypoxic pregnancy in sheep on the maternal cardiovascular and renal physiology. The data show that chronic hypoxia in the last third of gestation in sheep results in increased uteroplacental vascular resistance, associated with an increase in Rho kinase-dependent basal tone and increased reactivity to constrictor agonists in the uterine arteries in hypoxic ewes. We also report a shift in placental H₂S biology and angiogenic balance in the hypoxic placenta, which leads to elevated levels of sFlt-1 compared to VEGF and PIGF in the maternal circulation. These adverse effects are associated with a vasoconstrictor phenotype in the peripheral circulation, relative systemic hypertension and cardiac dysfunction in ewes undergoing hypoxic pregnancy. Despite elevated creatinine levels, there was no evidence of proteinuria or glomerular endotheliosis in hypoxic ewes. Combined, these data confirm that chronic hypoxia in late gestation in sheep can induce uteroplacental vascular dysfunction, and adverse consequences on the maternal heart and circulation, similar to those described in preeclampsia.
6. General discussion

The three experimental chapters in this thesis have shown that gestational hypoxia in the last third of pregnancy in sheep can recapitulate adverse placental, fetal and maternal symptoms that occur in women affected by preeclamptic pregnancy. Placental hypoxia promotes a state of placental oxidative stress, measurable in an increase in protein carbonylation (Yung et al., 2007). Post-translational protein carbonylation is also used as a biomarker of oxidative protein damage in preeclamptic placentae, and the level of protein carbonylation correlates well with the severity of the preeclamptic syndrome (Zusterzeel et al., 2001). Accumulation of damaged proteins triggers the activation of a powerful UPR to restore protein homeostasis (Schröder and Kaufman, 2005). In the present study, the UPR was activated in the ER, cytosol and mitochondria, along with activation of MAPKs, which mediate several molecular cascades in response to cellular stress (Tang et al., 2014). These molecular changes were accompanied by morphological changes to cellular organelles, including ER expansion and changes to mitochondrial cristae shape and size. Cellular proteotoxic stress is involved in the pathogenesis of several complications in pregnancy, and many of these molecular cellular markers have been reported in placentae from women suffering from preeclampsia (Burton et al., 2009; Yung et al., 2012; Yung et al., 2014; Charnock-Jones, 2016; Yung et al., 2019). Placental mitochondria from hypoxic pregnancies also showed compensatory changes in respiratory activity to limit mitochondrial generation of ROS while maintaining total respiratory capacity. Recent evidence suggests that activation of the UPRmt may underlie the changes in cellular respiration patterns observed in preeclamptic placentae (Vaka et al., 2018; Yung et al., 2019). Hypoxic placentae also showed altered energy metabolism to decrease fatty acid metabolism and increase glucose uptake, which is likely in favour of
glycogen deposition to maintain a longer term fetal resource allocation of glucose (Akison et al., 2017). There were also marked changes to the placental gene expression profile in hypoxic ewes, including the increased expression of the pro-inflammatory cytokine TNFα, the adipocytokine adiponectin and the anti-angiogenic factors sEng and sFlt-1.

These alterations in placental signalling and mitochondrial function in the present study occurred together with adverse effects in the maternal physiology in ewes undergoing hypoxic pregnancy. In contrast to control ewes, sheep undergoing hypoxic pregnancy failed to reduce their uterine vascular resistance and maternal arterial blood pressure with advancing gestation. These effects on the maternal physiology of chronic hypoxia are similar to those induced by high altitude hypoxia in animals and humans. Pregnancy at high altitude reduces uterine blood flow and impairs the fall in maternal arterial blood pressure with advancing gestation in women and in sheep (Zamudio et al., 1985; Palmer et al., 1999; Giussani et al., 2007; Hu et al., 2017b; Ducsay et al., 2018). These findings may, at least partly contribute to the increased risk of FGR and preeclampsia at high altitude (Keyes et al., 2003; Julian et al., 2008).

Data in the present study show that chronic hypoxia during the last third of gestation in sheep led to an angiogenic imbalance in the maternal plasma driven by an increase in sFlt-1 concentration. Thus, increased expression of antiangiogenic factors in the placenta may contribute to angiogenic imbalance and endothelial dysfunction in the maternal circulation, impairing both the fall in uterine vascular resistance and arterial blood pressure with advancing gestation, while also increasing cardiac afterload, driving left ventricular dysfunction. Maternal endothelial dysfunction may also contribute to alterations in glomerular filtration, increasing the concentration of maternal plasma creatinine levels and reducing estimated glomerular filtration in
hypoxic ewes, as measured in preeclamptic women (Lafayette, 2005).

Downstream, the asymmetric FGR in chronically hypoxic pregnancies was represented by more severe growth restriction of the fetal trunk compared to the fetal brain, yielding an increase in relative fetal brain weight. This is consistent with our previous studies, which revealed that chronic fetal hypoxia promotes a sustained redistribution of blood flow away from non-essential peripheral circulations towards the brain, the so-called “fetal brain sparing effect” (Allison et al., 2016; Giussani, 2016). Asymmetric FGR is commonly present in both human clinical studies and animal models of gestational hypoxia (Dashe et al., 2000; Swanson and David, 2015).

Combined, therefore, the data show that chronic hypoxia during pregnancy in sheep provides a link between placental stress, FGR with evidence of fetal brain sparing and maternal cardiovascular dysfunction, as in human pregnancy complicated by preeclampsia. These findings are of both scientific and clinical significance, as there is still no treatment for preeclampsia, except for delivery of the fetus and placenta (Uzan et al., 2011). An effective intervention against preeclampsia would thus not only have a significant impact on fetal health, but also on maternal healthcare provision. For instance, in a recent epidemiological analysis in the United States, preeclampsia increased the risk of adverse events in mothers from 4.6% to 10.1% and in infants from 7.8% to 15.4%, which was associated with a total cost burden of $2.18 billion for the first 12 months after birth (Stevens et al., 2017). Not included in these costs is the additional burden of increased long-term risks of cardiovascular disease for both mother and child. However, progress in this field is limited the disappointing outcome of several clinical trials and by the lack of appropriate animal models, in which candidate therapy can be tested (Szalai et al., 2015; Aouache et al., 2018).
Strikingly, the animal model presented in the current thesis combines aspects of both late-onset gestational insults and symptoms of early-onset gestational compromise. This supports the concept that both early- and late-onset preeclampsia are not distinct diseases, but rather two extreme presentations within a spectrum of preeclamptic disorders that share malperfusion and diffuse placental hypoxia as a common denominator (Redman, 2017). Distinguishing strictly between placental and maternal cardiovascular aetiologies of preeclampsia risks drawing unjustified attention to a single measurable outcome, such as fetal growth, uterine blood flow or maternal blood pressure. Furthermore, high yield experimental models, such as those involving cell culture and rodent models often strive to identify a precise pathway or a precise target for intervention. However, maternal, placental and fetal compromise are unlikely to occur in isolation and cannot be measured by extrapolating the consequences of a singular insult at one specific timepoint within a distinct pathway. The clinical picture will vary along the continuum of the preeclamptic syndrome, developing on the background of a combination of maternal co-morbidities, placental immunocompatibility and fetal genetic predispositions (Roberts and Escudero, 2012; Bartsch et al., 2016; Lokki et al., 2018; Galaviz-Hernandez et al., 2019; Al-Rubaie et al., 2020). In addition, environmental factors, such as maternal diet, socioeconomic status and pollution will affect disease presentation, severity and progression (K et al., 2014; Rosen et al., 2018; Dasinger et al., 2020). It is thus increasingly important to take into consideration the wider picture, viewing preeclampsia in the context of the maternal-placental-fetal unit at the level of the whole organism. This current thesis presents an ovine model of gestational hypoxia, which recapitulates functional, cellular and molecular signatures of preeclampsia in the fetus, placenta and mother, mimicking the syndrome of preeclampsia. Therefore, we have not only shown that
gestational hypoxia is a risk factor and important mechanism promoting preeclampsia, but that this model provides a useful tool to investigate the pathophysiology of preeclampsia from a holistic angle. This presents an opportunity to identify and test candidate therapy by moving towards a more comprehensive approach.
6.1 Maternal antioxidant therapy for preeclampsia

Many investigations have confirmed that the placental ischaemia that underlies placental dysfunction in preeclampsia is associated with elevated oxidative stress in both the placental and the systemic circulation (Walsh, 1998; Tsukimori et al., 2005; Tsukimori et al., 2007). It is thought that placental oxidative stress and antioxidant imbalance in the maternal circulation mediate key aspects of endothelial dysfunction (Noris et al., 2004; McCord et al., 2006; Ramirez et al., 2006; Murphy et al., 2012). Given that several antioxidants are reduced in women suffering from preeclampsia, including vitamins A, C and E, glutathione and β-carotene, clinical studies have progressed into testing the effects of maternal treatment with antioxidants to ameliorate the symptoms of preeclampsia (Raijmakers et al., 2004; Lamarca, 2012).

On the one hand, the use of antioxidants, such as melatonin and vitamins C and E, has been shown to improve placental function in women at risk of pre-eclampsia and also rescued FGR and associated pathologies in the short- and long-term in animal models of adverse pregnancy (Chappell et al., 2002; Richter et al., 2012; Miller et al., 2014; Brain et al., 2019). These have the potential to reduce placental oxidative and ER stress, improve placental PPARγ signalling and protein synthesis (Chappell et al., 2002; Kasimanickam and Kasimanickam, 2011; Richter et al., 2012; Miller et al., 2014). On the other hand, supplementation with vitamins C and E has not been effective against preeclampsia in randomised double-blind multicentre clinical trials, and antioxidant treatment may paradoxically have adverse effects by interfering with the normal physiological functions of ROS (Podmore et al., 1998; Poston et al., 2006; Halliwell and Gutteridge, 2007; Roberts et al., 2010; Cindrova-Davies, 2014; Brain et al., 2019). ROS are not merely cellular stressors, but are also involved in numerous intracellular signalling cascades, acting as second messengers with important roles in
the regulation of cell survival, growth and proliferation (Thannickal and Fanburg, 2000). In addition, antioxidant supplementation may generate an antioxidant excess with “pro-oxidant” effects (Podmore et al., 1998; Rehman et al., 1998). By relieving oxidative stress, NO may be released from its interaction with the superoxide anion, increasing its bioavailability. Since NO is also a substrate for peroxynitrite, excess NO may itself promote oxidative stress, increasing oxygen-radical-mediated DNA damage (Podmore et al., 1998; Rehman et al., 1998; Halliwell and Gutteridge, 2007; Radi, 2013; Brain et al., 2019).

The inability of maternal supplementation with vitamin C to ameliorate preeclampsia in human clinical trials has been a topic of continuous debate, ever since the negative findings were first published (Poston et al., 2006). Although there have been several clinical trials in different countries, all have used the same dose and dosing regimen (Rumbold et al., 2008). Some have argued that this intervention occurs too late in pregnancy and/or that this dose of vitamin C is insufficient in an attempt to explain the lack of benefit conferred by vitamin C supplementation against preeclampsia (Brain et al., 2019). Therefore, with the incentive of clarifying some of the complex effects of maternal antioxidant intervention on healthy and adverse pregnancy, we have performed preliminary investigations using maternal antioxidant treatment with vitamin C in the same ovine model of gestational hypoxia introduced in this thesis.

**6.1.1 Treatment with vitamin C**

Ascorbic acid, or vitamin C, is well known for its antioxidant properties, and an essential component of the mammalian diet (Bánhegyi et al., 1997). Its functional importance for vascular health and pregnancy is well documented, which has led to investigations into the suitability of vitamin C as a free radical scavenger to treat
compromised pregnancy (Kolb et al., 1991; Baydas et al., 2002). In both human and ovine pregnancy, for example, vitamin C administration is associated with the promotion of a dilator phenotype in the fetal circulation, an increase in umbilical blood flow, and protection from FGR (Thakor et al., 2010a; Thakor et al., 2010b; Parraguez et al., 2011; Brain et al., 2019; Sales et al., 2019). In addition, vitamin C deficiency increases the myogenic tone in pregnant rats, but not in non-pregnant animals (Ramirez et al., 2006). It is likely that these effects are rooted in reducing hypoxia-associated oxidative stress, increasing NO bioavailability and nitric-oxide-dependent vasodilatation, and thus maintaining nutrient and oxygen delivery to the fetus (Parraguez et al., 2011; Richter et al., 2012). Given these observations, several studies have been carried out to assess the potential of vitamin C to rescue gestational syndromes associated with placental ischaemic injury and oxidative stress, including preeclampsia. Vitamin C is able to prevent hypoxia-reoxygenation induced sFlt-1 secretion in placental explants in vitro, and the use of vitamins during pregnancy is associated with a decreased risk of preterm birth and FGR (Catov et al., 2007; Cindrova-Davies et al., 2007). However, protective effects of vitamin C are yet to be observed in clinical practice. This may be, because vitamin C must be administered at high doses, designed to achieve a concentration that exceeds that of NO by a factor of 100,000 in order to acquire effective antioxidant properties in vivo (Jackson et al., 1998; Thakor et al., 2010b).

In pilot studies from our laboratory, we trialled intervention with vitamin C in the same sheep model as used in the present thesis. A dose of 200 mg.kg\(^{-1}\).day\(^{-1}\) vitamin C was chosen based on previous experiments in our group, to reach the required concentration for vitamin C to compete effectively with NO in scavenging superoxide in vivo (Jackson et al., 1998; Thakor et al., 2010b). Vitamin C treatment was able to
rescue hypoxia-associated FGR and increased umbilical blood flow via NO-dependent vasodilatation (Thakor et al., 2010a; Brain et al., 2019). In addition, vitamin C promoted a rightward shift in the maternal oxygen-haemoglobin dissociation curve (Brain et al., 2019). Combined, these effects of vitamin C may allow the fetus to compensate for the reduction in oxygen delivery, thereby ameliorating the effects of hypoxia on fetal growth (Brain et al., 2019). While this provided proof of principle that maternal supplementation with vitamin C can protect from FGR, this dose of vitamin C is incompatible with human treatment, and nothing is known about the effects of this dose on placental function. Thus, we further performed a preliminary assessment to test the hypothesis that vitamin C can ameliorate oxidative stress-induced placental dysfunction. While treatment with vitamin C had no effects on placental weight and placental efficiency, vitamin C surprisingly further increased markers of oxidative stress and activation of the UPR^ER, UPR^Cyt and UPR^mit, and was associated with increased activation of MAPKs (Figure 6.1). These preliminary results indicate that the beneficial effects of vitamin C on fetal weight and developmental programming are independent of amelioration of placental stress; vitamin C treatment may even exacerbate activation of placental stress responses. In addition, while our previous studies have shown protective effects of vitamin C on fetal body weight, it was also associated with an increase in fetal hepatic nitrotyrosine in the hypoxic cohort, which was not observed in normoxic fetuses treated with vitamin C (Brain et al., 2019). The same was also the case for placental protein carbonylation and placental levels of the ER chaperone GRP78 (Figure 6.1D and E).

Combined, therefore these findings suggest that there may be an interaction between vitamin C and chronic hypoxia that promotes both fetal and placental oxidative stress. This warrants more detailed investigations into the effects of vitamin C on the fetus
and placenta in preeclamptic pregnancy, and calls for further assessments of the effects of vitamin C supplementation in pregnancy complicated by chronic hypoxia.

**Figure 6.1. Effects of vitamin C treatment on hypoxic pregnancy.**

Values are mean ± S.E.M. for fetal weight (A), placental weight (B), fetal to placental weight ratio (C), the relative ratio of placental levels of post-translational protein carbonylation (D), GRP78 (E), PDI (F), HSP27 (G), HSP70 (H), phosphorylated compared to total ERK (I), phosphorylated compared to total JNK (J), HSP60 (K) and TID1 (L) at 138 dGA. Groups are N (○, n=5), H (●, n=5), HC (■, n=5) and NC (□, n=5). Significant differences (p<0.05) are for the main effect of hypoxia and for the main effect of vitamin C treatment; when there was an interaction, significant differences (p<0.05) are denoted by different letters; two-way ANOVA with Tukey’s post hoc test.
6.1.2 Future perspectives of maternal antioxidant therapy

Our data on the effects of maternal vitamin C supplementation on placental stress may, at least in part, explain the negative results from several clinical trials (Rumbold et al., 2008; Conde-Agudelo et al., 2011; Rumbold et al., 2015; Oh et al., 2020). However, the fact remains that preeclamptic women consistently show signs of oxidative stress and reduced antioxidant capacity, and therefore the search for alternative antioxidant therapies suitable for clinical practice continues (Aouache et al., 2018). Mitochondria become major sources of ROS under conditions of low oxygen tension due to the nature of electron transfer during cellular respiration and the generation of superoxide anions (Aljunaidy et al., 2017; Burton et al., 2017b). Thus, protecting mitochondria specifically from oxidative damage may provide a more successful therapeutic strategy (Smith et al., 2008; Smith and Murphy, 2010). However, conventional antioxidants are unable to penetrate the mitochondrial membranes, calling for the use of mitochondria-targeted antioxidants, such as MitoQ (Smith et al., 2008; Smith and Murphy, 2010). MitoQ is a ubiquinol antioxidant attached to a lipophilic triphenyl phosphonium cation, which allows the drug to enter mitochondria in the absence of a specific uptake mechanism (Murphy, 2008; Smith and Murphy, 2010). The mitochondrial membrane potential concentrates MitoQ several hundredfold in mitochondria, where it is reduced to its active ubiquinol form, preventing lipid peroxidation and mitochondrial damage. Not only has MitoQ been shown to be beneficial in both animal models and human clinical trials in improving cardiovascular function, but it is also safe for administration in vivo with no pro-oxidant effects in non-pregnant subjects (Graham et al., 2009; Gane et al., 2010; Rodriguez-Cuenca et al., 2010; Snow et al., 2010). More recently, several groups, including our own laboratory, have shown that maternal treatment with MitoQ in animal models of gestational
hypoxia is capable of improving placental perfusion and rescuing placental mitochondrial stress, FGR and developmental programming of cardiovascular and neurodegenerative diseases (Aljunaidy et al., 2017; Phillips et al., 2017; Aljunaidy et al., 2018; Nuzzo et al., 2018; Botting et al., 2020). In order to understand how MitoQ confers these beneficial actions on placental function and fetal development, it will be instrumental to expand this investigation by assessing its effects on the activation of placental stress pathways, mitochondrial energy metabolism and maternal cardiovascular function.
6.2 Future directions

6.2.1 Targeting mitochondrial metabolism

The data presented in chapter 4 shows that placental mitochondria undergo a number of changes in response to chronic gestational hypoxia in late gestation. This opens the debate whether any of these changes can be used as biomarkers for the early detection of preeclampsia, or whether they may be suitable targets for intervention. It is, however, important to note that many mitochondrial functional changes are adaptive rather than pathological in nature. For example, alterations in mitochondrial morphology are well characterised and evolutionarily conserved responses to hypoxia across a range of tissues (Neary et al., 2014; Fuhrmann and Brüne, 2017). The decrease in mitochondrial complex I activity observed in the current study is successfully compensated for by changes in mitochondrial complex abundance, ultimately maintaining total OXPHOS capacity. Furthermore, decreased relative usage of products of fatty acid metabolism in the placenta was associated with a decrease in ACC abundance and an increase in CPT1 expression, both of which favour fatty acid transport into the cell for β-oxidation (Wakil and Abu-Elheiga, 2009). Within this complex regulatory network governing mitochondrial respiration, decreased activation of AMPK was associated with increased placental expression of the upstream “rescue hormone” adiponectin, which is a key player in the control of lipid utilisation (Achari and Jain, 2017). Even though these changes were unable to prevent the development of fetal growth restriction and maternal cardiovascular function, it would be premature to label these responses as maladaptive and to assume that all metabolic alterations represent underlying causes in the pathology of preeclampsia. Our data suggests that mitochondria harbour a striking ability to self-regulate their functional and metabolic milieu, allowing them to adapt to their respective environment. One of the markers that
has gained traction in the past decade is the hypoxamir miR-210, which drives many of the mitochondrial responses to hypoxia (Chan et al., 2009). MiR-210 may be suitable as a predictive serum biomarker for preeclampsia, aiding the identification and monitoring of women at risk of developing the disease (Anton et al., 2013; Nikuei et al., 2016; Koushki et al., 2018; Tkachenko et al., 2020). Furthermore, AMPK agonists, such as AICAR, have potential in improving pregnancy outcomes, as shown in some animal models of preeclampsia (Banek et al., 2013; Kumagai et al., 2018; Lane et al., 2020). However, this aspect of the current literature calls for a more detailed investigation into the nature of each of these pathways and requires careful evaluation of their functional relevance at the mitochondrial, cellular, tissue and whole organism level.

6.2.2 Effects of chronic hypoxia in non-pregnant ewes

Our model closely resembles the symptoms of placental insufficiency that occurs with many complications of pregnancy, including preeclampsia, presenting with a vicious cycle of increased uteroplacental vascular resistance, reduced uteroplacental perfusion, placental stress and placental dysfunction that culminate in both FGR and maternal cardiovascular dysfunction (Tong and Giussani, 2019). However, in contrast to preeclampsia, this model of complicated pregnancy creates a state of maternal-placental-fetal hypoxia. While evidence from non-pregnant animals and evidence from RUPP models suggest that the presence of the stressed placenta is the key driver in maternal cardiovascular dysfunction during hypoxic pregnancy, it will be vital to repeat the investigations performed in the present study on non-pregnant ewes (Vilar et al., 2008; Li et al., 2012; Zhou et al., 2013; LaMarca et al., 2016). Additional experiments, such as carunculectomies, uterine artery ligations and umbilical cord occlusions in
pregnant animals will also allow us to isolate the individual effects of maternal, placental and fetal hypoxia, while controlling for species differences as well as the degree, timing and duration of the hypoxic insult.

6.2.3 Post-partum maternal studies

It is well known that women with pregnancy complications are at greater risk of developing cardiometabolic diseases and chronic kidney disease many years post-partum (Cunningham and LaMarca, 2018). While cardiovascular disease is already a leading cause of death in women, the risk further increases in women with acute or past cases of preeclampsia (Jónsdóttir et al., 1995; Hannaford et al., 1997; Agatisa et al., 2004; Germain et al., 2007; Mosca et al., 2011; Vaccarino, 2019). It has been hypothesised that additional haemodynamic challenges to the maternal cardiovascular system during pregnancy, such as preeclampsia and hypertension, can highlight pre-existing cardiovascular and renal pathologies (Chung and Leinwand, 2014; Cunningham and LaMarca, 2018). For example, it was demonstrated that women, who subsequently develop preeclampsia, have decreased cardiac output and increased peripheral vascular resistance even pre-conception compared to women, who develop healthy pregnancies (Foo et al., 2018; Thilaganathan and Kalafat, 2019). An opposing line of thought suggests that preeclampsia can cause irreversible changes to the maternal cardiorenal system, which predispose to de novo cardiovascular events and end-stage renal disease with advancing age. For example, administration of the VEGF inhibitor sunitinib to rodents resulted in hypertension, which returned to baseline a few days after treatment, while renal histopathology and urinary ET-1 excretion persisted even after sunitinib withdrawal (Kappers et al., 2010; Kappers et al., 2011). Combined evidence from several prospective cohort studies
suggests that the true picture is an amalgamation of both effects. It is now known that women, who subsequently develop preeclampsia have asymptomatic diastolic dysfunction, and that these women show persistent left ventricular dysfunction associated with an increased risk of developing essential hypertension and symptomatic heart failure for several years post-partum (Lykke et al., 2009; Mongraw-Chaffin et al., 2010; Melchiorre et al., 2011; Melchiorre et al., 2013). In the present study we observed changes to maternal systemic cardiovascular function, which are akin to maternal cardiovascular dysfunction during preeclampsia. It will be instrumental to assess the reversibility of these markers in the same ovine model longitudinally post-partum.

6.2.4 Sex differences

There is growing evidence that sexual dimorphisms in pregnancy outcome are mediated by sex-specific functions of the placenta, triggering divergent adaptive responses in the face of adverse prenatal conditions (Clifton, 2010). In the past decade, a plethora of evidence from clinical investigations and rodent models has emerged, indicating that female conceptuses are relatively protected from adverse intrauterine conditions, possibly mediated by greater placental antioxidant defences and mitochondrial biogenesis (Borrás et al., 2003; DiPietro and Voegtline, 2017; Evans and Myatt, 2017; Jiang et al., 2017; Kalisch-Smith et al., 2017; Song et al., 2018). In contrast, male fetuses are more vulnerable to prenatal and perinatal challenges due to faster rate of growth, and are found to be 20% more likely to experience adverse outcomes in complicated pregnancies (Vatten and Skjærven, 2004). In preeclampsia, for example, female fetuses are more likely to present with significant FGR compared to males, which often show a normal growth trajectory (Stark et al., 2009). There is
also evidence that women suffering from preeclampsia carrying a male fetus show more prominent adverse peripheral microvascular function and vasoconstrictor phenotypes (Stark et al., 2006). These finding indicate that male fetuses may be attempting to follow a normal growth trajectory by redirecting blood flow away from the maternal periphery, while the female fetus attempts to limit its energy requirements by restricting its rate of growth (Stark et al., 2009; Clifton, 2010). Taking into account the limited sample size, sex differences were neither studied nor controlled for in the present study. Future studies should supplement the pre-existing cohort and examine the sex-specific effects of hypoxic pregnancy on the fetal, placental and maternal phenotype.

6.2.5 Intergenerational studies

Intrauterine development is crucial in determining health in later life, and exposure to adverse conditions during fetal development leads to long-term changes that persist into adulthood, which may even affect future generations without further suboptimal exposure (Gluckman et al., 2008; Aiken and Ozanne, 2014). Intrauterine stressors during the development of the reproductive tract can affect the inheritance of programming defects, both through epigenetic mechanisms in the F2 generation, but also beyond the F2 generation through de novo propagation via the maternal line or through extra-genomic components (Aiken and Ozanne, 2014). For example, both maternal diet and gestational hypoxia greatly affect offspring reproductive function, including ovarian perfusion, oestrous cycling and follicular reserve (Connor et al., 2012; Aiken et al., 2013; Chan et al., 2015a; Chan et al., 2015b; Aiken et al., 2019; Pampanini et al., 2019). Furthermore, exposure to a low-protein grandmaternal diet in rats leads to adverse ovarian function in granddaughters, and there is even evidence

General discussion
that epigenetic programming can be transmitted as far as the F4 generation (Rakyan and Whitelaw, 2003; Anway et al., 2005; Aiken et al., 2015). While we know from studies in the same sheep model as in the present study that gestational hypoxia in the last third of gestation programs cardiovascular dysfunction in adult offspring, which was ameliorated by maternal vitamin C and MitoQ supplementation, we have not yet assessed its effects on reproductive capacity (Botting et al., 2016; Brain et al., 2019; Botting et al., 2020). Future studies should investigate the transgenerational effects of placental stress on reproductive function in the offspring and further generations, and whether hypoxia-mediated mitochondrial dysfunction adversely affects progression of the maternal line. Interestingly, while both vitamin C and MitoQ were successful in rescuing fetal growth and offspring cardiovascular programming, our pilot data indicate that vitamin C had no beneficial effects on placental stress (Botting et al., 2016; Brain et al., 2019; Botting et al., 2020). Comparing the transgenerational effects of antioxidant supplementation during pregnancy will shed light on whether placental dysfunction contributes to developmental programming, or whether the adverse effects are mostly mediated by the reduction in fetal weight.
6.3 Closing remarks

The work presented in this PhD thesis adopted an integrative approach to address the effects of gestational hypoxia on placental and maternal physiology in the sheep. This was achieved by combining experiments in vivo with those at the isolated organ and molecular levels. We found that gestational hypoxia leads to significant placental dysfunction, including activation of the placental unfolded protein response, alterations in mitochondrial respiration and energy metabolism, and angiogenic imbalance. These changes were linked to adverse consequences on downstream fetal development and on upstream maternal cardiovascular function, as in preeclampsia. Therefore, this ovine model of gestational hypoxia provides a useful model to investigate the pathophysiology of preeclampsia and to assess various strategies of intervention. This thesis has also raised several new questions and provided pilot data for exciting future investigations.

* * *
Appendix

Cambridge ewe diet composition

Complementary feed for feeding to pregnant and lactating ewes, supplied by Manor farm feed Ltd., UK.

Composition: Sugar beet pulp, wheat feed, barley culms, distillers dark grains, oat feed, alfalfa, chopped straw, rye, palm kernel expeller, wheat, calcium carbonate, sodium chloride, magnesium chloride, sunflower extract, soya bean hulls and glucose.

Analytical constituents:

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<th>Constituent</th>
<th>Amount</th>
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</thead>
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<tr>
<td>Energy</td>
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<tr>
<td>Crude oils and fats</td>
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<tr>
<td>Crude protein</td>
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<tr>
<td>Crude fibre</td>
<td>14%</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>Phosphorus</td>
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<tr>
<td>Magnesium</td>
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</tr>
<tr>
<td>Vitamin E</td>
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Appendix

Publications

Topical review:


Journal papers:


Conference abstracts accepted for oral presentation:


**Conference abstracts accepted for poster presentation:**


**Future publications:**


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Bibliography


Bibliography


Bibliography


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