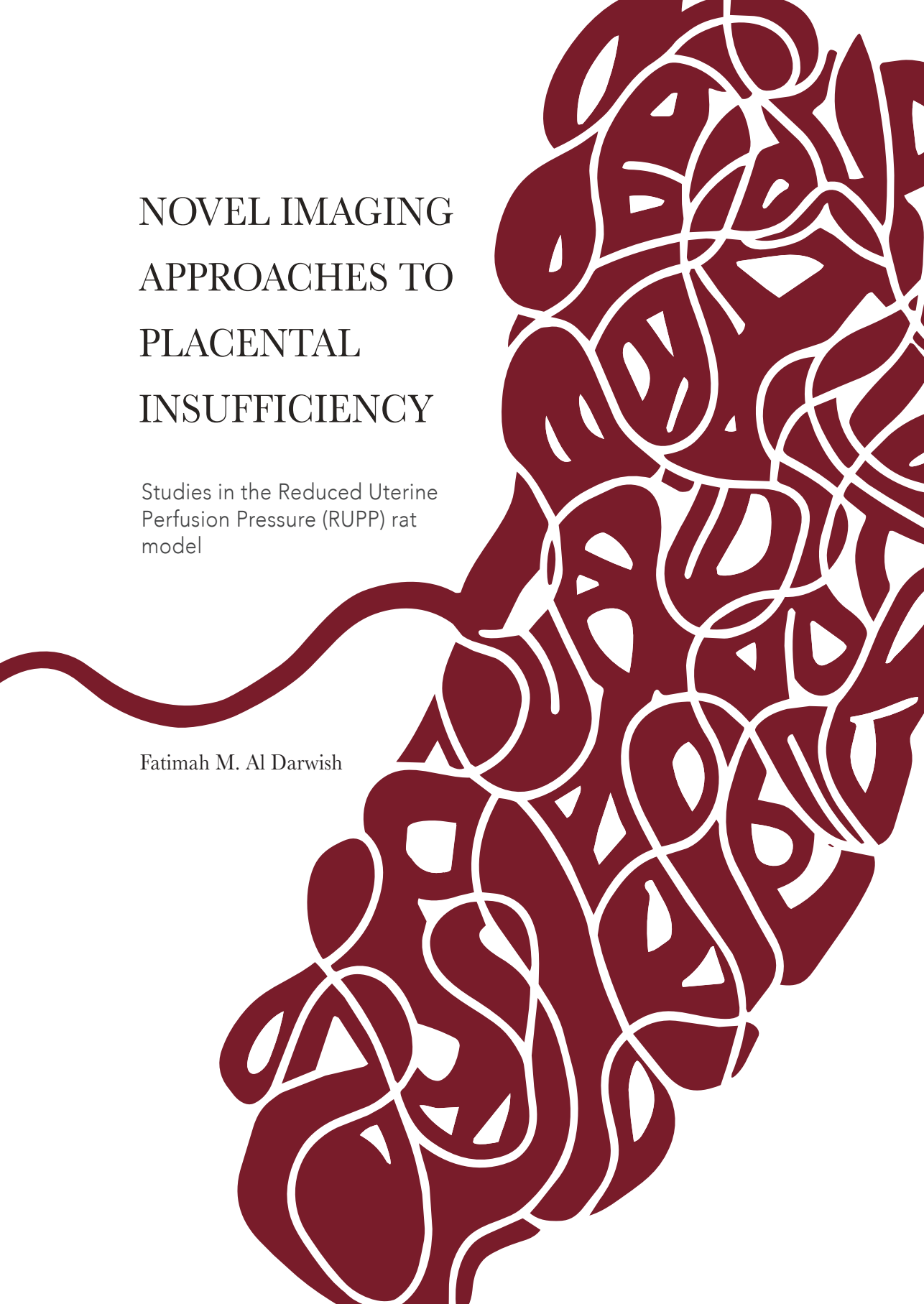


# NOVEL IMAGING APPROACHES TO PLACENTAL INSUFFICIENCY

Studies in the Reduced Uterine  
Perfusion Pressure (RUPP) rat  
model

Fatimah M. Al Darwish





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## **Novel Imaging Approaches to Placental Insufficiency**

*Studies in the Reduced Uterine Perfusion Pressure (RUPP) rat model*

Thesis, University of Amsterdam, with a summary in Dutch

Proefschrift, Universiteit van Amsterdam, met een samenvatting in het Nederlands

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Cover illustration: Fatimah M. Al Darwish

Layout: Mohammed Huraiz

Print: ProefschriftMaken

ISBN/EAN: 978-94-6534-617-5

The research described in this thesis was kindly supported by The Netherlands Organisation for Health Research and Development (ZonMw).

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Novel Imaging Approaches to Placental Insufficiency  
Studies in the Reduced Uterine Perfusion Pressure rat model

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor  
aan de Universiteit van Amsterdam  
op gezag van de Rector Magnificus  
ten overstaan van een door het College voor Promoties ingestelde commissie,  
in het openbaar te verdedigen in de Agnietenkapel  
op donderdag 8 oktober 2026, te 10.00 uur

door Fatimah Mahdi A Al Darwish  
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# 1

## Chapter

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**General introduction**

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## Placental insufficiency: clinical burden and unmet needs

Placental insufficiency is a placenta-centered failure to establish or sustain an adequate maternal–fetal exchange capacity, and it sits upstream of several major obstetric syndromes. Clinically, its most consequential manifestations include preeclampsia (PE), defined as new-onset hypertension at  $\geq 20$  weeks' gestation accompanied by at least one of the following: proteinuria, maternal end-organ dysfunction, or uteroplacental dysfunction<sup>[1,2]</sup>. Another key manifestation is fetal growth restriction (FGR), a pathological failure to reach growth potential. FGR is suspected when estimated fetal weight or abdominal circumference is below the 10<sup>th</sup> centile for gestational age, especially when accompanied by abnormal Doppler findings or reduced growth velocity<sup>[3,4]</sup>. Together, PE and FGR drive substantial perinatal morbidity and mortality and account for a large share of medically indicated preterm birth. Growth-restricted fetuses are over-represented among stillbirths and are associated with increased neonatal complications, largely due to the prematurity<sup>[5]</sup>. Beyond the perinatal period, adverse intrauterine growth environments are linked to long-term cardiometabolic and neurodevelopmental risk in the offspring<sup>[6]</sup>.

The shared biological origin of PE and FGR is increasingly traced to early placental development. In normal pregnancy, extravillous trophoblast invasion remodels uterine spiral arteries from narrow, high-resistance vessels into high-capacity, low-resistance conduits<sup>[7]</sup>. In parallel, endovascular trophoblasts form transient luminal occlusions that regulate the timing and pattern of maternal blood entry into the intervillous space<sup>[8]</sup>. This sequential establishment of uteroplacental perfusion occurs alongside marked shifts in the placental oxygen environment during the first trimester<sup>[9]</sup>. When spiral artery remodeling is shallow or incomplete, the placenta may be exposed to reduced and maldistributed perfusion, intermittent flow, and oxidative stress. These perturbations can damage trophoblast and fetal vascular endothelium and promote the release of pro-inflammatory and anti-angiogenic signals<sup>[1,7]</sup>. Depending on the balance between placental adaptation and maternal susceptibility, the downstream phenotype may be predominantly fetal or systemic maternal<sup>[1]</sup> (**Figure 1**).

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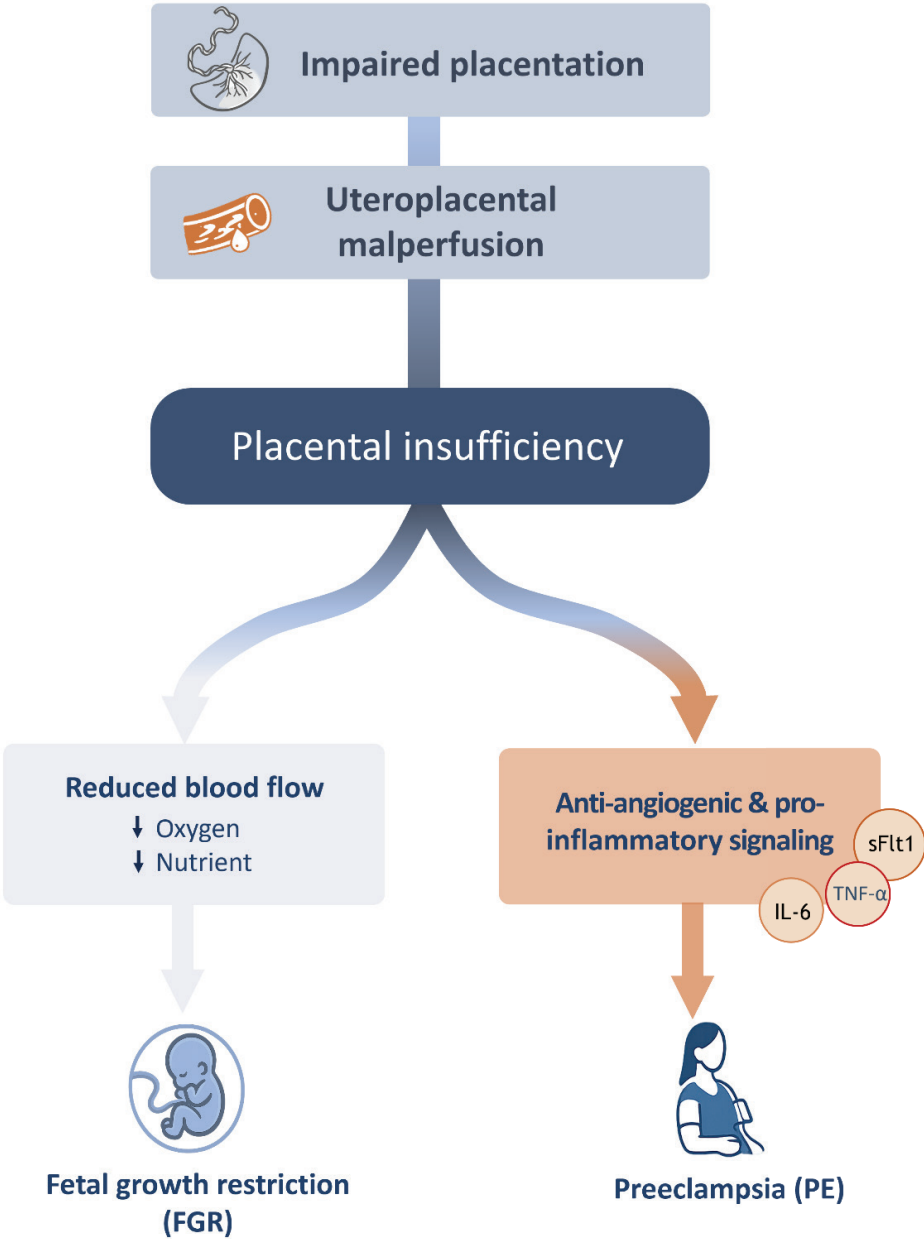


**Figure 1. Placental insufficiency cascade from impaired placentation to PE and FGR.** Shallow trophoblast invasion and incomplete spiral artery remodeling lead to uteroplacental malperfusion and disturbed oxygen dynamics, leading to placental insufficiency. Placental insufficiency promotes the release of anti-angiogenic and pro-inflammatory signaling, it also reduces blood flow to the fetus resulting in predominantly maternal (PE) and/or fetal (FGR) phenotypes.

A major clinical challenge is the indirect nature of current monitoring methods. Standard surveillance relies on ultrasound derived fetal biometry combined with Doppler indices of blood flow in the uteroplacental and fetoplacental vessels. While these tools provide valuable prognostic information, they focus on specific flow patterns in selected vessels. In other words, they do not directly measure how well the placenta is functioning. In the absence of a widely effective therapy capable of restoring placental function once insufficiency is established, clinical management remains largely limited to surveillance and the timing of delivery<sup>[10,11]</sup>. Consequently, decision-making focuses on balancing ongoing intrauterine compromise against the risks of prematurity<sup>[10]</sup>.

The monitoring and therapy gap reinforce each other. Without tools that can directly measure placental function, it is difficult to define precise actionable treatment targets, verify whether potential interventions are affecting the intended targets *in vivo*, and determine whether an intervention improves placental function early enough to alter clinical trajectories.

These limitations motivate the development of functional biomarkers capable of assessing placental health more directly and, in parallel, experimental frameworks that support the testing of new therapies. Because direct experimentation in human pregnancy is ethically constrained, animal models remain indispensable for advancing pathophysiological understanding and for preclinical evaluation of candidate interventions.

### **Animal models for placental insufficiency–induced PE/FGR: rationale for the RUPP model**

Over the years, a wide range of models has been developed to study PE and FGR. These include strategies involving vascular ligation, nitric oxide inhibition, anti-angiogenic factor infusion, immune system modulation, nutritional restriction, hypoxia exposure, and genetic modifications<sup>[12]</sup>. Each model captures specific aspects of the syndrome and serves different experimental goals. Some are better suited for studying immune regulation, others for vascular dysfunction or metabolic shifts. However, only a small subset of PE/FGR models are primarily initiated by placental insufficiency itself, including the reduced uterine perfusion pressure (RUPP) model<sup>[13]</sup>, single umbilical artery ligation (SUAL)<sup>[14]</sup>, and the Dahl salt-sensitive (Dahl SS) rat<sup>[15]</sup>.

Among these, the RUPP rat model is widely used because it reproduces key features observed in PE and FGR phenotypes and is initiated by a defined hemodynamic insult. The model is induced surgically by partially restricting uterine perfusion by placing a silver clip on the abdominal aorta above the iliac bifurcation and additional clips on the ovarian arteries (**Figure 2**). This restricts uterine blood flow while limiting compensatory pathways producing a placenta centered reduction in uterine perfusion at a known gestational time point, which is

advantageous for studying temporal dynamics and for designing intervention studies around a defined insult window<sup>[16]</sup>.

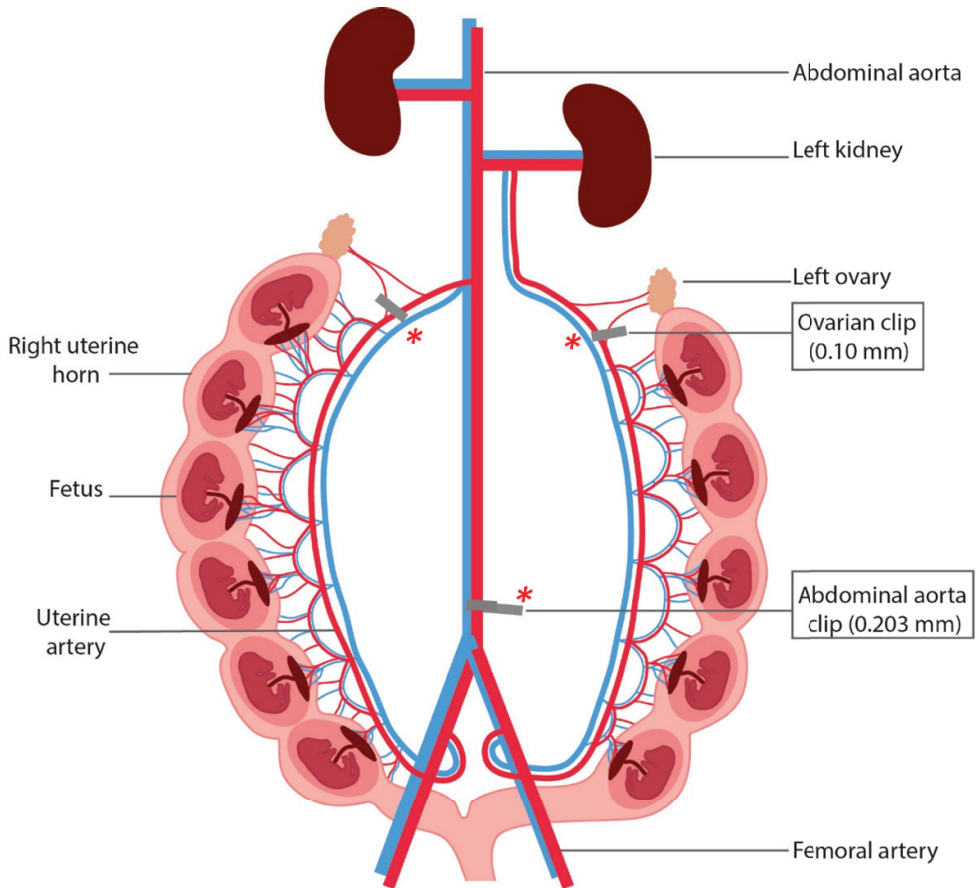


**Figure 2. Schematic overview of the reduced uterine perfusion pressure (RUPP) model in the pregnant rat.** The diagram shows the gravid bicornuate uterus and uterine vasculature. Uteroplacental perfusion is reduced by placing a clip (\*) on the abdominal aorta below the renal arteries, and by applying clips to the right and left ovarian arteries near the uterine arcade, just proximal to the first segmental artery, to limit compensatory increases in uterine blood flow via the ovarian circulation. Reproduced from van Kammen et al. 2024 (doi:10.1152/ajpheart.00056.2024)

Functionally and systemically, RUPP induces a maternal syndrome characterized by elevated arterial pressure and renal hemodynamic impairment within late gestation<sup>[13]</sup>. This pattern is consistent with a placental ischemia driven maternal response. In earlier studies, RUPP initiated at GD14 increased arterial pressure by ~20–25 mmHg and reduced renal plasma flow and GFR

in pregnant rats. These findings highlight RUPP's reliability as a pregnancy-specific model for inducing hypertensive and renal stress<sup>[17]</sup>. Importantly, RUPP also reproduces downstream pathways frequently linked to PE and FGR biology. These include angiogenic imbalance (increased circulating sFlt-1 with reduced free PlGF/VEGF) and inflammatory signaling (e.g., elevated IL-6 and TNF- $\alpha$ )<sup>[18–20]</sup>. Oxidative stress has similarly been implicated as a mediator of the hypertensive phenotype in this model<sup>[21]</sup>.

At the same time, the RUPP model is not fully understood in terms of how functional impairments evolve over time after surgery. Despite extensive use, including studies of placental metabolism<sup>[22]</sup>, oxidative stress<sup>[21]</sup>, inflammation<sup>[19]</sup>, and potential interventions<sup>[23,24]</sup>, functional characterization of placental perfusion trajectories and their relationship to structural remodeling and oxygenation remains incomplete. This knowledge gap is significant: without time specific functional targets, it becomes difficult to design interventions that are well-timed and mechanistically targeted. Studies may fail not because the intervention is ineffective, but because the window of opportunity was missed.

## Functional imaging readouts

To address the time-dynamic knowledge gap in the RUPP model, this thesis prioritizes functional imaging readouts that can be assessed in vivo and compared across gestation. The emphasis is on outcomes that reflect organ-level function rather than isolated vessel waveforms. Two functional dimensions are central: *perfusion*, which describes maternal delivery and transit through the uteroplacental exchange bed, and *oxygenation*, which reflects the oxygenation-related state of the placental microvascular environment.

### Perfusion

In preclinical pregnancy research, dynamic contrast enhanced (DCE) MRI has been used to interrogate uteroplacental perfusion and has demonstrated sensitivity to experimentally induced reductions in perfusion in controlled rodent settings<sup>[25]</sup>. In this thesis, we developed and implemented a 3D DCE-MRI protocol to assess placental perfusion, and analyzed the resulting enhancement time courses using semi-quantitative metrics that capture distinct aspects of delivery and transport over time, including measures of enhancement magnitude, timing (time to peak), and integral delivery/retention over defined windows (area under the curve). Semi-quantitative DCE metrics of this type are commonly reported in the broader DCE literature as practical, interpretable descriptors of dynamic uptake behavior, particularly when the goal is longitudinal comparison and robust ranking of perfusion related differences across conditions.

To complement MRI-based perfusion readouts with a tracer and sensitivity profile that is strongly microvascular, this thesis also incorporates contrast enhanced ultrasound (CEUS). CEUS has been used to visualize and quantify intervillous-space perfusion in nonhuman primates, including destruction–replenishment paradigms that yield parameters related to microvascular blood volume and replenishment rate<sup>[26]</sup>, and it has been reviewed as a practical approach for probing placental development and function in vivo<sup>[27]</sup>.

## Oxygenation

Perfusion alone is not sufficient to define placental function. Oxygenation sensitive readouts therefore provide a second functional axis. In this thesis, oxygenation is primarily assessed using T2\* mapping, which exploits the fact that deoxyhemoglobin increases local magnetic susceptibility and accelerates transverse signal decay, producing lower T2\* values in blood and tissues with higher deoxyhemoglobin fraction<sup>[28]</sup>. Importantly, T2\* is sensitive not only to oxygenation but also to aspects of the microenvironment (e.g., tissue composition)<sup>[28]</sup>. Human placental MRI studies consistently show strong gestational-age dependence of T2\* and report lower placental T2\* in pregnancies complicated by placental dysfunction phenotypes, including hypertensive disease and FGR, supporting its use as a functional biomarker that can be tracked across gestation and related to outcome<sup>[28,29]</sup>.

To complement MRI-based oxygenation with a modality grounded in optical hemoglobin contrast, this thesis also incorporates photoacoustic imaging (PAI). PAI estimates oxygenation-related indices by spectrally separating oxy- and deoxyhemoglobin absorption, enabling spatially resolved measures such as sO<sub>2</sub> in vivo<sup>[30]</sup>. In placental research, PAI has been used to track oxygenation changes during controlled maternal gas challenges and to detect functional hypoxia in experimental placental insufficiency settings, including RUPP model and therapy-response studies<sup>[23,31]</sup>.

## Thesis outline

The work in this thesis aims to advance the mechanistic, time-specific understanding of placental insufficiency in the RUPP rat model by integrating in vivo functional imaging of placental perfusion and oxygenation with biological readouts of uteroplacental remodeling. The broader goal was to define temporally informed functional readouts that can support future mechanistic studies and therapy testing. **Chapter 2** sets the clinical and translational rationale by reviewing molecular and imaging biomarkers linked to placental hypoxia and insufficiency, and by highlighting the limitations of current monitoring approaches that motivate the need for robust in vivo functional biomarkers. **Chapter 3** establishes and applies quantitative imaging readouts at late gestation (GD19), using T2\* mapping to interrogate placental and fetal oxygenation

alongside 3D DCE-MRI-derived measures of perfusion, thereby framing oxygenation and perfusion as complementary dimensions of placental function. **Chapter 4** then anchors these functional signals to structure by evaluating how closely the RUPP model captures key vascular remodeling mechanisms relevant to human disease, including temporal changes in placental morphology, uNK cell composition, trophoblast invasion, and spiral artery remodeling. Building on this framework, **Chapter 5** presents a longitudinal 3D DCE-MRI study that shows the time course of functional impairment after RUPP from GD14 to GD18, explicitly separating acute post-surgical effects from later gestational changes and introducing voxel-wise analyses to quantify within-litter heterogeneity. Finally, **Chapter 6** extends the functional characterization into a multimodality setting at mid-gestation (GD16) by combining DCE-MRI and T2\* mapping with CEUS and PAI to test whether independent perfusion and oxygenation sensitive readouts converge on consistent conclusions at this stage.

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# 2

## Chapter

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### **From Molecules to Imaging:** Assessment of Placental Hypoxia Biomarkers in Placental Insufficiency Syndromes

*Cells* 2023 August; 12(16):2080. doi:10.3390/cells12162080

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**Abstract:** Placental hypoxia poses significant risks to both the developing fetus and the mother during pregnancy, underscoring the importance of early detection and monitoring. Effectively identifying placental hypoxia and evaluating the deterioration in placental function requires reliable biomarkers. Molecular biomarkers in placental tissue can only be determined post-delivery and while maternal blood biomarkers can be measured over time, they can merely serve as proxies for placental function. Therefore, there is an increasing demand for non-invasive imaging techniques capable of directly assessing the placental condition over time. Recent advancements in imaging technologies, including photoacoustic and magnetic resonance imaging, offer promising tools for detecting and monitoring placental hypoxia. Integrating molecular and imaging biomarkers may revolutionize the detection and monitoring of placental hypoxia, improving pregnancy outcomes and reducing long-term health complications. This review describes current research on molecular and imaging biomarkers of placental hypoxia both in human and animal studies and aims to explore the benefits of an integrated approach throughout gestation.

**Keywords:** placental hypoxia; molecular biomarkers; non-invasive imaging.

# Introduction

Placental insufficiency is associated with pregnancy complications, including pre-eclampsia (PE), fetal growth restriction (FGR), and stillbirth <sup>[1]</sup>. The pathophysiology of placental insufficiency is not entirely clear. It involves many factors, including anti-angiogenic <sup>[2,3]</sup>, pro-inflammatory <sup>[4,5]</sup>, and hypoxic factors <sup>[6]</sup>, representing a complex web of interactions that profoundly affects the delicate balance required for optimal fetal development (Figure 1).

Placental hypoxia is a central factor in the development of placental insufficiency <sup>[7]</sup>. During placentation, the trophoblast cells differentiate and invade the maternal tissues, establishing the vital placental vascular network that facilitates nutrient and gas exchange between the mother and fetus. Any disruption in this process can lead to inadequate oxygen supply to the placenta and subsequently contribute to placental insufficiency and related pregnancy complications <sup>[1,7]</sup>. Therefore, assessing placental oxygenation or hypoxia is essential for understanding part of the pathophysiology of placental insufficiency.

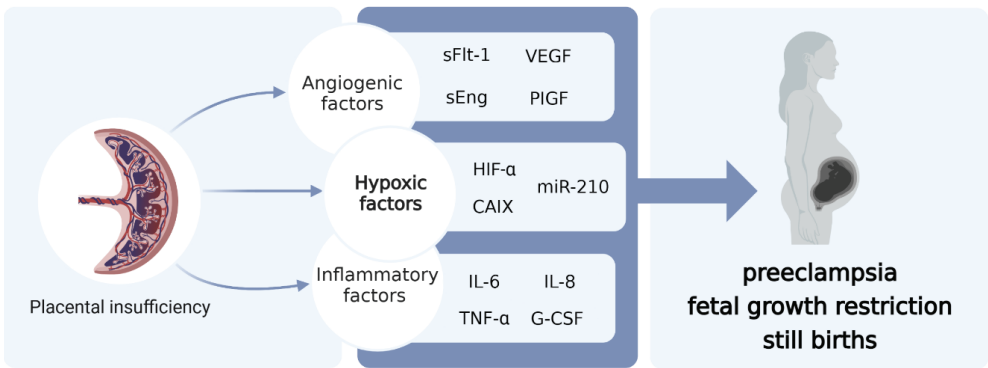


**Figure 1. Interplay of factors contributing to placental insufficiency:** Examples of potential biomarkers related to each factor presented in the blue box. sFlt-1: Soluble fms-like tyrosine kinase-1, VEGF: Vascular endothelial growth factor, sEng: Soluble endoglin, PlGF: Placental growth factor, HIF- α: Hypoxia-inducible factor –alpha, CAIX: Carbonic Anhydrase IX, miR-210: microRNA-210, IL6: Interleukin 6, IL8: Interleukin 8, TNF-α: Tumor necrosis factor-alpha, G-CSF: Granulocyte colony-stimulating factor.

The current approach to monitoring placental function involves ultrasound biometry, Doppler measurements, and cardiotocography to assess fetal wellbeing <sup>[8]</sup>. Despite their utility, the current biomarkers fall short in detecting early signs of placental insufficiency and precisely predicting adverse pregnancy outcomes. Approximately half of FGR cases remain undetected during pregnancy, leading to missed opportunities for timely interventions. Furthermore, once FGR is detected, existing monitoring strategies may inadequately identify fetal compromise in

a timely and reliable manner <sup>[9]</sup>. Therefore, the incorporation of reliable biomarkers becomes imperative, especially in determining the optimal timing for intervention and delivery, a crucial consideration given the delicate balance between the risks of stillbirth and those associated with preterm birth <sup>[10]</sup>.

One way to identify placental hypoxia is the use of molecular biomarkers, which can be detected in maternal blood during pregnancy or in placental tissue after delivery. Circulating biomarkers in maternal blood can provide valuable information about the overall health status. On the other hand, ex vivo investigations of placental tissue can be more specific, as it allows the direct examination of the placenta; however, it provides only a static representation of placental function at a specific time, disregarding the dynamic changes in placental development throughout gestation. Nevertheless, the assessment of the placenta after delivery becomes apparent too late to intervene, underscoring the importance of alternative methods to monitor placental health in real-time.

The assessment of dynamic changes of the in utero condition can be facilitated by non-invasive imaging techniques that can safely and effectively assess the placental and fetal condition over time. Non-invasive imaging offers a more comprehensive understanding of the underlying biological processes that drive placental disease development and progression during multiple gestational phases. Fortunately, recent advances in imaging technologies such as photoacoustic imaging (PAI) and magnetic resonance imaging (MRI) have shown great promise in detecting and monitoring placental hypoxia <sup>[11,12]</sup>. These imaging techniques have the potential to complement molecular biomarkers, offering a more thorough understanding of placental function throughout gestation.

While several studies have individually investigated the utility of molecular and imaging biomarkers, there is an emerging imperative to establish a connection between these two approaches. In this narrative review, we therefore aimed to examine the current research on molecular and imaging biomarkers in human and animal studies and identify studies that have adopted an integrated approach of incorporating both biomarker types to assess placental hypoxia.

## Molecular Biomarkers of Hypoxia

Molecular biomarkers of hypoxia are proteins and genes that are activated in response to reduced oxygen levels. Several molecular biomarkers, including HIF-1 $\alpha$ , CAIX, and miR-210, are involved in the cellular response to hypoxia <sup>[13–15]</sup>. These biomarkers can be measured in placental tissues or maternal blood samples using various techniques, such as immunohistochemistry, ELISA, and qPCR. Elevated levels of these biomarkers have been associated with placental hypoxia and adverse pregnancy outcomes. It is important to note that there are ad-

ditional downstream biomarkers, particularly angiogenic factors, that provide further insight into vascular and endothelial dysfunction <sup>[2]</sup>. However, discussing these factors falls beyond the scope of this narrative review, which focuses specifically on hypoxia.

## **Hypoxia Inducible Factor (HIF)- $\alpha$**

Hypoxia Inducible Factor (HIF)- $\alpha$  is a transcription factor that plays a critical role in the cellular response to hypoxia or low oxygen levels. It regulates the expression of genes involved in various physiological processes, including angiogenesis, metabolism, and cell survival <sup>[16]</sup>. HIF is composed of two subunits: HIF- $\alpha$  and HIF- $\beta$ . The HIF- $\alpha$  subunit is highly regulated by oxygen availability and it degrades rapidly under normoxia, while it stabilizes under hypoxia. Under hypoxic conditions, the stabilization of HIF- $\alpha$  results in its accumulation. Subsequently, HIF- $\alpha$  binds with HIF- $\beta$  to form an active HIF complex, which triggers the transcription of target genes, including VEGF. This process aims to enhance the delivery of oxygen to the hypoxic region of tissues <sup>[1]</sup>. To gain insights into the significance of HIF- $\alpha$  in placental insufficiency, several studies have explored its expression and activity in both human and animal models.

### ***Human Studies***

During the first trimester of pregnancy, the levels of HIF-1 $\alpha$  protein in the maternal blood serum, determined through ELISA testing, have demonstrated effectiveness in predicting preeclampsia (PE). Without the use of uterine artery Doppler, the sensitivity and specificity of HIF-1 $\alpha$  levels were found to be 66.7% and 71.5%, respectively. When combined with Doppler, the sensitivity increased slightly to 74.2%. This study concludes that the measurement of HIF-1 $\alpha$  protein levels using ELISA, either alone or in combination with uterine artery Doppler, is an effective approach for predicting PE during the first trimester of pregnancy <sup>[17]</sup>. Tianthong et al. showed that serum HIF-1 $\alpha$  levels can distinguish between early-onset and late-onset PE, with higher expression observed in early-onset cases compared to late-onset and normal control samples <sup>[17]</sup>. In another prospective longitudinal study in high cardiovascular risk pregnancies, HIF-1 $\alpha$  mRNA levels in plasma were able to predict later-stage development of PE, with elevated levels observed until 23 weeks of gestation <sup>[18]</sup>. Others also showed that HIF-1 $\alpha$  expression is elevated in prenatal blood samples taken at the diagnosis of PE, as well as in postnatal blood samples from pregnancies affected by PE when compared to control samples <sup>[19]</sup>. After delivery, studies conducted on ex vivo placental tissue have consistently demonstrated elevated levels of HIF-1 $\alpha$  and HIF-2 $\alpha$  in pregnancies affected by PE and FGR <sup>[20–25]</sup>. Nevertheless, the number of studies simultaneously examining both HIF-1 $\alpha$  and HIF-2 $\alpha$  is limited, but the available research suggests that HIF-2 $\alpha$ , rather than HIF-1 $\alpha$ , is prominently induced in placentas from patients with FGR and PE <sup>[26,27]</sup>. Comparing to blood samples, ex vivo analysis of HIF-1 $\alpha$  has not been successful in differentiating between early-onset and late-onset PE <sup>[25]</sup>. This suggests that although HIF-1 $\alpha$  levels are altered in ex vivo placenta samples of PE, they may not be a suitable standalone marker for discriminating between early and late-onset PE cases.

Furthermore, elevated HIF-1 $\alpha$  levels were recorded in pregnant women living in high altitudes, which is associated with an increased risk of PE and FGR [28,29]. Analysis of placental tissue from women residing at high altitudes (>2700 m) revealed increased HIF-1 $\alpha$  mRNA and protein levels, suggesting the activation of the hypoxia-inducible factor pathway in response to chronic hypoxia. This upregulation of HIF-1 $\alpha$  is likely to facilitate placental oxygen transport by promoting erythropoiesis and placental angiogenesis, which are critical for supporting fetal growth and development under low oxygen conditions [28]. These findings provide further evidence of the impact of placental hypoxia on pregnancy outcomes and highlight the relevance of the high-altitude model in understanding the molecular mechanisms underlying FGR and PE.

### ***Animal Models***

In animal models of placental insufficiency, HIF- $\alpha$  expression has been primarily assessed *ex vivo* in placental tissue. These studies have consistently reported increased levels of HIF-1 $\alpha$  mRNA in various models of placental insufficiency, including modified Reduced uterine perfusion pressure (RUPP) (created by ligation of the ovarian arteries only) in mice [30], L-NAME rats (treated with N $\omega$ -nitro-L-arginine methyl ester) [31], and rats on a low-sodium diet [32]. Additionally, elevated HIF-1 $\alpha$  protein levels have been observed in different models such as RUPP rats and mice [16,33–36], L-NAME rats and mice [31,37], LPS-treated rats [38], and hypoxia-induced models in rats and mice [16]. In the case of both modified RUPP and selective RUPP (which involves the ligation of both ovarian and uterine arteries), elevated HIF-2 $\alpha$  mRNA levels have been found compared to sham. However, HIF-1 $\alpha$  was found to be reduced specifically in the case of selective RUPP [39].

These findings suggest that measurements of HIF-1 $\alpha$  and HIF-2 $\alpha$  hold promise as potential biomarkers for the prediction and identification of placental insufficiency. However, further research is needed to fully understand their clinical significance and effectiveness in predicting conditions such as PE, FGR and stillbirth. Additionally, exploring the combination of these biomarkers with other relevant markers to develop a robust prediction model is essential.

### **Carbonic Anhydrase IX (CAIX)**

Carbonic Anhydrase IX (CAIX) is a transmembrane protein that belongs to the carbonic anhydrase family and plays a crucial role in the regulation of acid-base balance in cells and highly expressed in hypoxic conditions [40]. In normal condition, CAIX is expressed in various tissues, including the gastrointestinal tract, kidney, and reproductive organs. In the placenta, CAIX expression can be observed in the villous cytotrophoblast during early gestation, indicating its involvement in the placental structure and function at this stage. Additionally, CAIX is consistently present in the chorionic plate mesenchymal cells throughout the entirety of a healthy gestation [41]. In placental insufficiency, CAIX have primarily been investigated in clinical research studies.

### *Human Studies*

CAIX serum levels have been investigated as a potential biomarker for predicting the onset of pregnancy complications. A recent longitudinal study has examined the possibility of using CAIX serum level as a biomarker to predict the onset of pregnancy complications in women with a higher risk of developing PE [13]. The study found that CAIX levels were significantly increased in women who later developed PE, starting from the 28th week of gestation, with a high level of sensitivity and specificity (100% and 81.82%, respectively) [13]. This suggests that CAIX could be a reliable predictive biomarker for the onset of PE. Also, CAIX serum level was elevated in PE patients during the third trimester compared to normal pregnancies [42]. In addition, in a study comparing oxidative stress and hypoxia markers in HELLP syndrome, a subtype of severe PE, CAIX levels were significantly higher in HELLP syndrome patients [43]. These findings suggest that hypoxia induces CAIX and plays a significant role in the pathophysiology of these pregnancy complications.

### **miR-210**

MicroRNAs (miRNAs) are a type of small non-coding RNA that play a critical role in regulating gene expression at a posttranscriptional level and they function as a regulator of cell activities including growth, differentiation and apoptosis. Thus, they can serve as molecular biomarkers for various pathological conditions. Among the many miRNAs identified, miR-210 has emerged as a promising hypoxia biomarker in many diseases including cardiovascular diseases, cancer and PE hypoxia [44,45]. Overexpression of miR-210 appears to negatively impact cell migration and trophoblast invasion, which are crucial for normal placental development [45].

### *Human Studies*

Overexpression of miR-210 has been detected in the plasma of PE patients [46–48]. In the second trimester (12–14 weeks of gestation), miR-210 was upregulated in the plasma of pregnant women who later developed PE [14]. Later in pregnancy, both severe (sPE) and mild PE (mPE) patients exhibited significantly higher plasma miR-210 expression levels compared to controls [46,47]. Notably, a study investigating maternal exosomal miR-210 levels demonstrated that the highest expression was observed in sPE, followed by mPE, while there were no significant differences between gestational and chronic hypertension compared to normal pregnancy. This finding emphasizes the role of a hypoxic placenta in PE development [47]. Furthermore, a PCR array of miR-210 target genes revealed that downregulated genes in both severe and mild PE cases were associated with immunosuppression, apoptosis, cell growth, signaling, angiogenesis, and DNA repair which can be associated with PE risk and severity [46]. In addition, studies have shown that miR-210 is upregulated in ex vivo placental tissue of patients with PE, both with and without small-for-gestational age neonates [49]. A recent review reported upregulation in

the placental tissue of PE patients in the majority of studies but downregulation in one study, suggesting inconsistency of the results <sup>[48]</sup>.

### ***Animal Studies***

In a mouse model of PE induced by toll-like receptor 3 agonist, miR-210 was upregulated in the placenta, which is partly driven by HIF-1 $\alpha$  and nuclear factor  $\kappa$ B (NF- $\kappa$ B) <sup>[50]</sup>. The expression level of miR-210 was upregulated in the uterine arteries of sheep exposed to high altitude hypoxia <sup>[51]</sup>. These results imply that miR-210 is a promising molecular biomarker for placental hypoxia in PE. It has the potential of early prediction of the disease. Its expression in the placenta and plasma of PE patients, as well as its impact on downstream gene expression, suggest its potential utility in clinical practice.

## **Imaging Biomarkers of Hypoxia**

Imaging biomarkers are quantitative or qualitative measurements or features derived from images that provide information about biological processes or functions of a tissue or organ. These biomarkers can be used to assess disease progression, monitor treatment response, and aid in diagnosis. In pregnancy, direct and dynamic measures of placental function are possible through non-invasive imaging techniques such as MRI and photoacoustic imaging (PAI) <sup>[11,12]</sup>.

### **T2\* MRI**

MRI has emerged as a non-invasive tool to assess placental function in vivo. Among various MRI sequences, T2\* weighted MRI has shown promising results as an imaging biomarker of placental hypoxia <sup>[52]</sup>. T2\* is a magnetic resonance imaging parameter that reflects the decay of the MRI signal intensity over time due to factors such as magnetic field inhomogeneities caused by the presence of substances like deoxygenated hemoglobin in the tissue <sup>[53]</sup>. This sensitivity to changes in the local magnetic field makes T2\* MRI useful as an indicator of tissue hypoxia. Changes in T2\* values can provide valuable insights into alterations in oxygenation levels within the tissue, making it a critical imaging biomarker for studying conditions like placental hypoxia.

Baseline quantitative T2\* values can be directly related to the oxygenation status of the tissue. Based on T2\* MRI, two functional parameters have been used to assess the change in signal intensity in response to a gas challenge, such as hyperoxia or hypercapnia. One is delta T2\*, which is based on quantitative T2\* values, and this parameter estimates the change in placenta T2\* value from two T2\* scans obtained at different conditions. The other parameter is Blood Oxygen Level Dependent (BOLD) MRI, which is a relative measure of T2\* signal change over time in response to a challenge. Delta T2\* and delta BOLD under gas challenge reflects the hemodynamic status of the tissue.

### **Human Studies**

In a prospective observational study of 100 singleton pregnancies, placental  $T2^*$  was found to predict low birth weight better than uterine artery Doppler [54]. Placental  $T2^*$  was also able to distinguish between FGR and small-for-gestational-age (SGA) fetuses, both of which were lower than the control group [55]. Additionally, placental  $T2^*$  values were significantly lower in FGR cases validated with ex vivo placental pathology showing necrosis and fibrosis [56]. Moreover, in a cohort of SGA fetuses with normal fetal Doppler flows,  $T2^*$ -weighted placental MRI was found to be a significant predictor of placenta-related outcomes such as placental vascular malperfusion, SGA at birth, PE, and preterm delivery [57]. A recent large prospective longitudinal study of 316 pregnancies shows the potential of  $T2^*$  to identify pregnancies at risk starting from early to mid-gestation (10–20 weeks) [58].

Limited studies used oxygenation challenge with MRI to assess the placental condition in abnormal pregnancies. In cases diagnosed with FGR, the peak delta  $R2^*$  ( $1/T2^*$ ) value, representing a change in oxygen saturation levels, during hyperoxia was lower in FGR cases compared to healthy controls [59]. However, another study found no difference in delta  $T2^*$  values in the placenta [60]. These findings indicate that further research is needed to better understand the relationship between placental oxygenation and abnormal pregnancies, as well as to determine the reliability and consistency of oxygenation challenge with MRI as a diagnostic tool.

### **Animal Studies**

In a mouse model of PE induced by fetoplacental overexpression of STOX1A, a transcription factor, MRI revealed a reduction in  $T2^*$  of the PE placentas [61]. Similarly, in a cohort of pregnant Rhesus macaques, placental oxygenation assessed by  $T2^*$  was shown to be reduced [62]. While in the model of FGR induced by ligation of left uterine vascular pedicle, baseline  $T2^*$  was not different between FGR placentas and control, however, employing BOLD MRI and evaluating delta  $T2^*$ , a significant decrease in delta  $T2^*$  in response to hyperoxygenation was observed in FGR placentas compared to controls [63,64]. Similarly, BOLD-MRI showed impaired placental responses to hypercapnia in a hypoxic mouse model of placental insufficiency [65].

### **Photoacoustic Imaging (PAI)**

PAI is a novel imaging technique that combines optical and ultrasound imaging using non-ionizing nanosecond pulses [66]. This technique can measure placental oxygenation directly as oxyhemoglobin and deoxyhemoglobin absorb light at different wavelengths. With this information, it becomes possible to calculate the oxygen saturation levels and makes. PAI is a promising technique for placental insufficiency particularly due to its ability to provide absolute measurements of placental oxygenation, which allows for objective tracking of trends in oxygenation throughout gestation. However, its use in humans is hampered by the imaging

depth limit, which is, however, an area of ongoing development <sup>[66]</sup>. Therefore, the application of PAI in pregnancy research is currently limited to animals.

### *Animal Studies*

During the transitional phase towards clinical utility, PAI already has been proven a valuable tool for evaluating placental and fetal oxygenation in animal models. Several studies reported a reduction in placental oxygen saturation (sO<sub>2</sub>) measured by PAI in response to placental hypoxia induced by L-NAME treatment or ACE2 knockout followed over time <sup>[35,37]</sup>. Additionally, longitudinal assessment of placental oxygenation revealed a reduction followed by a slight recovery over time in placental sO<sub>2</sub> in the RUPP rat model <sup>[35]</sup>. Using the RUPP model of placental insufficiency, PAI has demonstrated promising potential in testing the therapeutic response on placental oxygenation <sup>[67]</sup>. In addition, the reduced placental oxygenation detected by PAI was validated with tissue hypoxia biomarkers, HIF-1 $\alpha$ , confirming placental hypoxia <sup>[35,67]</sup>.

## **Discussion: The Integration of Molecular and Imaging Biomarkers and Its Challenges**

Placental insufficiency in pregnancy complications such as PE and FGR can be better understood through the examination of biomarkers. These biomarkers are associated with key factors involved in the pathophysiology of placental insufficiency, with a particular focus on hypoxia in this review. The studies reviewed consistently demonstrate elevated levels of HIF- $\alpha$  in cases of PE and FGR, both in human blood samples and placental tissue, suggesting their potential as biomarkers for predicting and identifying placental insufficiency. Alongside HIF- $\alpha$ , CAIX and miR-210 have also emerged as promising biomarkers for placental hypoxia. Clinical studies have shown significant increases in CAIX serum levels in women who later developed PE, while miR-210 expression has been detected in plasma and tissue samples from patients. These findings underscore the potential value of these biomarkers in improving our understanding and detection of placental hypoxia in pregnancy complications (Figure 2).

Imaging biomarkers, including T2\* MRI and PAI, provide valuable non-invasive approaches to evaluate placental function. T2\* MRI shows promise in identifying pregnancies affected by placental insufficiency, enabling the prediction of low birth weight and differentiation between FGR and small-for-gestational-age fetuses. Although PAI is currently limited to animal studies, it has the potential in assessing placental and fetal oxygenation (Figure 2). To maximize the potential of non-invasive imaging biomarkers, it is crucial to utilize them longitudinally over time, offering more detailed insights into the condition of the feto-placental unit. While Doppler ultrasound has been widely used, there is a need to introduce new diagnostic tools that harness the potential of more sensitive and direct imaging techniques. Despite the current challenges associated with PAI in human applications, such as limited depth penetration and

safety concerns<sup>[66]</sup>, ongoing technological advancements and research efforts aim to overcome these limitations and enhance its clinical utility.

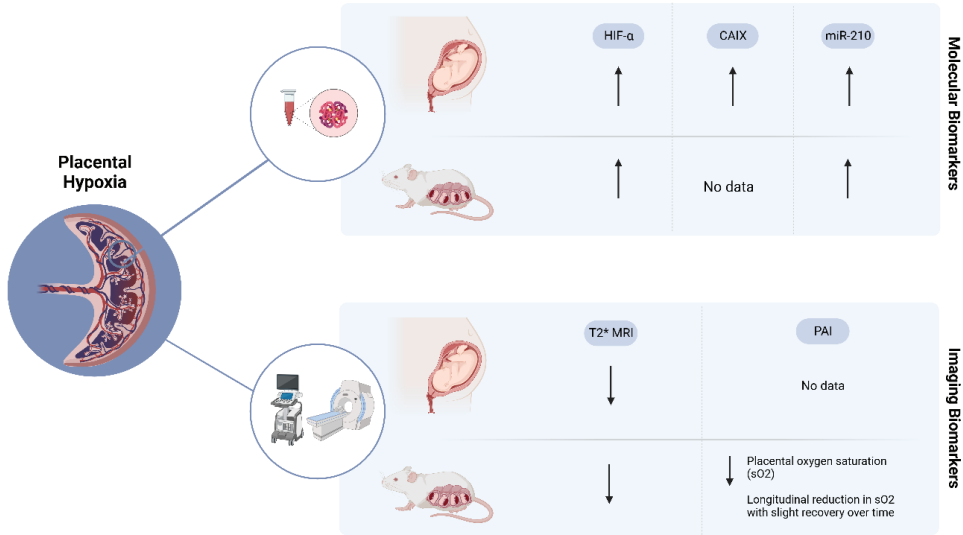


**Figure 2. Overview of the molecular and imaging biomarkers of placental hypoxia.** HIF-  $\alpha$  [16-25, 28, 30, 38], CAIX [13, 42, 43], miR-210 [14, 46-50], T2\* MRI [16, 55, 56, 59, 62-64], PAI [35, 37, 67]

Studying molecular or imaging biomarkers solely has established consistent findings in associating placental hypoxia with an increase in molecular biomarkers such as HIF $\alpha$ , CAIX, and miR-210 levels or a decrease in imaging biomarker values obtained by T2\* MRI and PAI. On the other hand, few preclinical studies using PAI have explored the integration of molecular and imaging biomarkers to validate their findings. For example, oxygen saturation values were compared to ex vivo HIF- $\alpha$  levels in placental tissue, revealing a negative correlation between the two<sup>[35,37,67]</sup>. This suggests that the levels of oxygen saturation and HIF- $\alpha$  expression may provide complementary information about placental hypoxia. Clinical studies have demonstrated that ex vivo pathological examinations of affected placentas reveal abnormalities associated with vascular malperfusion, which are consistent with the outcomes obtained from T2\* MRI within the same study<sup>[54,56,68]</sup>. However, it is important to note that, to our knowledge, no clinical studies have integrated hypoxia molecular biomarkers with imaging biomarkers in the assessment of placental insufficiency. The integration of these biomarkers in future clinical studies holds great potential for a more comprehensive and accurate evaluation of placental hypoxia.

However, the integration of molecular and imaging biomarkers in detecting placental hypoxia presents certain challenges that need to be addressed. One major challenge is the standardization of biomarker assessment across different imaging facilities and laboratories to ensure consistent and reliable results. Additionally, the assessment of both molecular and imaging biomarkers within the same study can be time-consuming. However, this approach is crucial to avoid potential false assumptions that may arise when connecting the results of molecular biomarkers from one study with imaging biomarkers from another study conducted under different conditions. By integrating both types of biomarkers in a single study, we can enhance the accuracy and reliability of placental hypoxia detection.

## Conclusions

Based on this review, HIF- $\alpha$  as the molecular biomarker and T2\* MRI as the imaging technique hold promise for predicting and identifying placental-insufficiency-related conditions. Together, they may provide a comprehensive approach to assess placental function and guide interventions. Nonetheless, further research and validation studies are warranted to fully elucidate the clinical significance and effectiveness of this molecular biomarker and imaging technique pair in improving maternal and fetal outcomes in cases of placental insufficiency.

The integration of molecular and imaging biomarkers has the potential to revolutionize the detection and monitoring of placental hypoxia. However, the current utilization of this integrated approach remains limited to a few studies. To fully understand the clinical utility of this approach, future investigations should consider several key factors. These include evaluating biomarkers at early gestational ages, conducting assessments at multiple time points, examining well-defined patient populations, and utilizing preclinical animal models. By addressing these factors, researchers can thoroughly investigate the potential benefits of this approach and validate their clinical utility in the management of placental insufficiency by finding the optimal indication for timing of pre-term deliveries.

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# 3

## Chapter

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Assessment of feto-placental oxygenation and perfusion in a rat model of placental insufficiency using T2\* mapping and 3D dynamic contrast-enhanced MRI

*Placenta* 2024 June; 151:19–25. doi:10.1016/j.placenta.2024.04.008

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## Abstract

**Introduction:** Placental insufficiency may lead to preeclampsia and fetal growth restriction. There is no cure for placental insufficiency, emphasizing the need for monitoring fetal and placenta health. Current monitoring methods are limited, underscoring the necessity for imaging techniques to evaluate fetal-placental perfusion and oxygenation. This study aims to use MRI to evaluate placental oxygenation and perfusion in the reduced uterine perfusion pressure (RUPP) model of placental insufficiency.

**Methods:** Pregnant rats were randomized to RUPP (n=11) or sham surgery (n=8) on gestational day 14. On gestational day 19, rats imaged using a 7T MRI scanner to assess oxygenation and perfusion using T2\* mapping and 3D-DCE MRI sequences, respectively. The effects of the RUPP on the feto-placental units were analyzed from the MRI images.

**Results:** RUPP surgery led to reduced oxygenation in the labyrinth ( $24.7 \pm 1.8$  ms vs.  $28.0 \pm 2.1$  ms,  $P=0.002$ ) and junctional zone ( $7.0 \pm 0.9$  ms vs.  $8.1 \pm 1.1$  ms,  $P=0.04$ ) of the placenta, as indicated by decreased T2\* values. However, there were no significant differences in fetal organ oxygenation or placental perfusion between RUPP and sham animals.

**Discussion:** The reduced placental oxygenation without a corresponding decrease in perfusion suggests an adaptive response to placental ischemia. While acute reduction in placental perfusion may cause placental hypoxia, persistence of this condition could indicate chronic placental insufficiency after ischemic reperfusion injury. Thus, placental oxygenation may be a more reliable biomarker for assessing fetal condition than perfusion in hypertensive disorders of pregnancies including preeclampsia and FGR.

**Key words:** fetal growth restriction, RUPP, placental oxygenation, hypoxia, placenta imaging

## Introduction

Placental insufficiency plays a key role in the development of most pregnancy complications, including preeclampsia (PE) and fetal growth restriction (FGR). Placental vascular insufficiency arises from various factors, including abnormal remodeling of maternal spiral arteries and deficiencies in feto-placental blood vessel formation. Each of these can independently contribute to the condition, affecting nutrient and oxygen delivery to the feto-placental unit <sup>[1]</sup>. As a result, redistribution of fetal circulation in favor of the fetal brain might occur to promote survival. However, it puts both the mother and the fetus at risk, resulting in increased perinatal morbidity and mortality <sup>[2]</sup>.

With no treatment available, the assessment of the fetal and placental condition becomes critical in deciding the optimal timing of delivery. Currently, Doppler ultrasound forms the gold-standard for monitoring placental insufficiency in clinical practice <sup>[3]</sup>. While it provides valuable information on the impedance of uteroplacental and umbilicoplacental circulations, flow assessment in a single vessel is an indirect measure of placental function that fails to represent the whole feto-placental circulation. Instead, the assessment of feto-placental oxygenation and perfusion using non-invasive imaging techniques offer a direct window on the feto-placental condition <sup>[4]</sup>.

Quantitative T2\*-MRI and dynamic contrast-enhanced (DCE-) MRI have been employed to investigate placental oxygenation and perfusion. Quantitative T2\*-MRI has been used to measure placental oxygenation by utilizing the paramagnetic properties of deoxyhemoglobin, providing insights into oxygen levels <sup>[5]</sup>. Clinical studies showed lower placental oxygenation values in later stages of normal pregnancy, cases of PE, and FGR <sup>[6,7]</sup>. T2\* values also revealed reduced response to maternal respiratory challenge in affected placentas compared to controls, as observed in both human and rat studies <sup>[8,9]</sup>. Meanwhile, DCE-MRI has demonstrated its utility in assessing perfusion abnormalities in the placenta of growth-restricted fetuses <sup>[10]</sup>, quantifying maternal placental vessel perfusion in non-human primates <sup>[11]</sup>, monitoring placental perfusion changes throughout gestation in mice <sup>[12]</sup>, and identifying decreased perfusion parameter in various rodent models under different conditions like acoustic stress <sup>[13]</sup>, pharmacological stress <sup>[14]</sup>, hypoxia <sup>[15]</sup>, and mechanically induced ischemia <sup>[16]</sup>.

Non-invasive imaging offers insights into the alterations in placental function during development and following intervention. The Reduced Uterine Perfusion Pressure (RUPP) rat model stands out as a prominent model for studying placental insufficiency, particularly due to its induction method suited for studying the consequences of impaired blood supply, including vascular dysfunction and FGR <sup>[17]</sup>. Despite the significance of non-invasive imaging, limited research investigates placental function after the RUPP surgery <sup>[18,19]</sup>. Therefore, in this hypothesis generating study, we explored the long-term functional response of the feto-placental unit to the mechanically induced reduction of the uterine blood flow in a rat model for placental insufficiency induced FGR using T2\* mapping and 3D DCE-MRI.

## Material and Methods

All experimental procedures conducted in this study were approved by the Amsterdam University Medical Centers Animal Ethics Committee and were in accordance with the ARRIVE guidelines and European Union guidelines for the welfare of laboratory animals (Directive 2010/63/EU).

### Animals

Twelve-week-old timed pregnant Sprague Dawley (n=21) were supplied by Envigo (Envigo RMS B.V, Horst, the Netherlands) at gestational day five or six. Animals were housed in pairs in conventional cages at regular 12 h light/dark cycle, and constant room temperature (22°C) and humidity (50%) with free access to food and water. An eight-day acclimation period preceded the experiment.

### RUPP procedure

We randomly assigned the pregnant dams to either the RUPP or sham surgery groups. The surgeries were performed by two experienced surgeons. Isoflurane anesthesia (Zoetis Inc., Parsippany, NJ, USA); 3-4% was used for induction using an induction chamber, and 1-2% was used for maintenance delivered via a cone mask. A midline abdominal incision allowed exteriorization of the uterine horns to visualize the utero-placental vessels. For RUPP surgery, a silver clip, AG000450 (Goodfellow Cambridge, Pittsburgh, PA, USA), (1 cm length, 1.5 mm width, and 0.203 mm inner diameter) was placed around the abdominal aorta above the bifurcation of the iliac arteries. Two more clips (1 cm length, 1.5 mm width, and 0.100 mm inner diameter) were placed around the right and left ovarian arteries to avoid compensatory blood flow to the uterine horns. The sham surgery was comparative to the RUPP surgery, but the clips were placed randomly in abdominal fat or connective tissue. For pain management, rats received subcutaneous injection of carprofen (Zoetis Inc., Parsippany, NJ, USA) (5 mg/kg body weight) minimally thirty minutes prior to surgery and every 12 h post-operatively for 24 h. After surgery, animals were housed solitary until sacrifice. The surgery considered successful when it results in partial fetal reabsorption. Rats were excluded when they were not pregnant or when the RUPP procedure resulted in total fetal reabsorption.

### MRI Protocol

On gestational day 19, MRI was performed (7.0T, MR Solutions Ltd., United Kingdom) under isoflurane anesthesia (3-4% induction, 1-2% maintenance). A heated MRI cradle was used to stabilize body temperature at 37°C, whereas the respiratory rate was kept around 50 breaths/min. Temperature and respiration were monitored during scanning. Following calibration scans, an anatomical multi-slice T2-weighted turbo-spin-echo sequence with respiratory gating

was acquired in coronal view to localize fetuses and placentas. Subsequently, a 2D multi-slice multi-gradient echo sequence with respiratory gating was applied for T2\* mapping to assess oxygenation in the placentas and fetal organs of several fetuses. Finally, to investigate placental perfusion, a 3D DCE-MRI gradient-echo acquisition covering the entire abdomen was performed. Data was acquired continuously without respiratory gating starting 1 min before and up to 11 min after 0.025 mmol contrast agent (Dotarem, 500 mmol Gd/L, Guerbet, Villepinte, France) injection via a tail vein catheter using an infusion pump (4 ml/min). To facilitate high-temporal resolution reconstructions, k-space filling was performed using a pseudo-radial scheme in two phase-encoding directions and a tiny golden-angle increment of 16.95° between successive spokes. Detailed sequence parameters are given in **Table 1**.

| Imaging parameter              | T2W       | T2*W      | 3D-DCE        |
|--------------------------------|-----------|-----------|---------------|
| Acquisition plane              | Coronal   | Coronal   | -             |
| Repetition time, ms            | 2500      | 130       | 8             |
| Echo time, ms                  | 30        | 2.2       | 2             |
| Echo-spacing, n                | -         | 2.2       | -             |
| Number of echoes, n            | -         | 8         | -             |
| Flip angle, °                  | 90        | 30        | 25            |
| Matrix size, pixels            | 256 x 256 | 192 x 192 | 128 x 96 x 96 |
| Field of view, mm <sup>2</sup> | 90 x 90   | 60 x 60   | 60 x 60       |
| Slice thickness, mm            | 1.2       | 1.2       | 60            |
| Slice gap, mm                  | 0.5       | 0.2       | -             |
| Signal average, n              | 4         | 8         | 1             |
| Total acquisition time, min    | 10        | 3-5       | 11            |

**Table 1. MRI sequences parameter.** T2W, T2-weighted sequence; T2\*W, T2\*-weighted multi echo sequence; 3D-DCE, 3-dimensional dynamic contrast enhanced.

## Conceptus Measurements

Maternal weight gain was measured between gestational days 14 and 19. Following MRI, dams were euthanized by isoflurane over-dose (5%). The viable and reabsorbed fetuses in each dam were counted placental and fetal weight and fetal crown-rump length for each viable fetus were measured, which were then averaged per litter. Following the measurements, the fetuses were decapitated.

Fetal weight was presented as uncorrected and corrected for differences in maternal weight and litter size by the following equation: fetal weight/(maternal weight at GD 19/number of fetuses in horn).<sup>[20]</sup>

## Data Processing

T2\* maps were produced by fitting each pixel with a mono-exponential decay function using in-house developed software (Matlab, The MathWorks Inc., Natick, MA, USA). As oxygenation parameter, average T2\* values were obtained by drawing regions of interest (ROIs) to visually identifiable fetal (high intensity) and maternal (low intensity) sides of the placenta (3 to 6 placentas per dam). To this end, only placentas on the sagittal plane were measured. As an indicator of fetal hemodynamic redistribution, we also assessed oxygenation values within the fetal brain and liver (3 to 6 per dam) (**Figure 1C**).

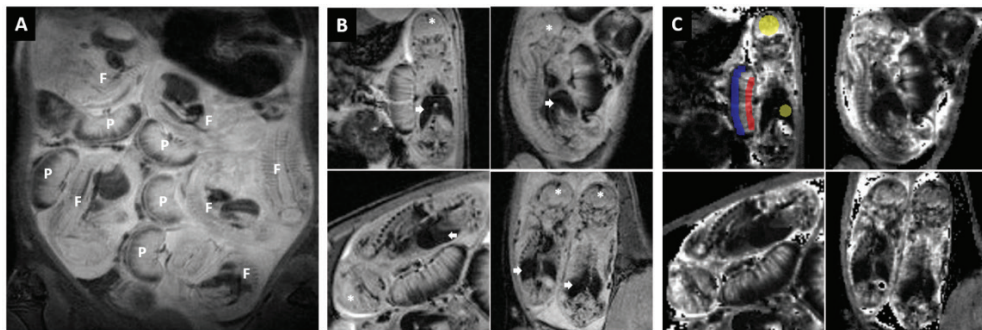


**Figure 1. MRI of a pregnant rats at gestational day (GD) 19.** A) T2-weighted anatomical coronal image showing several fetuses and placentas. B) Zoomed-in T2\*-weighted images (at TE=9.4 ms) showing fetuses and placentas from different rats. C) Zoomed-in T2\* maps from corresponding locations in (B) with example of the ROIs in placenta (labyrinth (red) and junctional zone (blue)), brain and liver (yellow). Placentas are indicated with P. Fetuses are indicated with F. Fetal brains are indicated with asterisk. Fetal livers are indicated with arrow.

Undersampled 3D-DCE data was reconstructed with a temporal resolution of 15 s using a compressed-sensing reconstruction algorithm with total-variation regularization in the temporal domain. 3D Slicer (<http://www.slicer.org>)<sup>[21]</sup> was used for drawing ROIs on the labyrinth and junctional zones of the placenta. Signal intensity-time curves from each ROI were used for semi-quantitative assessment of placental perfusion. This involved calculating several key parameters: the area under the curve (AUC), providing a measure of the extent of contrast agent distribution and washout over time; the maximum signal enhancement, indicating the peak of perfusion; the time to peak (TTP), reflecting the rate at which the tissue reaches maximal perfusion; and the inflow slope of the fitted curve, offering insights into the efficiency of

blood delivery to the placenta. Placental volume was obtained by drawing ROIs around the whole placenta.

### Statistical analysis

Statistical analysis were performed in GraphPad Prism (9.1.0, GraphPad Software, San Diego, CA, USA) and IBM SPSS Statistics for Windows (version 28.0, IBM Corp., Armonk, NY, USA). Continuous data were tested for normality with Shapiro–Wilk test. Differences between RUPP and sham rats were analyzed using unpaired Student's t-test in case of normality, otherwise, Mann-Whitney test was used. A nested t-test was performed when only part of the feto-placental units was analyzed per dam. A two-sided  $p$  value  $< 0.05$  was considered statistically significant. Data are presented as mean  $\pm$  SD when assuming a normal distribution, or as the median in instances of non-normality.

## Results

### Animals

Two rats were excluded since one rat appeared not pregnant and one rat showed total fetal reabsorption after the RUPP surgery; resulting in analysis of a total of nineteen rats ( $n=11$  RUPP and  $n=8$  sham).

### Conceptus Morphometric

RUPP pregnancies showed a higher reabsorption rate of fetuses ( $30.7 \pm 23.4\%$  vs.  $0.7 \pm 2.0\%$ ,  $P < 0.01$ ) and a lower maternal weight gain between GD14 and 19 ( $18.2 \pm 11.5$  g vs.  $42.3 \pm 5.7$  g,  $P < 0.01$ ) compared to the sham rats. While length of RUPP fetuses was reduced ( $4.3 \pm 0.6$  cm vs.  $4.7 \pm 0.6$  cm,  $P = 0.03$ ), fetal and placental weights were similar between the groups ( $2.5 \pm 0.1$  g vs.  $2.4 \pm 0.2$  g and  $0.5 \pm 0.1$  g vs.  $0.5 \pm 0.01$  g respectively). However, when corrected for differences in maternal weight and litter size<sup>[20]</sup>, fetal weight was reduced in RUPP compared to sham ( $0.03 \pm 0.01$  g vs.  $0.05 \pm 0.01$  g,  $P < 0.01$ ).

### T2-weighted MRI and T2\*-mapping: oxygenation

Anatomical T2-weighted scans and corresponding T2\* maps showed high spatial resolution and clear visualization of several feto-placental units with distinct fetal organs, such as brain, heart, and liver (**Figure 1**). In our T2\* maps, we identified two distinct areas of the placenta: the labyrinth and the junctional zone<sup>[22]</sup>. These different T2\* values reflect distinct oxygenation values. Compared to the shams, RUPP rats showed reduction of average placental T2\* values

on both the fetal ( $24.7 \pm 1.8$  ms vs.  $28.0 \pm 2.1$  ms,  $P=0.002$ ) and maternal ( $7.0 \pm 0.9$  ms vs.  $8.1 \pm 1.1$  ms,  $P=0.04$ ) sides (**Figure 2A**). However, no differences between RUPP and sham rats were detected in the oxygenation value of the fetal brain or liver (**Figure 2B**). It's important to note that the analysis of placenta, liver, and brain was not possible in one animal each due to artifacts.

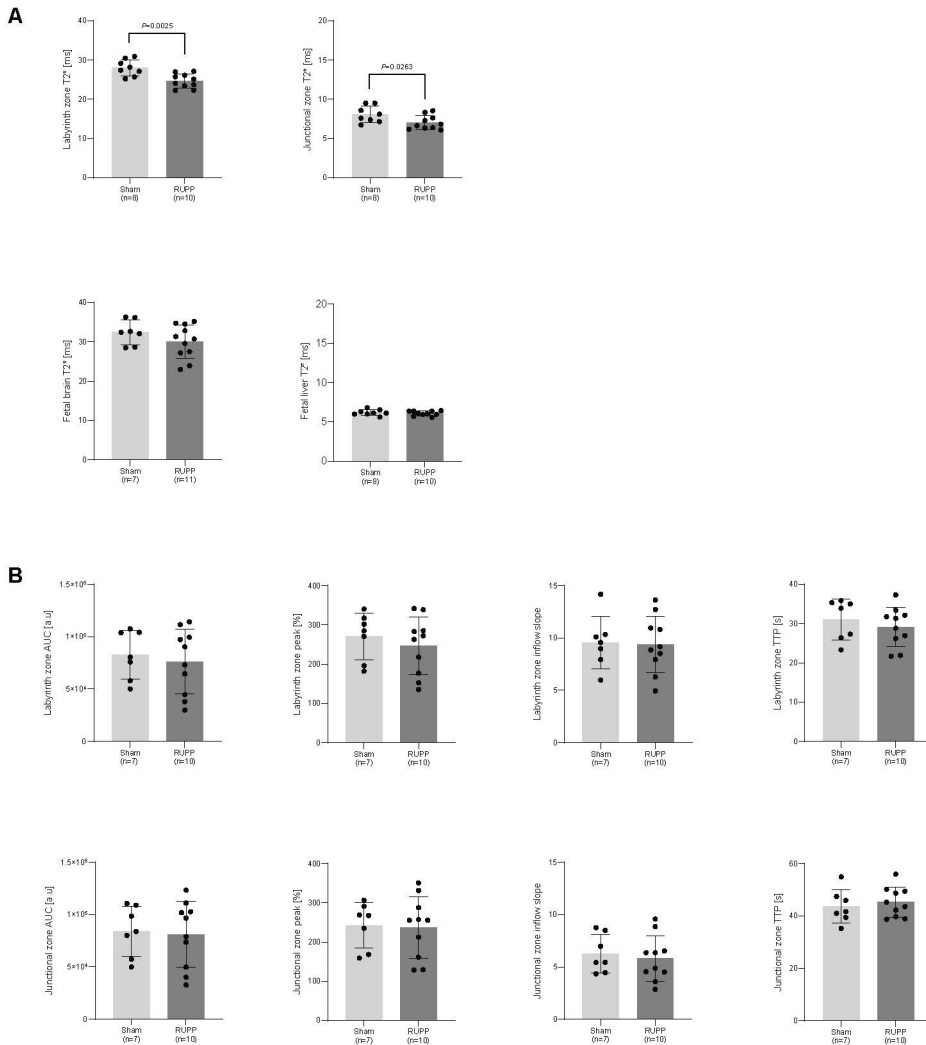


**Figure 2. MRI outcomes in Reduced Uterine Perfusion Pressure (RUPP) and sham rats.** A) Oxygenation parameter: T2\* in the placenta (labyrinth and junctional zone) and fetal organs (brain and liver); B) Perfusion parameters: Semi-quantitative parameters derived from dynamic contrast enhanced (DCE) time curves in the placenta (labyrinth and junctional zone); All graphs show mean  $\pm$  SD analyzed with Student's t-test. P values in panel A analyzed with nested t-test due to the inability to visualize all placentas and fetuses. AUC, area under the curve; TTP, time to peak.

### 3D DCE-MRI: perfusion

Two DCE-MRI recordings were excluded due to technical problems resulting in analyses of seventeen rats (n=10 RUPP and n=7 sham). The 3D technique provided full coverage of the rat uterus with isotropic resolution (**supplementary video**), enabling the visualization of the complex arrangement of all placentas including the placentas of reabsorbed fetuses. The relatively high temporal resolution allowed for visualization of the enhancement patterns of the placental flow through the maternal canal to the labyrinth and back to the junctional zone. No differences in perfusion parameters between the RUPP and sham placentas were detected (**Figure 2B**). The perfusion and enhancement pattern of the placentas of the reabsorbed fetuses were visually distinctly different. For the non-absorbed fetuses, no differences in placental volumes were found (**Figure 3**).

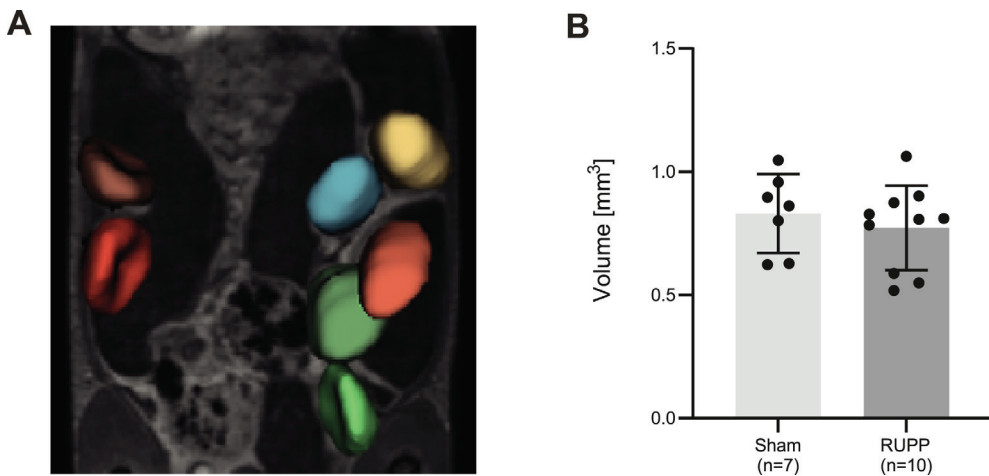


**Figure 3. Placental volume obtained from 3D dynamic contrast-enhanced MRI (DCE-MRI).** A) Three-dimensional segmentation of the placentas overlaid a T1-weighted coronal view of a rat abdomen; B) Placental volume in RUPP and sham animals.

## Discussion

### A. Principal of Finding

Currently, obstetrics is challenged in deciding the optimal timing of delivery of growth restricted fetuses as no treatment is available. The development of novel techniques and animal models to monitor feto-placental condition and test interventions are highly needed. To our knowledge, this is the first study to use multimodality MRI to assess the function of the feto-placental unit in the RUPP rat model. Interestingly, our findings revealed a reduction in placental ox-

ygenation in RUPP rats, a critical indicator of compromised placental function, yet this was not mirrored in the fetal organs. This suggests a potential compensatory mechanism at play, maintaining fetal organ oxygenation despite placental challenges. Additionally, the observation that perfusion in the feto-placental unit was comparable to that in sham rats opens new avenues for understanding the intricate balance in feto-placental circulation in compromised pregnancies. These insights not only contribute to our understanding of the pathophysiology of FGR but also underscore the potential of multimodality MRI as a valuable tool in prenatal diagnostics and intervention strategies.

## **B. What is known**

### **Placental insufficiency secondary to RUPP reduces oxygenation in placenta but not fetuses: a mild FGR phenotype?**

Even though no other studies exist on the use of MRI to measure feto-placental oxygenation in the RUPP animal model, our observation on chronically reduced placental oxygenation is in line with another animal study using photoacoustic imaging two and four days after RUPP surgery<sup>[19]</sup>. This reduction in oxygenation may indicate hypoxia in the RUPP placenta which has been found previously by the increased expression of hypoxia-inducible factor (HIF1- $\alpha$ )<sup>[19,23]</sup>. Moreover, a reduction in placental T2\* values was reported in human PE and FGR<sup>[6,7,24]</sup>. Oxygenation in fetal organs remained unchanged in the RUPP fetuses, which suggests a mild FGR phenotype without brain-sparing. This is in accordance with a reduction in fetal length and litter size but not weight in RUPP animals in our study. This finding is consistent with some studies, which reported no decrease in fetal weight after the RUPP surgery<sup>[19,25]</sup>.

### **Placental insufficiency induced by RUPP leaves perfusion in feto-placental unit unchanged: possible adaptation?**

The RUPP procedure has been shown to result in a 40% reduction of aortic blood flow, resulting in decreased uterine perfusion pressure in the acute setting after the insult<sup>[17,26]</sup>. However, using DCE MRI, the gold standard for perfusion evaluation, we did not observe any difference in placental perfusion five days after the surgery. When using the FGR mechanical model produced by ligation of the left uterine vascular pedicle, lower placental perfusion measured with DCE-MRI was reported in the ligated horn compared to control<sup>[16]</sup>. Aside from the differences in the animal model, perfusion assessment in the aforementioned study was performed in 2D slices with higher temporal resolution (<1 s). Despite the lower temporal resolution, we believe our DCE-MRI protocol would have detected large perfusion differences if present. It is possible that five days after surgery the placental and fetal perfusion has already adapted to the adverse *in utero* condition. The ability of the placenta to recover from the acute insult of the RUPP surgery has indeed also been reported in oxygenation measured with photoacoustic imaging<sup>[19]</sup>.

### **C. Clinical implications of the discrepancy of reduction in placental oxygenation unaccompanied by reduction in placental perfusion: unfortunate timing or sign of a chronically unhealthy placenta?**

While placental hypoxia is believed to be secondary to a reduced oxygen supply by the acute reduced placental perfusion as part of the RUPP surgery, the persistence of low placental oxygenation might be a sign of a chronically ill placenta as part of ischemic-reperfusion injury. The reduction in placental oxygenation despite unaltered perfusion five days after the RUPP procedure suggest a different underlying mechanism for placental insufficiency in the acute versus chronic phases. In the acute phase, shortly after the RUPP surgery, it is speculated that oxygen supply to the feto-placental unit reduced as a result of reduced perfusion. In the chronic phase, reperfusion after ischemic injury might follow.

Based on existing knowledge, we anticipate that the relative hypoxic environment triggers trophoblast proliferation, enhancing blood flow and oxygen supply to the placenta. This adaptive response has been noted in modified RUPP mouse model, where trophoblast proliferation increased in the acute phase, leading to unchanged placental weight between days 1 and 4 post-surgery<sup>[27]</sup>. Whether this still exists at GD 19 remains to be elucidated. Another possible explanation could be the redistribution of blood flow to the surviving fetuses. However, these compensatory mechanisms might be insufficient to counteract chronic placental hypoxia represented by increased hypoxia markers expressions including HIF-1a and its downstream target TGF- $\beta$ -3<sup>[27]</sup>. We did not observe differences in ex-vivo HIF-1a expression in placentas collected from an additional experiment (shown in the **Supplementary methods, Figure S1 and S2**). While these finding do not corroborate reduced placental oxygenation at histological level, it does not disqualify the efficacy of MRI in measuring placental oxygenation either. It is conceivable that our observations reflect a transient phenomenon that failed to induce significant lasting histological alterations.

Our result of unaltered fetal perfusion in the presence of reduced placental oxygenation might support these compensatory mechanisms to protect the fetus. Considering the potential adaptive response of perfusion, placental oxygenation could be a more valuable and reliable imaging biomarker to determine fetal condition. Nevertheless, it is important to understand that adaptation is a dynamic process that depends on time and intensity of the insult and cannot be fully explained from a single time point. Therefore, longitudinal assessment, using non-invasive imaging methods, is highly warranted<sup>[22]</sup>.

### **D. Research implications**

Potentially, the measurement of placental oxygenation rather than perfusion (or flow as currently done in clinical practice) provides a more promising approach to determine optimal timing of delivery of growth restricted fetuses which in turn might prevent perinatal morbidities, stillbirth,

or non-communicable developmental disorders in later life. With limited studies available on assessment of fetoplacental oxygenation and perfusion on the RUPP model and other animal models, further assessments are essential for a complete understanding of the placental insufficiency pathophysiology leading to FGR. Future research should focus on longitudinal measurements of the fetoplacental unit to assess both the acute and chronic phases. Besides optimization of the MRI, multimodality imaging could also include the use of photoacoustic imaging<sup>[18]</sup> and contrast-enhanced ultrasound<sup>[28]</sup> as complementary techniques of oxygenation and perfusion measurements. In addition, it's crucial to recognize the already established value of longitudinal, multimodal imaging studies in pregnant nonhuman primate models, which have provided significant insights into fetoplacental oxygenation and perfusion<sup>[29]</sup>. The development of imaging biomarkers for fetoplacental function in animal models provide the opportunity to evaluate the effect of novel therapeutic interventions.

### E. Strengths and Limitations

This is the first study to use multimodality MRI as a promising technique to assess the chronic alterations in oxygenation and perfusion of the fetoplacental unit in a rat model of placental insufficiency. The MRI protocols used in this study produced high quality T2\* maps and high-temporal resolution 3D DCE-MRI images that facilitate a quantitative evaluation of placental oxygenation and perfusion parameters in pregnant rats. Additionally, the use of an animal model offers opportunities for investigations that are not possible in pregnant women including the use of different contrast agents, multiple imaging modalities, and new therapies.

We also acknowledge some limitations. Due to the intricate and complex nature of placental circulation, effectively distinguishing between fetal and maternal circulations via MRI imaging poses significant challenges. Therefore, in our analysis, we focused on the labyrinth and junctional zone as regions of interest.

Furthermore, our oxygenation analysis did not include all fetoplacental units per dam. Knowing that the position of the fetus in the uterine horn is a possible source of variation, locating the fetal position would add valuable information. However, it proved challenging to determine the relative position of the fetus within the horn from the MRI, especially in late gestation. Another limitation is that oxygenation and perfusion measurements were obtained at a single time point, five days post-surgery. Therefore, a longitudinal assessment is warranted to assess earlier changes in fetoplacental units that stay viable and fetoplacental units that get reabsorbed.

Moreover, differences in MRI outcome parameters could be more distinct in more severe FGR since our morphometric data suggested a mild phenotype of FGR. Most previous studies showed reduction in fetal weight<sup>[30,31]</sup>, while others, similar to our finding reported the lack of fetal weight differences<sup>[19,25]</sup>. In general, this inconsistency can be due to the fact that fetal

weight after the RUPP surgery is influenced by many factors, including litter size, placental weight, fetal sex, and fetal position in the uterine horn <sup>[32]</sup>, of which some are less relevant in the human situation. However, the drastic difference in maternal weight gain supports the RUPP phenotype <sup>[31,33]</sup>. Nevertheless, even in a mild phenotype, we were able to observe differences in MRI derived placental oxygenation measures, which are likely to be more pronounced in a more severe FGR/PE phenotype.

## **F. Conclusion**

MRI enables non-invasive assessment of oxygenation and perfusion of the feto-placental unit. A reduced placental oxygenation was observed, while fetal oxygenation and placental perfusion remained unchanged five days after the RUPP surgery of placental insufficiency. The persistence of lower oxygenation unaccompanied by a difference in perfusion might be a sign of a chronically ill placenta after ischemic reperfusion injury. Considering the potential adaptive response of (re)perfusion, this suggests that measurements of placental oxygenation might be more valuable to monitor fetal condition than the currently used Doppler flow measurements. Longitudinal assessment would contribute in demonstrating feto-placental adaptation to the RUPP surgery which is expected to vary based on the timing of assessment following the surgery.

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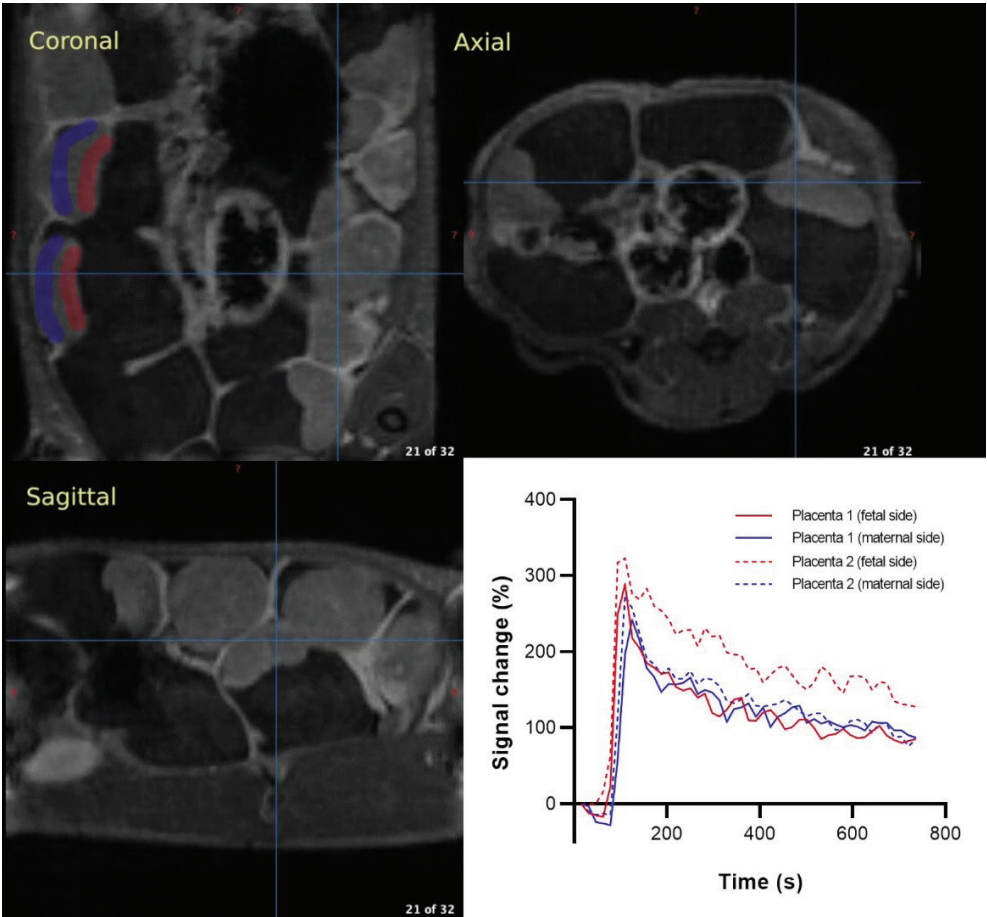
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Supplementary material



**Supplementary video: Three Dimensional Dynamic contrast enhancement MRI.** DCE MRI video in three planes (coronal, axial and sagittal) showing the enhancement pattern of the placentas through the maternal canal to the fetal side and then to the maternal side. Upper left: Example of ROIs drawn on the labyrinth (red) and junctional zone (blue) of the placenta. Lower right: Signal intensity time curve during contrast injection at selected placentas.

### Supplementary methods: Quantification of HIF-1 $\alpha$

For ex-vivo assessment, placental tissue were obtained from a separate experiment that was performed for a different research question. Enzyme-linked immunosorbent assay (ELISA) and immunohistochemical assessments were performed on one placental from each animal (n=8 RUPP and n=8 sham).

#### *ELISA*

Protein lysates were prepared from frozen tissue samples by homogenizing with RIPA lysis buffer. Protein content was subsequently quantified utilizing the BCA assay (Thermo Fisher, United States). Placental concentrations of HIF-1 $\alpha$  were determined using an ELISA kit obtained from LifeSpan BioSciences, Inc (Shirley, MA, USA), in accordance with the manufacturer's protocols. Samples were tested in duplicate, and the absorbance was measured at 450 nm using a microplate reader (Byonoy GmbH, Hamburg, Germany). Accurate concentrations of HIF-1 $\alpha$  within the samples were determined by comparing their respective absorbencies with those obtained for the reference standards, which were plotted on a standard curve. The results were expressed as pg/mL.

#### *Immunohistochemistry Analysis*

Placentas were initially fixed in formalin, followed by embedding in paraffin blocks and sectioning into 4  $\mu$ m slices. Subsequently, slides underwent a sequential process including deparaffinization in xylene, rehydration through a series of descending ethanol concentrations, antigen retrieval in citrate buffer, treatment with phosphate buffered saline (PBS), and then blocking serum.

The primary antibody targeting HIF-1 $\alpha$  (abcam, Cambridge, MA) was diluted in blocking serum (1:200 dilution) and allowed to incubate overnight. On the following day, slides were rinsed in PBS and subjected to incubation with a goat anti-rabbit secondary antibody (abcam, Cambridge, MA), followed by 3,3'-diaminobenzidine (DAB) for color development over a 10-minute period. Subsequently, slides were rinsed in Milli-Q water for 5 minutes and counter-stained with hematoxylin.

Microscopic examination was carried out using Philips Ultra Fast Scanner 1.6 (Philips, Eindhoven, the Netherlands), and images were transferred to QuPath<sup>[1]</sup> version 0.5.0 for analysis. Manual segmentation was performed to delineate the whole placenta, labyrinth zone, junctional zone with the decidua. Color deconvolution technique was applied to distinguish the DAB and hematoxylin signals. Quantification of HIF-1 $\alpha$  expression was determined as the percentage area fraction of DAB staining relative to the total area of the placental segment.

## Results:

The concentration of HIF-1 $\alpha$  quantified by ELISA did not show significant differences between the groups (Figure S1). Similarly, immunohistochemically analysis revealed no significant differences in the percentage of HIF-1 $\alpha$  expression in the placenta of the RUPP group compared to the Sham group (Figure S2).

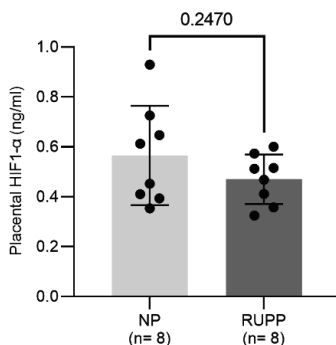


**Figure S1. Placental concentrations of HIF-1 $\alpha$  analyzed by ELISA.** NP = placentas from non-treated rats, RUPP = placentas from RUPP rats.

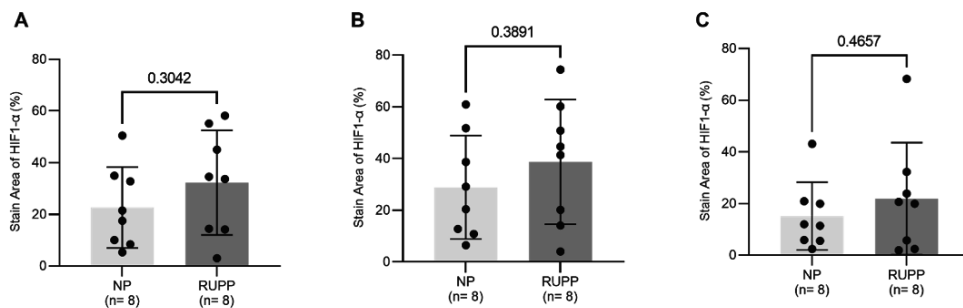


**Figure S2. Quantification of HIF-1 $\alpha$  staining in the whole placenta.** (A), labyrinth zone (B) and junctional zone + decidua (C). NP = placentas from non-treated rats, RUPP = placentas from RUPP rats.

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# 4

## Chapter

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Impaired placental vascular remodeling and persistent uNK cells in the RUPP model: A time-dependent perspective

*Placenta* 2026 February; 174:1–8. doi: 10.1016/j.placenta.2025.11.008

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## Abstract

**Background:** Placental insufficiency underlies major obstetric complications such as preeclampsia and fetal growth restriction (FGR). The reduced uterine perfusion pressure (RUPP) model is widely used to mimic PE and FGR; however, its impact on placental structure, immune cell regulation, and vascular remodeling in the mesometrial triangle remains poorly examined. This study investigates these processes to clarify the model's relevance for human placenta dysfunction pathology.

**Methods:** Maternal, fetal, and placental parameters were assessed in pregnant rats at gestational day (GD) 14, 16, 18, and 19, comparing normal pregnant and RUPP groups. Placental morphology was assessed using hematoxylin and eosin staining. Within the mesometrial triangle, uterine natural killer (uNK) cell distribution was evaluated using ANK61 staining, while trophoblast invasion and spiral artery remodeling were assessed by pancytokeratin and  $\alpha$ -SMA staining, respectively.

**Results:** Placental macrostructural architecture was preserved in RUPP. However, uNK cell migration from the mesometrial triangle was impaired, with higher ANK61-positive cell presence persisting at GD19. This was accompanied by a reduction in trophoblast invasion depth. Spiral artery remodeling was impaired in RUPP placentas, with a greater proportion of unremodeled vessels and fewer highly remodeled arteries by GD19.

**Conclusion:** While gross placental structure remains intact in the RUPP model, the key functional adaptations uNK cell migration, trophoblast invasion, and vascular remodeling, are impaired. These findings support the RUPP as a relevant model to study the pathophysiological mechanisms of placental insufficiency. Future research should focus on temporal molecular profiling to elucidate the mechanisms behind impaired uNK cell retention and shallow trophoblast invasion.

## Introduction

Placental insufficiency is the key driver of pregnancy complications such as preeclampsia (PE) and fetal growth restriction (FGR). These conditions significantly affect maternal and perinatal morbidity and mortality worldwide <sup>[1–3]</sup>. PE affects 2–8% of pregnancies and is clinically defined by new-onset hypertension combined with proteinuria or other maternal end-organ dysfunction or FGR after 20 weeks of gestation <sup>[4]</sup>. While the precise mechanisms remain unclear, impaired placentation early in pregnancy is widely recognized as a major underlying cause <sup>[5–7]</sup>.

In PE, shallow and aberrant invasion of fetal trophoblast cells into the uterine wall and spiral arteries results in incomplete remodeling of these vessels <sup>[8]</sup>. This results in narrowed arteries with high resistance, causing placental ischemia and oxidative stress. The stressed placenta then releases inflammatory and anti-angiogenic factors into the maternal circulation, triggering systemic endothelial dysfunction <sup>[9–11]</sup>. Moreover, dysfunctional uterine natural killer (uNK) cells and macrophages, along with altered cytokine profiles, further contribute to defective placentation and spiral artery remodeling <sup>[12,13]</sup>.

To investigate the mechanisms of defective placentation in PE and develop strategies to improve placental function, the use of animal models is essential. Similar to human pregnancies, rat pregnancies involve hemochorial placentation, in which fetal trophoblasts invade the uterine wall and contribute to the remodeling of maternal spiral arteries. A process regulated in part by uterine immune cells, such as natural killer (uNK) cells <sup>[12,14]</sup>. While these processes are present in both species, there are notable differences in timing. In humans, trophoblast invasion starts early in pregnancy and is largely complete by mid-gestation. In contrast, in rats, trophoblast invasion into the mesometrial triangle begins around mid-gestation, at day 13–14, and continues through late pregnancy <sup>[15]</sup>.

The Reduced Uterine Perfusion Pressure (RUPP) rat model is the most widely used animal model that closely mimics the human situation, as it recapitulates many clinical features of PE, including hypertension, placental ischemia, and fetal growth restriction <sup>[16,17]</sup>. The fact that RUPP is induced at mid-gestation raises questions about its suitability for studying the in human early placental events, such as trophoblast invasion and immune modulation <sup>[16,18]</sup>. However, considering the different timing of placental development in rats, it remains plausible that the RUPP model may still influence those processes.

Despite the frequent use of the RUPP model, little is known about how well it resembles the in human early pathological features of the placenta. This represents a critical gap, as the effective translation of in vivo-tested interventions to improve placental health depends on the alignment between the experimental model and human pathology.

Therefore, this study evaluates how closely the RUPP model mirrors key vascular mechanisms of human placental pathology by assessing temporal changes in placental morphology of the whole placenta, and uNK cell composition, trophoblast invasion, and spiral artery remodeling in the mesometrial triangle relative to controls.

## Material and Methods

### Ethical considerations

This study was conducted following institutional guidelines for the care and use of laboratory animals of Utrecht University and the University Medical Center Utrecht, and all animal procedures were approved by the local Animal Welfare Body (AWB; Utrecht, The Netherlands) under an Ethical license provided by the national competent authority (Centrale Commissie Dierproeven, CCD, The Netherlands), securing full compliance with the European Directive 2010/63/EU for the use of animals for scientific purposes. All efforts were made to report the animal experiments according to the ARRIVE guidelines (**Table S1**).

### Animals

Twelve to thirteen-week-old time-pregnant virgin Sprague Dawley rats at GD 5, 6, or 8 were delivered by Envigo (the Netherlands). All sixty-four dams were group housed for an acclimatization period. Room conditions of 12h light/dark cycle and a monitored room temperature (22°C) and humidity (50%). All animals had free access to food and water. To minimize potential confounders, animals were randomly assigned to groups, cage location was randomly mixed across different groups and timepoints, and measurements were performed in a randomized order. Caregivers were blinded during allocation and conducting experiments; for researchers, blinding was not feasible for the group assignment and intervention, as group allocation was visibly apparent based on factors such as body weight and the presence of surgical wounds.

### Induction of PE/FGR model

On GD14, rats were randomized to either the RUPP or NP group (n=32 per group) and allocated to a timepoint for euthanasia (n=8 per timepoint), using a non-deterministic method. We chose NP animals instead of sham as controls as this provides a clear physiological baseline to assess deviations from normal placental development and no differences compared to sham were shown in previous literature <sup>[19]</sup>. RUPP surgery was performed as described by Granger et al. <sup>[20]</sup>. Under isoflurane anesthesia (3% for induction and 2–2.5% maintenance), a midline abdominal incision was made to expose the uterine horns, providing clear access to the uteroplacental vasculature. A silver clip (AG000450, Goodfellow) with dimensions of 15 mm in length, 1.5 mm in width, and an inner diameter of 0.203 mm was positioned around

the abdominal aorta just above the iliac artery bifurcation. To prevent compensatory blood flow to the uterus, two clips (10 mm × 1.5 mm, inner diameter 0.100 mm) were placed around both the left and right ovarian arteries. Carprofen (5 mg/kg body weight) was administered subcutaneously as analgesia 30 minutes before surgery and 12 and 24 hours post-operatively.

### **Euthanasia and organ collection**

Rats were euthanized on GD 14, 16, 18, and 19 to collect the placentas; other tissues were used for another study. Rats were euthanized by total bleeding under 5% isoflurane. Fetuses and placentas, including the mesometrial triangle, were counted and weighed individually. One placenta from each uterine horn, preferably from the 3<sup>rd</sup> position, was submerged in JB fixative (Tris buffered calcium acetate (0.05%), zinc acetate (0.5%), and zinc chloride (0.5%)) for 24 hours at room temperature. After fixation, the placentas were kept in 70% ethanol at 4°C overnight and then dehydrated and submerged in paraffin in the Vacuum Infiltration Tissue Processor, according to standard protocol. Placentas were paraffin-embedded and sectioned into 5 µm slices along the mesometrial triangle–fetal axis.

### **Histology and analyses**

Three slides per placenta containing the maternal channel were selected for staining. One slide per placenta was stained with hematoxylin and eosin (H&E) to assess tissue morphology. The other samples were stained for ANK61 to visualize uterine natural killer (uNK) cells. The ANK61 antibody also binds to T cells. Therefore, a CD3 staining was performed to exclude T cells from the ANK61-positive cell population. Lastly, a cytokeratin and α-SMA staining was performed to identify trophoblasts and smooth muscle cells, respectively. For each analysis, one placenta section was selected, and all analyses were performed blinded.

#### ***H&E staining***

The 5 µm placenta sections were deparaffinized and stained with H&E according to standard protocol, with 5 minutes in hematoxylin and 2 minutes in eosin, and mounted with Depex.

#### ***Uterine Natural Killer cell (uNK) and T cell staining***

After deparaffinization (2 × 5 min in xylene, 2 × 5 min in 100% ethanol), sections were treated with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 20 min to block endogenous peroxidase. Sections were rehydrated through graded ethanol (96%, 70%, 50%, 30%, 2 min each) and rinsed in demineralized water (2 min). Antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) at 95°C for 3 min, followed by 30 min cooling and a PBS wash (5 min). To block nonspecific binding in biotin staining, sections were incubated with 5% normal horse serum (NHS) in PBS for 30 min at 37°C. Primary antibody (Mouse-anti-NK cell activation structures, anti ANK61, 1:200)

or (rabbit anti-CD3 (2GV6), RTU) were applied overnight at 4°C in PBS with 2% NHS. The next day, slides were washed (2× 5 min in PBS) and incubated with horse-anti-mouse biotin (Vector, 1:100 in PBS) or brightvision Goat anti-Rabbit HRP for 45 min at 37°C. For ANK61 staining, the Vectastain ABC complex was prepared during this step and applied for 30 min after two PBS washes. Sections were then rinsed in 0.05 M Tris-HCl (pH 7.6) for 5 min. For ANK61 staining, DAB staining was performed for 5 min (50 mg DAB in 100 mL Tris-HCl with 30 µL 30% H<sub>2</sub>O<sub>2</sub> added just before use). CD3 staining was developed with NovaRED substrate, for 10 min until red signal appeared. Slides were rinsed in water (5 min), counterstained with hematoxylin (1–2 min), and rinsed in demineralized water (10 min). Sections were dehydrated (2× 10 s in 96% and 100% ethanol), cleared in xylene (2× 10 s), and mounted with DEPEX.

### ***Trophoblasts and α smooth muscle actin staining***

After deparaffinization, the sections were blocked with 10% Donkey serum (Jackson ImmunoResearch) in 1% bovine serum albumin, phosphate buffered saline (1% BSA-PBS). According to protocol to double stain for trophoblasts and α smooth muscle actin, sections were incubated at 4°C overnight with Rabbit anti-pan cytokeratin (Novus Biologicals, 1:75) and Mouse anti α-SMA (Abcam, 1:5000). The next day, after washing, the secondary antibodies (IgG(H+L), Alexa Fluor 488 Donkey anti Rabbit and IgG(H+L), Alexa Fluor 594 Donkey anti Mouse) were applied in a 1:500 dilution. The secondary antibodies were incubated for 1 hour at room temperature. The nuclei were then counterstained with 4',6-diamidino-2-phenylindol (DAPI, 5 minutes incubation at room temperature, 1:5000). The sections were mounted using Fluor Save reagent and cover glasses.

All stained sections were scanned in the pathology department using a NanoZoomer S60 Digital slide scanner (Hamamatsu). For the IF scans, three channels were used: FITC (495 – 520 nm), Texas Red (586 – 603 nm), and DAPI (350 – 470 nm). The exposure times were set to 19 ms, 63 ms, and 66 ms. All analyses were performed in QuPath 0.5.1 <sup>[21]</sup>.

### ***Morphological analysis of the placenta layers***

Area of the total placenta, labyrinth zone (LZ), junctional zone (JZ), decidua, and mesometrial triangle (MT) were measured in the H&E. The different placental layers were distinguished in the H&E sections based on their visual characteristics as described in literature <sup>[22]</sup>. The LZ consists primarily of trophoblast cells, which can be recognized as a layer of dark pink cells, densely packed together. The JZ consists of spongiotrophoblast cells, which present as pale pink in the H&E staining. On top of the JZ is a thin layer of decidua, consisting of densely packed, elongated decidual cells. The MT consists of a mixture of trophoblasts, decidual cells, and maternal arteries.

### ***Uterine Natural killer cell recruitment analysis***

The uNK cell recruitment was determined by the percentage of ANK61-positive cells within the MT. The presence of uNK cells and T cells was determined using a manually defined threshold

based on the intensity of positive brown or red staining. The same threshold was applied consistently across all images to ensure comparability. To exclude T cells from the ANK61-positive cell population, the percentage of ANK61 – CD3-positive area relative to the total MT area was calculated as percentage of positive pixels in the MT and presented as percentage positive pixels ANK61 – CD3 in the MT.

### ***Trophoblast invasion analysis***

Trophoblast invasion analyses were performed by applying a manually set threshold to identify cytokeratin-positive staining within the MT. To assess the pattern of invasion, the maximal depth of the cytokeratin-positive region was measured, normalized to the total depth of the MT, and presented as the percentage depth of trophoblast invasion in the MT.

### ***Spiral artery remodeling analysis***

Spiral artery remodeling was analyzed within the entire MT. Remodeling was assessed based on the extent of  $\alpha$ -SMA loss in the arterial wall as previously described [23,24]. Spiral arteries were classified into four categories according to the percentage of  $\alpha$ -SMA disappearance (**Figure 3F**): unremodeled (0–3% disappearance), slightly remodeled (3–33% disappearance), moderately remodeled (33–67%  $\alpha$ -SMA disappearance), and highly remodeled (67–100% disappearance).

## **Measurement of sFlt-1**

Protein lysates were prepared from one placenta per uterine horn, minced on ice, and homogenized in 2 ml ice-cold RIPA buffer with protease inhibitors. After 2 hours on ice, lysates were centrifuged (12,000 rpm, 20 min, 4 °C), and supernatants were collected. Total protein concentration was measured using the Qubit™ Protein Broad Range Assay Kit (Thermo Fisher Scientific) and adjusted to 1,000  $\mu$ g/ml. For circulating sFlt-1, maternal blood was collected into EDTA tubes, centrifuged to obtain plasma, and stored at –80 °C until analysis. Tissue and plasma sFlt-1 levels were quantified using the Mouse sVEGFR1/Flt-1 DuoSet ELISA Kit (R&D Systems) per the manufacturer's instructions. Samples were run in triplicate, and absorbance was measured at 450 nm.

### ***Dropouts and exclusion criteria***

The exclusion criteria were standardized before the experiment to reduce heterogeneity. Three animals were excluded from participation in the experiment because they were not pregnant or had total resorption. One dam was considered a dropout in the human endpoints criteria, due to complications during the RUPP surgery. Four dams were excluded from the study for all outcomes with a cut-off value of litter size <4 or >16, as very small or large litter sizes increase variability in fetal weight [25]. In histological evaluation, four placentas were excluded because the MT was not visible or damaged. Resulting in 52 inclusions for histological analysis. A total of 46 animals were included in both the placental and circulating sFlt-1 analyses, as samples with undetectably low sFlt-1 levels were excluded from the outcomes.

### Statistical analysis

The statistical analyses were performed in GraphPad Prism version 9.0 for Windows (GraphPad Software, San Diego, California, USA). The dam was considered the experimental unit. If data were normally distributed, a two-way ANOVA was performed, followed by Tukey's post hoc test. When data were not normally distributed, group comparisons were performed using the Mann-Whitney U test with a Bonferroni correction. Data are presented as mean  $\pm$  standard deviation (SD). A two-sided p-value  $< 0.05$  was considered statistically significant.

## Results

### Group characteristics and phenotype

We assessed maternal, fetal, and placental parameters across gestational days (GD) 14, 16, 18, and 19 (**Table 1**). Maternal weight gain was reduced in RUPP dams at GD16 and GD19, indicating impaired gestational progression. The number of implantation sites was similar between NP and RUPP groups at all points. The percentage of fetal resorptions was higher in RUPP pregnancies at GD16 and GD19. Fetal weight was significantly decreased in the RUPP group at GD19, and placental weight was significantly lower at GD18. The fetal-to-placental weight (FW: PW) ratio did not differ significantly between groups. Placental sFlt-1 levels showed no significant differences between NP and RUPP groups at any time point. Similarly, circulating sFlt-1 concentrations showed no significant differences (**Figure S1**).

|                                          | NP              |               |                |               | RUPP            |               |                 |                 |
|------------------------------------------|-----------------|---------------|----------------|---------------|-----------------|---------------|-----------------|-----------------|
|                                          | GD14<br>(n=7) § | GD16<br>(n=6) | GD18<br>(n=6)  | GD19<br>(n=7) | GD14<br>(n=6) § | GD16<br>(n=7) | GD18<br>(n=6)   | GD19<br>(n=7)   |
| Maternal weight gain GD14- end-point (g) | NA              | 5 $\pm$ 7     | 27 $\pm$ 7     | 69 $\pm$ 10   | x               | -10 $\pm$ 10  | 20 $\pm$ 12     | 35 $\pm$ 20***  |
| Implantation sites (#)                   | 12 $\pm$ 3      | 12 $\pm$ 1    | 11 $\pm$ 1     | 15 $\pm$ 2    | 14 $\pm$ 2      | 14 $\pm$ 2    | 14 $\pm$ 2      | 15 $\pm$ 1      |
| Resorptions (%)                          | 0 $\pm$ 0       | 8 $\pm$ 8     | 8 $\pm$ 13     | 0 $\pm$ 0     | 2 $\pm$ 4       | 38 $\pm$ 32** | 25 $\pm$ 22     | 40 $\pm$ 29***  |
| Fetal weight (mg)                        | 191 $\pm$ 29    | 543 $\pm$ 93  | 1727 $\pm$ 424 | 2204 $\pm$ 65 | 163 $\pm$ 9     | 467 $\pm$ 61  | 1572 $\pm$ 378  | 1908 $\pm$ 236* |
| Placenta weight (mg)                     | 282 $\pm$ 32    | 411 $\pm$ 46  | 552 $\pm$ 140  | 495 $\pm$ 24  | 260 $\pm$ 14    | 370 $\pm$ 39  | 432 $\pm$ 68 ** | 495 $\pm$ 26    |

|                             | NP              |               |               |               | RUPP            |               |               |               |
|-----------------------------|-----------------|---------------|---------------|---------------|-----------------|---------------|---------------|---------------|
|                             | GD14<br>(n=7) § | GD16<br>(n=6) | GD18<br>(n=6) | GD19<br>(n=7) | GD14<br>(n=6) § | GD16<br>(n=7) | GD18<br>(n=6) | GD19<br>(n=7) |
| FW: PW<br>(ratio)           | 0.7 ±<br>0.1    | 1.3 ±<br>0.3  | 3.3 ±<br>1.1  | 4.3 ±<br>0.3  | 0.6 ±<br>0.1    | 1.3 ±<br>0.2  | 3.6 ±<br>0.5  | 3.9 ±<br>0.3  |
| Placental sFlt-1<br>(pg/ml) | 496 ±<br>412    | 510 ±<br>200  | 651 ±<br>388  | 624 ±<br>432  | 583 ±<br>213    | 604 ±<br>618  | 825 ±<br>381  | 513 ±<br>136  |

**Table 1: Maternal and fetal outcomes in NP and RUPP groups across gestation.** Data are presented as mean ± SD. § dropouts because of undetectable concentration levels of placental sFlt-1 (included n=3 NP, n=4 RUPP). Asterisks indicate statistically significant differences between RUPP and time-matched NP controls: \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001. NP, normal pregnant; RUPP, Reduced uterine perfusion pressure; GD, gestational day; NA, not applicable.

### Morphological development of the placenta

Placental growth followed a similar developmental trajectory in both NP and RUPP groups from GD14 to GD19 (**Figure 1**), typical examples (**Figure S2 and S3**). Total placental surface area increased over time in both groups but did not differ significantly between NP and RUPP at any individual time point (**Figure 1B**). No significant differences were observed between groups in the absolute (**Figure 1B**) or relative (**Figure 1C**) areas of individual placental layers at any time point.

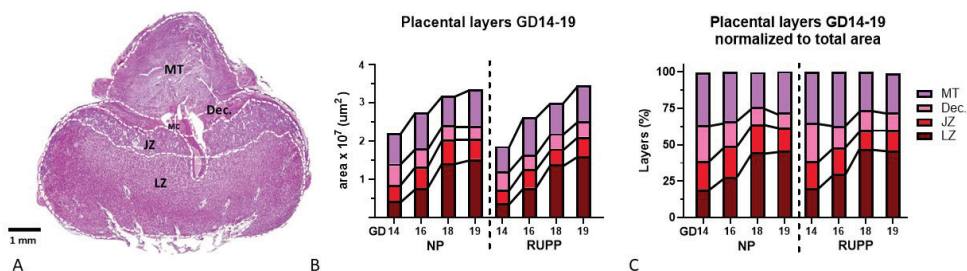


**Figure 1. Structural composition of the rat placenta across gestation.** (A) Representative overview of the placental layers in a normal pregnant rat at GD19. (B) Stacked bar graph depicting the absolute areas of individual placental zones at GD14, GD16, GD18, GD19. (C) Stacked bar graph showing the relative percentage contribution of each placental zone to the total placental area across gestation. Data are presented as means. MT, mesometrial triangle; Dec, decidua; JZ, junctional zone; LZ, labyrinth zone; MC, maternal channel; NP, normal pregnant; RUPP, Reduced uterine perfusion pressure; GD, gestational day.

### *Impaired uNK cell migration and trophoblast invasion in the mesometrial triangle*

Uterine NK cells (ANK-61 – CD3 positive cells) were distributed throughout the MT in both NP and RUPP groups at GD14 (**Figure 2A, B**) with limited trophoblast invasion (**Figure 2D**). As gestation progressed to GD16, GD18, and GD19, uNK cell decreased in both groups, with delayed migration in RUPP placentas. By GD19, a higher proportion of uNK cells remained in the mesometrial triangle of RUPP placentas compared to NP controls (**Figure 2A, C**). This impaired migration was accompanied by a reduction in trophoblast invasion depth (**Figure 2D, F**). At all timepoints, uNK cells were found near unremodeled spiral arteries, especially in RUPP placentas, where trophoblast invasion was limited.

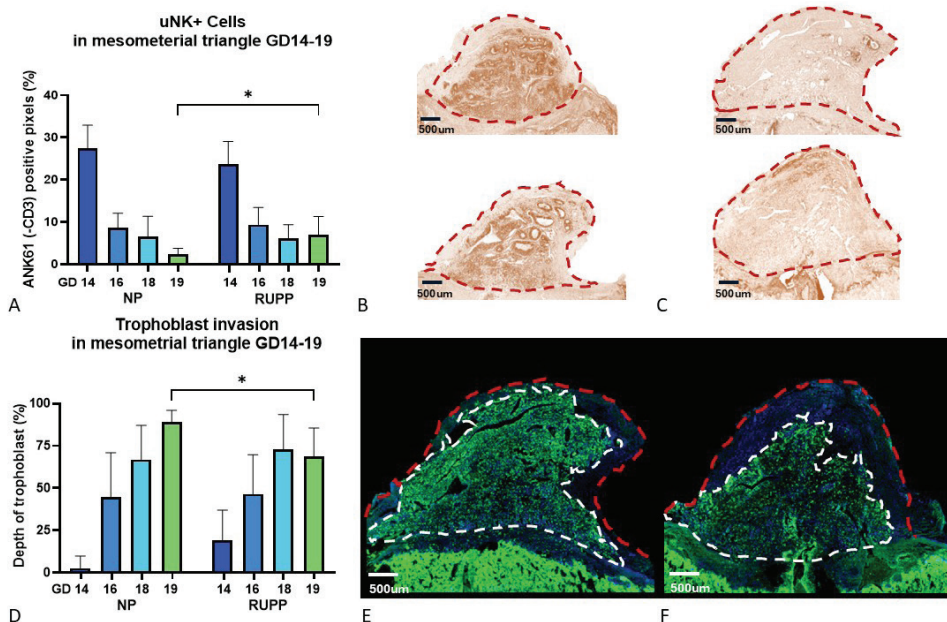


**Figure 2. Analysis of uNK cells and trophoblast invasion in the mesometrial triangle across gestation.** (A) Bar graph showing the percentage of uNK cells (positive ANK-61 pixel area) in the MT at GD14, GD16, GD18, GD19 (no interaction effect  $p=0.07$ ) (B, C) Representative images of uNK cell distribution within the MT at GD14 (B) and GD19 (C), with NP above, and RUPP below. Red dotted lines delineate the MT boundaries (D) Bar graph displaying percentage trophoblast invasion depth into the MT across gestational days (GD14, GD16, GD18, GD19, no interaction effect 0.09). (E, F) Representative image of trophoblast invasion in the MT at GD19 in NP (E) and RUPP (F), from trophoblast staining, pan-cytokeratin, green; nuclei, DAPI blue. Red dotted lines delineate the MT boundaries; the white dotted line delineates trophoblast cloud. Data are presented as mean  $\pm$  SD. Asterisks indicate statistically significant differences of each main effect (\*  $p<0.05$ ). uNK, uterine natural killer cell; MT, mesometrial triangle; NP, normal pregnant; RUPP Reduced uterine perfusion pressure; GD, gestational day.

### Spiral artery remodeling in mesometrial triangle

Spiral artery remodeling in the MT progressed over gestation in NP (**Figure 3**). At GD14, most spiral arteries in NP group showed little to no remodeling (**Figure 3A**). In contrast, in the RUPP group, a shift to slightly remodeled arteries was visible. By GD16 and GD18, NP placentas showed clear signs of remodeling progression, with fewer unremodeled arteries (**Figure 3B, C**). While RUPP animals showed a delayed progression at GD16 and 18, where arteries at GD16 remained largely unremodeled. At GD19, this difference became more pronounced (**Figure 3D**). While NP placentas showed advanced remodeling (16% unremodeled and 37% highly remodeled), the RUPP group showed a large proportion of unremodeled and less highly remodeled spiral arteries (42% unremodeled, 16% highly remodeled).

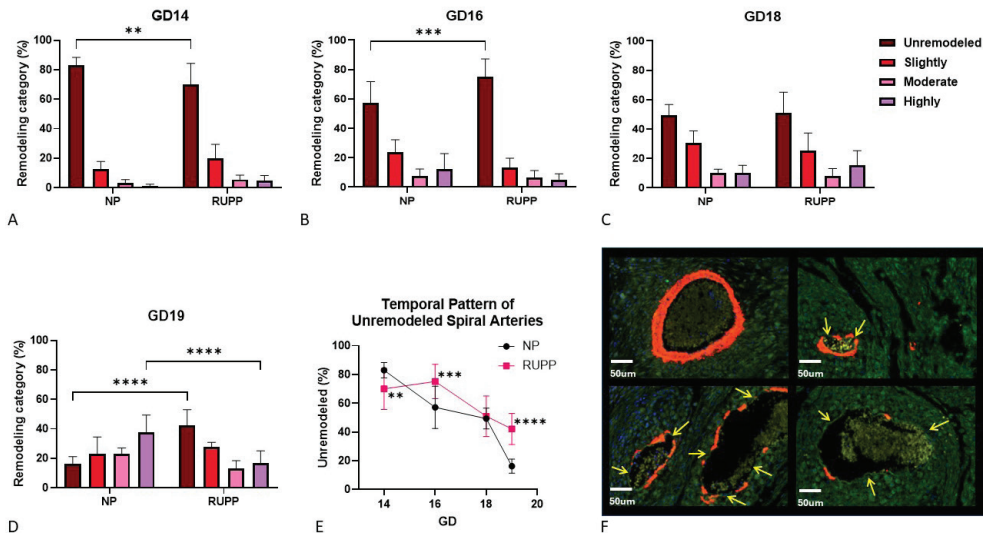


**Figure 3. Spiral artery remodeling in the mesometrial triangle across gestation.** (A–D) Spiral artery remodeling scores within the mesometrial triangle at GD 14(A), 16(B), 18(C), and 19(D).  $\alpha$ -SMA (alpha-smooth muscle actin) staining highlights vascular smooth muscle. Showing temporal loss of  $\alpha$ -SMA as an indicator of spiral artery remodeling. (E) Temporal pattern in the number of unremodeled spiral arteries per group from GD14 to GD19. (F) Representative examples of spiral artery remodeling categories based on  $\alpha$ -SMA staining: upper left unremodeled (0–3%  $\alpha$ -SMA disappearance), upper right slightly remodeled (3–33%  $\alpha$ -SMA disappearance), lower left moderately remodeled (33–67%  $\alpha$ -SMA disappearance), and lower right highly remodeled (67–100%  $\alpha$ -SMA disappearance). Data are presented as mean  $\pm$  SD. Asterisks indicate statistically significant differences \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , Respectively. MT, mesometrial triangle;  $\alpha$ -SMA, alpha-smooth muscle actin; NP, normal pregnant; RUPP, Reduced uterine perfusion pressure; GD, gestational day.

## Discussion

This study aimed to characterize the structural and cellular adaptations of the RUPP rat placenta throughout gestation, focusing on placental morphology, uNK cell presence, trophoblast invasion, and spiral artery remodeling. Our findings demonstrate that despite relatively preserved gross placental architecture and weight, RUPP causes significant time-dependent alterations in uNK numbers and impaired trophoblast invasion, leading to incomplete spiral artery remodeling from mid to late gestation in the rat placenta.

### Fetal and placental growth and the placental macrostructural architecture

Fetal weight remained comparable between groups until GD19, suggesting that RUPP-induced growth restriction may manifest only in late gestation or that compensatory mechanisms preserved fetal growth in mid-gestation. Placental weight was reduced at GD18, likely due to the placenta's increased vulnerability to ischemia during its rapid growth phase. By GD19, placental growth naturally plateaus in rats, which may explain why weight differences are no longer apparent [26]. The major placental structural organization remains intact, despite compromised placental perfusion. Studies using the RUPP model that report PE and FGR phenotypes have shown inconsistent findings regarding placental size in late gestation. These discrepancies likely reflect differences in the timing of RUPP induction and methodological variations between studies [27–29]. In human FGR placentas, a reduction in chorionic villi volume is observed, the human equivalent of the LZ [30]. The inconsistency in placental size findings, even in studies showing evident PE and FGR phenotype, indicates that functional placental insufficiency may occur without obvious macrostructural changes. Earlier reports demonstrated elevated placental and circulating sFlt1 in RUPP rat placenta [31–35]. Although tissue collection timing and sFlt-1 measurement methodology were comparable to previous studies, we did not observe increased placental sFlt-1, and circulating sFlt-1 displayed inter-animal variability. Notably, other groups have also reported no significant differences in placental sFlt-1 and substantial heterogeneity in circulating sFlt1 [17,36–38]. Implying that this outcome may be variable and possibly influenced by experimental conditions such as strain background, severity of placental ischemia, or methodological differences. This suggests that increases in this anti-angiogenic factor may not be a consistent feature of the RUPP model.

### Role of uNK-trophoblast interactions in vascular remodeling.

Our data revealed delayed uNK cell disappearance from the MT of RUPP placentas, accompanied by shallow end-gestational trophoblast invasion. These findings suggest impaired temporal coordination of key placental remodeling events under reduced perfusion, likely reflecting altered dynamics in the uNK cells and trophoblasts. This is also apparent from the persistent presence of perivascular uNK cell clusters around unremodeled spiral arteries in this model.

uNK cells support trophoblast invasion, while trophoblasts help regulate uNK cell behavior, both contributing to effective spiral artery remodeling. Therefore, our results may reflect a delayed withdrawal of uNK cells limiting trophoblast migration, or impaired trophoblast migration reducing the retreat of uNK cells <sup>[39,40]</sup>. We observed that such disruptions in uNK cell and trophoblast cell dynamics may result from direct effects of ischemia or secondary responses triggered by hypoxia or inflammation. We hypothesize that delayed uNK cell withdrawal plays a major role, since RUPP-induced placental ischemia has been shown to elevate local HIF-1 $\alpha$  levels and pro-inflammatory cytokines, potentially impairing uNK cell apoptosis or promoting dysregulation of other immunomodulatory cells, such as macrophages <sup>[14,41,42]</sup>.

Notably, previous work has demonstrated a significant increase in total and cytolytic NK cell populations in both the placenta and systemic circulation of RUPP rats, suggesting that ischemia promotes NK cell proliferation and shifts their activity toward a cytotoxic phenotype <sup>[43–46]</sup>. Importantly, heightened pro-inflammatory T-cell activity, particularly Th17 and CD4<sup>+</sup> subsets, has been shown to further amplify this cytotoxic shift by releasing cytokines such as IL-17, IFN- $\gamma$ , and TNF- $\alpha$ , which enhance NK cell activation and impair their normal vascular remodeling function. This T cell and NK cell activation contributes to our observation of impaired spiral artery remodeling. Our data show that spiral arteries in RUPP rats remain largely unremodeled at GD18/19, in contrast to the extensive remodeling observed in NP pregnancies, where most arteries have lost smooth muscle actin (SMA) and undergone physiological dilation. This presence of unremodeled arteries contributes to reduced uteroplacental perfusion and placental ischemia, a key feature of the RUPP model.

### **Impaired vascular remodeling: RUPP Compared to Animal Models and Humans**

The RUPP model demonstrates broader and more consistent disruption of placental vascular development compared to other placental insufficiency models, making it a more relevant model for studying human placental vascular adaptation. For instance, the ATP-induced albuminuria model shows less pronounced effects on spiral artery remodeling or trophoblast invasion <sup>[23]</sup>. Similarly, the transgenic model (hAngiotensinogen x hRenin), while reflecting aspects of gestational hypertension and some vascular remodeling, shows divergent patterns of trophoblast invasion compared to the human situation <sup>[47]</sup>. In mice, RUPP has been shown to cause altered trophoblast differentiation and reduced pericyte coverage in the placental labyrinth <sup>[28]</sup>. However, mice lack deep trophoblast invasion, a feature that is present in rats and more closely resembles the human placental structure. The rat RUPP model shows characteristics of uNK cell dynamics, trophoblast invasion, and spiral artery remodeling that are similar to the characteristics in human preeclampsia, and these processes are still actively ongoing at the time of RUPP induction (mid-gestation), allowing evaluation of these processes during

an active phase of placental remodeling <sup>[47]</sup>, and thus seems to be an excellent model to further study dysfunctional placentation in preeclampsia.

### Strengths and limitations

A key strength of this study is the comprehensive assessment of placental morphology and vascular remodeling across multiple gestational timepoints. By evaluating uNK cell localization and trophoblast invasion in parallel, and following spiral artery remodeling, we provide a detailed temporal picture of how immune and trophoblast elements respond to placental ischemia. Furthermore, the use of the MT in our histological analyses enabled us to evaluate a maternal compartment of the placenta that is often overlooked in rodent studies.

Several limitations should be acknowledged. Inevitably, histological analyses could not be performed on the same subject across timepoints, wherefore comparisons between different animals in a model with inherent variability may have influenced the strength of observed differences. Secondly, spiral artery remodeling was assessed using a blinded categorical scoring system rather than quantitative absolute measurements, which may affect the accuracy of comparisons between groups. Because of technical challenges, objective quantitative methods are not yet widely standardized for analyses of spiral artery remodeling, making categorical assessment a commonly used approach in preclinical spiral artery remodeling. Thirdly, the reliance on two-dimensional histological evaluation may not fully capture the three-dimensional complexity of placental architecture and vascular remodeling. Lastly, sex-specific effects were not evaluated, which may contribute to variability in placental development.

### Conclusion and Future Directions

We demonstrated that the RUPP model induces uNK cell retention, shallow trophoblast invasion, and reduced spiral artery remodeling, even in the presence of preserved gross placental structure. These observations support the relevance of the RUPP model for studying mid-gestational placental adaptation to ischemia in the rat. However, the timing difference between the rat and human remains an important consideration for translation. While the overarching mechanisms of placental invasion align between the human and rat, the earlier completion in humans warrants caution in directly extrapolating findings <sup>[48]</sup>.

Future studies should incorporate molecular analyses, including profiling of immune mediators and cellular populations, to elucidate the mechanisms underlying disrupted uNK cell dynamics and impaired trophoblast invasion in the RUPP placenta. Moreover, incorporating 3D imaging techniques could enhance spatial resolution and provide a more comprehensive understanding of placental vascular alterations.

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## Supplementary material



### The ARRIVE guidelines 2.0: author checklist

#### The ARRIVE Essential 10

These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

| Item                                    | Recommendation                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | Section/line number, or reason for not reporting                                                                                      |
|-----------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------|
| <b>Study design</b>                     | 1 For each experiment, provide brief details of study design including: <ol style="list-style-type: none"> <li>The groups being compared, including control groups. If no control group has been used, the rationale should be stated.</li> <li>The experimental unit (e.g. a single animal, litter, or cage of animals).</li> </ol>                                                                                                                                                                                                                                                           | Material and methods<br>animals<br><br>Material and methods, Statistical analysis                                                     |
| <b>Sample size</b>                      | 2 <ol style="list-style-type: none"> <li>Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used.</li> <li>Explain how the sample size was decided. Provide details of any <i>a priori</i> sample size calculation, if done.</li> </ol>                                                                                                                                                                                                                                                | Material and methods, euthanasia and organ collection<br><br>not done priori                                                          |
| <b>Inclusion and exclusion criteria</b> | 3 <ol style="list-style-type: none"> <li>Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established <i>a priori</i>. If no criteria were set, state this explicitly.</li> <li>For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so.</li> <li>For each analysis, report the exact value of <i>n</i> in each experimental group.</li> </ol> | Material and methods in Dropouts and exclusion criteria<br><br>Material and methods Dropouts and exclusion criteria<br><br>per figure |
| <b>Randomisation</b>                    | 4 <ol style="list-style-type: none"> <li>State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence.</li> <li>Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.</li> </ol>                                                                                                                                                  | Material and methods Induction of PE/FGF model<br><br>Material and methods, animals                                                   |
| <b>Blinding</b>                         | 5 Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).                                                                                                                                                                                                                                                                                                                                                                                              | Material and methods, animals and analyses                                                                                            |
| <b>Outcome measures</b>                 | 6 <ol style="list-style-type: none"> <li>Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes).</li> <li>For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.</li> </ol>                                                                                                                                                                                                                                                                              | Material and methods<br><br>not done priori                                                                                           |
| <b>Statistical methods</b>              | 7 <ol style="list-style-type: none"> <li>Provide details of the statistical methods used for each analysis, including software used.</li> <li>Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.</li> </ol>                                                                                                                                                                                                                                                                              | Material and methods, Statistical analysis<br><br>Material and methods, Statistical analysis                                          |
| <b>Experimental animals</b>             | 8 <ol style="list-style-type: none"> <li>Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight.</li> <li>Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.</li> </ol>                                                                                                                                                                                                         | Material and methods, Animals<br><br>Animals                                                                                          |
| <b>Experimental procedures</b>          | 9 For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including: <ol style="list-style-type: none"> <li>What was done, how it was done and what was used.</li> <li>When and how often.</li> <li>Where (including detail of any acclimatisation periods).</li> <li>Why (provide rationale for procedures).</li> </ol>                                                                                                                                                                                                  | Material and methods<br><br>Material and methods<br><br>Material and methods                                                          |
| <b>Results</b>                          | 10 For each experiment conducted, including independent replications, report: <ol style="list-style-type: none"> <li>Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range).</li> <li>If applicable, the effect size with a confidence interval.</li> </ol>                                                                                                                                                                                                                                        | Material and methods, Statistical analysis and per figure or table<br><br>not applicable                                              |

Table S1. ARRIVE guidelines

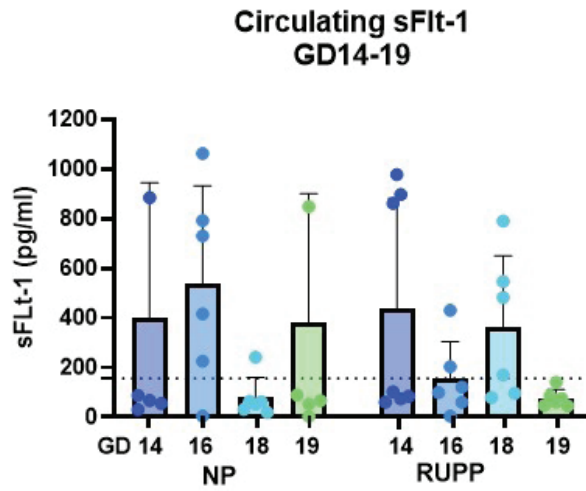


**Figure S1.** Circulating sFlt-1 in plasma (pg/mL) for NP and RUPP dams at GD14, 16, 18, and 19. Data are depicted as mean  $\pm$  SD; circles are individual dams. Measurements  $<156$  pg/mL are below the lowest standard (dashed line). Out-of-range readings were excluded. NP, normal pregnant; RUPP, reduced uterine perfusion pressure; GD, gestational day.

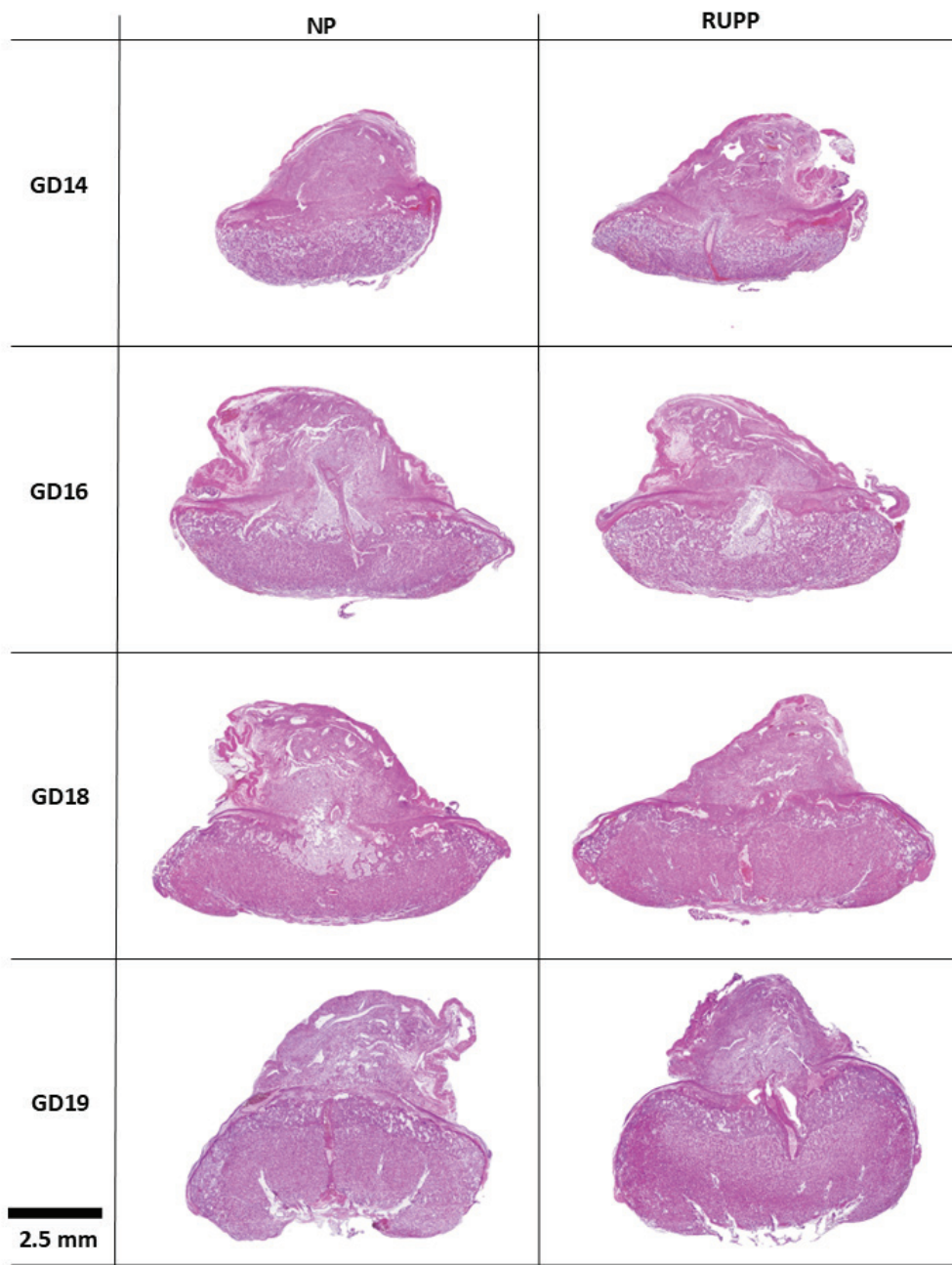


Figure S2. Typical examples HE-stained NP and RUPP placentas across timepoints. NP, normal pregnant; RUPP, Reduced uterine perfusion pressure; GD, gestational day.

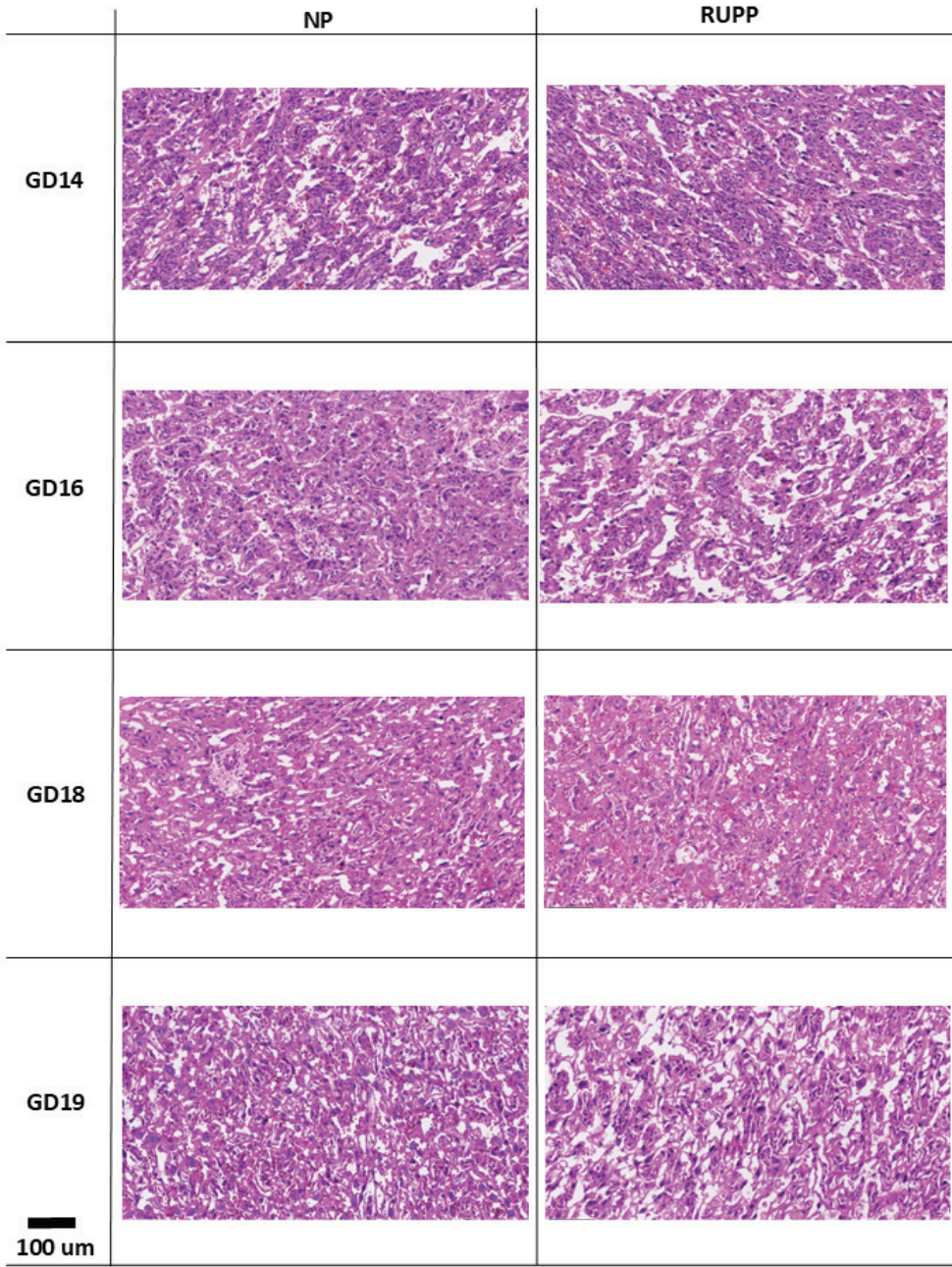


Figure S3. Typical examples HE-stained 20x objective in labyrinth zone of NP and RUPP placentas across time-points. NP, normal pregnant; RUPP, Reduced uterine perfusion pressure; GD, gestational day



# 5

## Chapter

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### Longitudinal 3D-DCE MRI Assessment of Placental Perfusion under Reduced Uterine Perfusion Pressure in Pregnant Rats

*NMR in Biomedicine* 2026 February; 39(4) :e70251. doi: 10.1002/nbm.70251

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## Abstract

Placental insufficiency is a major cause of preeclampsia and fetal growth restriction. The reduced uterine perfusion pressure (RUPP) rat model reproduces cardinal features of these disorders, but longitudinal changes in placental perfusion after RUPP are poorly documented. This study aimed to assess the acute and late effects of RUPP on placental perfusion between gestational day (GD) 14 and GD18 using 3D dynamic contrast-enhanced magnetic resonance imaging (3D-DCE MRI). Pregnant Sprague Dawley rats ( $n = 31$ ) were randomized to RUPP or normal pregnant (NP) groups on GD14. 3D DCE-MRI scans were performed on GD14, GD15, and GD16 or GD18. Perfusion parameters including time to peak (TTP), maximum enhancement (ME), wash-out percentage (WO%), initial area under the curve (iAUC), and total area under the curve (AUC) were derived from fitted signal–time curves. In addition, exploratory voxel-wise histogram analysis was performed to assess intra-litter variability. At GD14, RUPP placentas showed delayed TTP ( $p < 0.0001$ ), reduced ME ( $p = 0.03$ ), reduced WO% ( $p < 0.0001$ ), and elevated iAUC ( $p = 0.02$ ). Differences persisted for TTP ( $p = 0.02$ ) and WO% ( $p = 0.01$ ) at GD15 but resolved by GD16–GD18. Voxel-wise analysis revealed intra-litter heterogeneity, with 20% of analyzed RUPP placentas deviating from the NP template. These findings indicate that RUPP induces an acute but transient impairment of placental perfusion, with recovery by late gestation. GD14–GD15 emerge as a critical window for detecting acute perfusion impairment and evaluating interventions targeting acute injury.

**Keywords:** DCE-MRI, placental perfusion, placental imaging, RUPP

## Introduction

The placenta is a dynamic organ that facilitates nutrient and oxygen exchange between maternal and fetal circulations and provides essential regulatory support for fetal development. Placental insufficiency, associated with impaired blood flow, underlies major pregnancy complications such as preeclampsia (PE) and fetal growth restriction (FGR) that are associated with preterm birth and significant perinatal morbidity and mortality <sup>[1]</sup>. Currently, no effective treatments for placental insufficiency exist, and clinical assessment relies largely on indirect Doppler ultrasound indices (e.g., uterine and umbilical artery pulsatility and absent/reversed end-diastolic flow) rather than direct measures of placental function <sup>[2]</sup>. These unmet needs require improved monitoring strategies alongside the development and testing of new therapeutic interventions. As direct investigation of pregnancy disorders in humans is limited by ethical constraints and potential risks to mother and fetus, animal models are essential for advancing our understanding of placental function and for preclinical evaluation of therapeutic interventions.

Several animal models have been established to study PE, including approaches based on artery ligation, nitric oxide inhibition, renin-angiotensin system modulation, anti-angiogenic factor exposure, immunological manipulation, and transgenic strategies <sup>[3]</sup>. FGR has been induced through dietary restriction, heat exposure, artery ligation, hypoxia, embolization, or glucocorticoid administration <sup>[3]</sup>. Among these models, the reduced uterine perfusion pressure (RUPP) rat model is widely used because it replicates many hallmarks of human PE, including hypertension, proteinuria, endothelial dysfunction, and FGR <sup>[4]</sup>. The RUPP model is a mechanical model that involves partial clipping of the abdominal aorta to reduce uterine perfusion with additional clipping of the ovarian arteries to limit compensatory flow <sup>[4]</sup>.

The RUPP model has been used to study placental metabolism <sup>[5]</sup>, oxygenation <sup>[6]</sup>, oxidative stress <sup>[7]</sup>, inflammation <sup>[8]</sup>, and potential therapeutic interventions, including tempol <sup>[9]</sup>, sildenafil <sup>[9,10]</sup>, and vitamin D <sup>[11]</sup>. However, functional assessment of placental perfusion in this model is limited. In particular, little is known about how placental perfusion changes throughout gestation following RUPP surgery. Such information is critical to understand pathophysiological adaptations and define windows for therapeutic interventions.

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) enables non-invasive assessment of tissue perfusion using gadolinium-based contrast agents. Although its clinical use in pregnancy is restricted due to potential fetal risks, DCE-MRI is well suited for preclinical studies. We have previously demonstrated that 3D DCE-MRI can assess perfusion in all placentas in utero, highlighting its utility for longitudinal placental studies <sup>[12]</sup>. In that study, measurements were performed at gestational day (GD) 19 only. Notably, we observed no difference in placental perfusion between RUPP and control pregnancies. However, whether this restoration of placental perfusion occurs progressively over time remains unknown.

The objective of this study was therefore to investigate the acute and late gestational effects of RUPP surgery on placental perfusion from GD14 to GD18 using longitudinal DCE-MRI in pregnant rats. We hypothesize that placental perfusion would be impaired immediately after RUPP surgery, but would exhibit partial to full compensatory adaptations at later gestational stages.

## Material and Methods

All experimental procedures were approved by Amsterdam University Medical Centers Animal Ethics Committee and conducted and reported in accordance with the ARRIVE guidelines and European Union guidelines for the welfare of laboratory animals (Directive 2010/63/EU).

### Animals

Eleven to 13-week-old timed pregnant Sprague Dawley rats ( $n = 31$ ) were supplied by Envigo (Envigo RMS B.V, Horst, the Netherlands) at GD8. Animals were housed in pairs in conventional cages under a regular 12 h light/dark cycle, at a constant room temperature (22°C) and humidity (50%) with free access to food and water. A five-day acclimation period preceded the experiment. To minimize confounders, cage positions and the daily order of imaging were randomized.

### RUPP Procedure

Pregnant dams were randomly assigned by simple randomization to either the RUPP or normal pregnant (NP) group. The RUPP procedure was performed as previously described<sup>[12]</sup>. Briefly, animals were put under isoflurane anesthesia (3% induction, 2–2.5% maintenance), after which a midline laparotomy allowed exposure of the uterine horns. A silver clip (AG000450, Goodfellow Cambridge, Pittsburgh, PA, USA) with dimensions of 15 mm length, 1.5 mm width, and 0.203 mm inner diameter was placed around the abdominal aorta above the bifurcation of the iliac arteries, and two additional clips (10 mm length, 1.5 mm width, and 0.100 mm inner diameter) were placed around the right and left ovarian arteries to reduce uterine perfusion. NP animals were anesthetized for a similar duration but did not undergo surgery. NP animals were used as controls to provide a physiological baseline; prior studies report no differences from sham-operated controls<sup>[13]</sup>. All animals received carprofen (5 mg/kg, subcutaneous) 30 min before surgery and every 12 h for 24 h post-operatively. After surgery, dams were housed individually until sacrifice. Post-operatively, blinding of both animal care staff and researchers was not feasible because surgical wounds were visible on the animals' bodies. During image analysis, blinding was also compromised by clip-related artifacts on MRI scans.

## MRI Protocol

MRI was performed with a 7.0 T scanner (MR Solutions Ltd., Guildford, UK) under isoflurane anesthesia (3% for induction, 1%–2% for maintenance) using a quadrature rat body volume coil. Body temperature (36 °C –37 °C) and respiration (40–50 breaths/min) were continuously monitored and kept constant. After short initial scout scans, 3D DCE MRI was performed using a 3D RF-spoiled gradient-echo sequence with the following parameters: TR/TE = 8.0/2.0 ms, flip angle = 25°, FOV = 60 × 60 × 60 mm<sup>3</sup>, and matrix = 128 × 96 × 96. No respiratory triggering was performed. Data were acquired for 1 min prior to and 11 min after intravenous administration of Dotarem (0.025 mmol, 0.5mM Gd, Guerbet) via a tail vein catheter (3 ml/min) using a CHEMYX Inc., Fusion100 syringe pump. To facilitate retrospective high-temporal-resolution reconstructions, data were acquired using a pseudo-radial ky/kz-trajectory with a golden-angle increment of 16.95°. Raw data were processed using a publicly available in-house built MATLAB application<sup>[14]</sup>. The 12 min:22 s acquisition was binned into 60 time-frames of 12s:367ms/ frame. Image reconstruction was performed using the BART toolbox<sup>[15]</sup> with compressed sensing (BART pics command), employing wavelet regularization ( $\lambda = 0.001$ ) and temporal total variation regularization ( $\lambda = 0.1$ ).

Each animal underwent three MRI sessions (**Figure 1**): on GD14 immediately after surgery, GD15, and either GD16 or GD18. Animals were recovered after each imaging session, except after the final scan, when they were sacrificed.

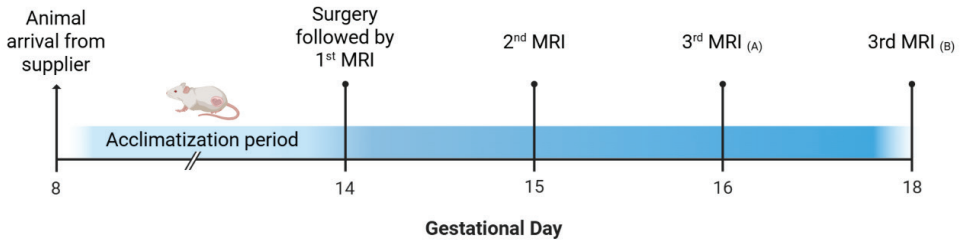


**Figure 1. Study timeline.** MRI sessions were performed on gestational day (GD)14 (surgery day), GD15, and either (A) GD16 or (B) GD18 following animal arrival on GD8 and a 5-day acclimatization period.

## Conceptus Measurements

Maternal weight was recorded before each imaging session. Dams were euthanized by isoflurane overdose (5%) after the final MRI. Viable and reabsorbed fetuses were counted; placental and fetal weights were measured for each viable fetus. At GD18, one to two fetuses per litter were dissected for measurement of fetal brain and liver weight; this was not feasible at GD16 due to their small size. Following the measurements, all fetuses were sacrificed by decapitation.

## Image Analysis

### *Region of Interest (ROI)-based DCE Analysis*

ROIs on a single sagittal slice per placenta were manually drawn in 3D Slicer (<http://www.slicer.org>)<sup>[16]</sup>. Signal–intensity time curves were converted to percent enhancement relative to baseline. These DCE curves were fitted using nonlinear least-squares optimization (MATLAB, lsqnonlin). Because the DCE profiles varied substantially in shape, three candidate models were tested for each curve: rise with exponential decay, double-sigmoid, and generalized gamma. Signal- and time-based weighting was applied to emphasize the enhancement peak, and the best model was selected based on the lowest root-mean-square error.

Fitted curves were then used for semi-quantitative assessment of perfusion parameters: time to peak (TTP), initial area under the curve until the peak (iAUC), total area under the curve (AUC), maximum enhancement (ME), and wash-out percentage (WO%) as illustrated in **Figure 2**. Placentas with substantial motion artifacts or associated with non-viable fetuses, identified by clearly reduced size, were excluded.

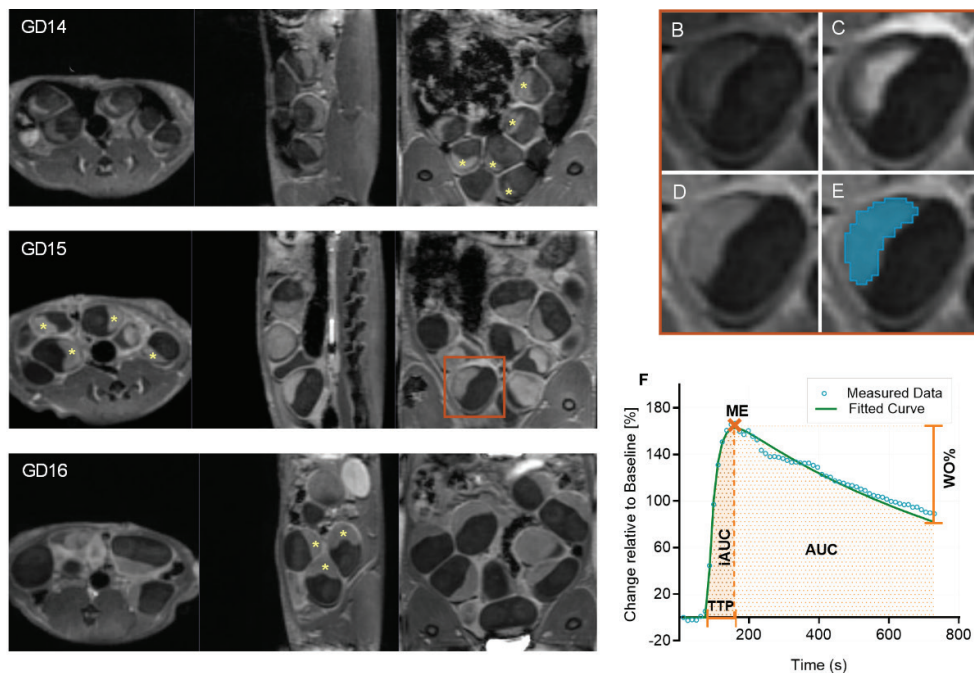


**Figure 2. DCE-MRI analysis workflow.** (A) Representative 3D DCE-MRI datasets of the rat placenta at gestational day (GD) 14, GD15, and GD16, displayed in three orthogonal planes. Placentas are indicated with asterisks. (B–E) Example of a single placenta across different phases: (B) precontrast baseline, (C) maximum enhancement, (D) wash-out phase, and (E) segmentation. (F) Signal intensity–time curve illustrating measured data (dots) and fitted curve (line). Semiquantitative parameters derived from the curve fit include time to peak (TTP), initial area under the curve to TTP (iAUC), total AUC (AUC), maximum enhancement (ME), and wash-out percentage (WO%).

### ***Voxel-Wise DCE Analysis***

In addition to the ROI-based analysis, we also performed an exploratory voxel-wise analysis in four placentas per scan at GD15. To this end, full 3D manual segmentations were performed, selecting two placentas from the upper and two from the lower uterine regions to capture potential spatial variability. Some placentas were excluded when small litter size or motion artifacts prevented segmentation.

From each 3D segmentation, voxel-wise total AUC values were extracted. As an initial voxel-wise summary, placenta-level mean and median voxel-wise AUC values were computed from the full voxel distributions and compared between NP and RUPP using linear mixed-effects models with dam as a random intercept.

To assess distributional differences beyond averaging, voxel-wise total AUC values were converted into normalized histograms (100 bins, range 0–350,000) to account for differences in placenta size. A reference histogram template was generated by averaging normalized NP histograms. Jensen–Shannon distance (JSD) was calculated to quantify divergence of individual RUPP placentas from the NP template<sup>[17]</sup>, with higher JSD values indicating greater deviation.

### **Exclusion Criteria**

Exclusion criteria were pre-specified before data collection to reduce heterogeneity. Animals or datasets were excluded for: total fetal resorption and substantial motion artifacts precluding reliable DCE analysis. Criteria were applied across groups and GDs.

### **Statistical Analysis**

Statistical analyses were performed in RStudio (version 4.2.0; R Core Team, Vienna, Austria). Continuous variables were assessed for normality using the Shapiro–Wilk test. For comparing fetal organ weights at GD18, the nonparametric Wilcoxon rank-sum exact test was used. For measurements that concerned the final time points only (resorption rate and maternal weight gain), two-way ANOVA was used when ANOVA assumptions were met; otherwise, a non-parametric factorial ANOVA using the aligned rank transform test was applied. Comparisons between RUPP and NP fetal parameters at multiple time points (placental weight, fetal weight, and perfusion parameters) were conducted using a multilevel mixed-effects model to account for fetal nesting within litters, provided all model assumptions were met. A two-sided p-value < 0.05 was considered statistically significant. Voxel-wise histogram analyses were performed in Python (version 3.13, Python Software Foundation, Wilmington, DE, USA). Graphs were created in GraphPad Prism (version 9.1.0, GraphPad Software, San Diego, CA, USA).

## Results

### Maternal Outcomes

The percentage of fetal resorption did not differ by group or GD and there was no group  $\times$  GD interaction ( $p = 0.87$ ). Maternal weight gain differed between groups, with RUPP dams gaining less weight than NP dams overall ( $p = 0.04$ ) and increased with gestational age in both groups ( $p < 0.001$ ), but without group  $\times$  GD interaction ( $p = 0.25$ ).

### Fetal and Placental Morphometric Outcomes

**Table 1** presents descriptive statistics for maternal, fetal, and placental outcomes by group and GD. Placental weight increased with gestational age in both NP and RUPP litters ( $p = 0.01$ ), but without a group  $\times$  GD interaction ( $p = 0.25$ ). Fetal weight increased with gestation in both NP and RUPP litters ( $p < 0.001$ ), without a group  $\times$  GD interaction ( $p = 0.70$ ). Fetal-to-placental weight ratio also increased with gestational age in both groups ( $p < 0.0001$ ), without a group  $\times$  GD interaction ( $p = 0.28$ ). At GD18, fetal brain and liver weights were similar between NP and RUPP fetuses ( $p = 0.54$  and  $0.22$ , respectively), but the brain-to-liver weight ratio was higher in RUPP fetuses ( $p = 0.038$ ).

|                                     | NP              |                 | RUPP            |                 |
|-------------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                     | GD16<br>(n = 7) | GD18<br>(n = 8) | GD16<br>(n = 8) | GD18<br>(n = 7) |
| Resorptions (%)                     | 8 (12)          | 4 (15)          | 27 (38)         | 15 (42)         |
| Maternal weight gain after GD14 (g) | 4 $\pm$ 7       | 20 $\pm$ 15     | -9 $\pm$ 9      | 16 $\pm$ 13     |
| Placental weight (mg)               | 417 $\pm$ 71    | 519 $\pm$ 110   | 380 $\pm$ 59    | 447 $\pm$ 73    |
| Fetal weight (mg)                   | 538 $\pm$ 100   | 1711 $\pm$ 431  | 510 $\pm$ 122   | 1653 $\pm$ 383  |
| Fetal brain (mg)                    | NA              | 97 $\pm$ 16     | NA              | 93 $\pm$ 17     |
| Fetal liver (mg)                    | NA              | 142 $\pm$ 30    | NA              | 122 $\pm$ 38    |
| FW:PW (ratio)                       | 1.3 (0.4)       | 3.1 (0.9)       | 1.3 (0.4)       | 3.7 (0.8)       |
| FB:FL (ratio)                       | NA              | 0.7 (0.1)       | NA              | 0.8 (0.1)       |

**Table 1: Maternal and fetal outcomes in NP and RUPP groups at GD16 and GD18.** Data are presented as mean  $\pm$  SD or median (IQR); n = number of dams. Abbreviations: GD, gestational day; NA, not available; NP, normal pregnant; RUPP, reduced uterine perfusion pressure.

## DCE-MRI Perfusion Parameters

Seventeen of a total of 93 DCE datasets needed to be excluded in the analysis, primarily due to the presence of fetal motion during acquisition (GD14:  $n = 4$  RUPP and  $n = 2$  NP; GD15:  $n = 5$  NP; GD16:  $n = 1$  RUPP and  $n = 1$  NP; and GD18:  $n = 1$  RUPP and  $n = 1$  NP). Additionally, one RUPP dam was excluded at GD15 and GD18 due to total fetal reabsorption. The final number of animals included in the analysis was as follows: GD14,  $n = 12$  RUPP and  $n = 13$  NP; GD15,  $n = 15$  RUPP and  $n = 10$  NP; GD16,  $n = 7$  RUPP and  $n = 6$  NP; and GD18,  $n = 6$  RUPP and  $n = 7$  NP.

Longitudinal changes in DCE parameters for RUPP and NP animals are illustrated in **Figure 3**. For most semi-quantitative perfusion parameters (TTP, iAUC, ME and WO%), a significant group  $\times$  GD interaction ( $p < 0.001$ ) was found. Specifically, at GD14, RUPP animals exhibited delayed TTP ( $p < 0.0001$ ), higher iAUC ( $p = 0.02$ ), reduced ME ( $p = 0.03$ ), and reduced WO% ( $p < 0.0001$ ) compared to NP. At GD15, differences persisted for TTP ( $p = 0.02$ ) and WO% ( $p = 0.01$ ), with a trend toward higher iAUC values ( $p = 0.06$ ). By GD16 and GD18, no significant differences were observed between groups. Total AUC did not differ between groups across GDs. **Video S1** provides representative DCE-MRI datasets, showing delayed and attenuated enhancement in RUPP at GD14 and normalization by GD16.

## Voxel-Wise Histogram Analysis

Placenta-level mean and median voxel-wise AUC did not differ between NP and RUPP at GD15 (mean: estimate  $-16,186$ ,  $p = 0.229$ ; median: estimate  $-16,337$ ,  $p = 0.247$ ).

In contrast, histogram-based analysis revealed marked variability in JSD within the RUPP group. Using a threshold defined as the NP mean  $+ 3$  SD, NP placentas clustered clearly below the threshold, whereas JSD values in the RUPP group showed marked intralitter variability, with some resembling NP and others deviating substantially (**Figure 4**). Overall, 20% of RUPP placentas (12 of 60 analyzed) exceeded the threshold. Two NP outliers exceeded this threshold and were excluded, as they corresponded to a single-fetus pregnancy and a dam reassigned from the RUPP group due to intraoperative bleeding.

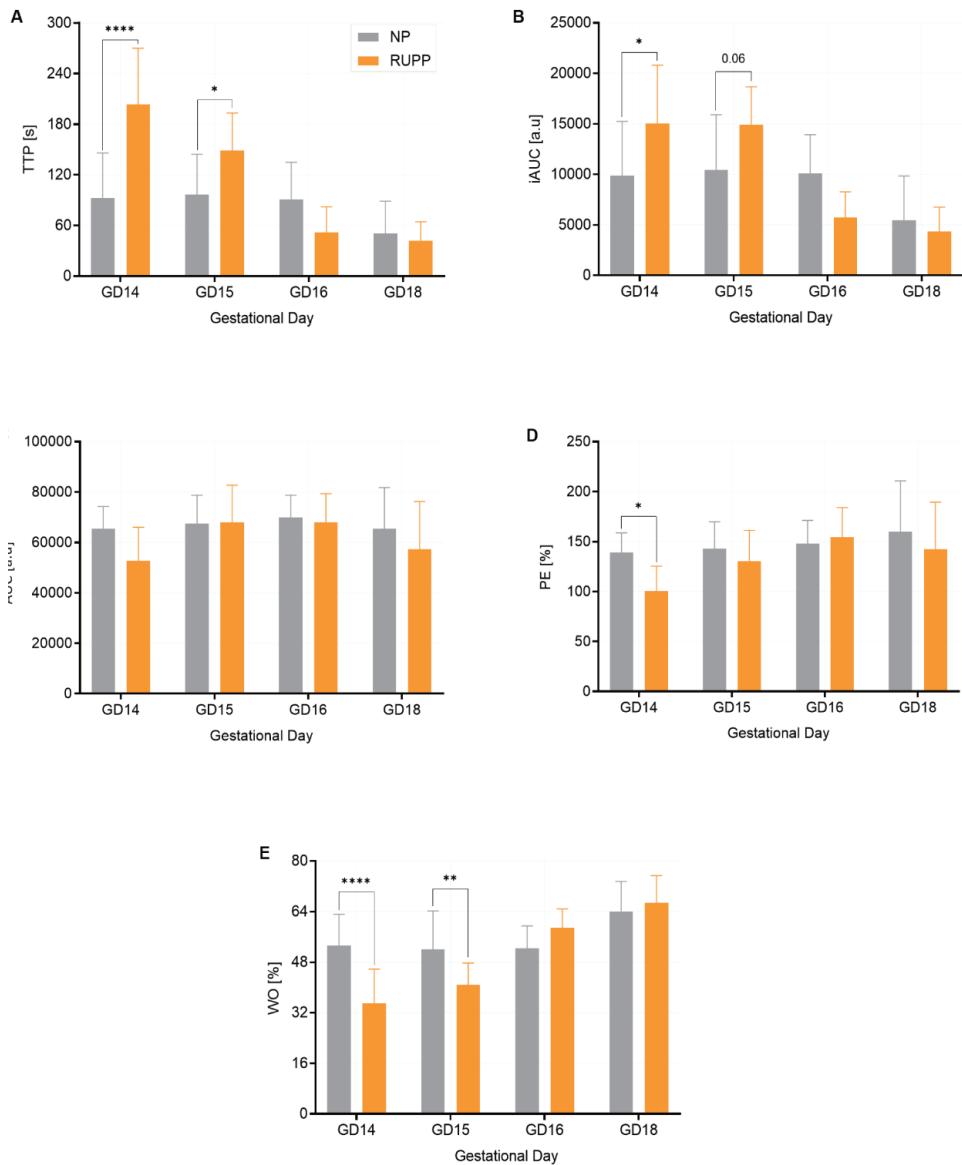


**Figure 3. Semiquantitative DCE-MRI perfusion parameters in RUPP and NP rats.** (A) Time to peak (TTP), (B) initial area under the curve to peak (iAUC), (C) total area under the curve (AUC), (D) maximum enhancement (ME), and (E) wash-out percentage (WO%) measured at GD14, GD15, GD16, and GD18. Data are shown as mean  $\pm$  SD. Asterisks indicate post hoc pairwise comparisons between RUPP and NP at each gestational day (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).

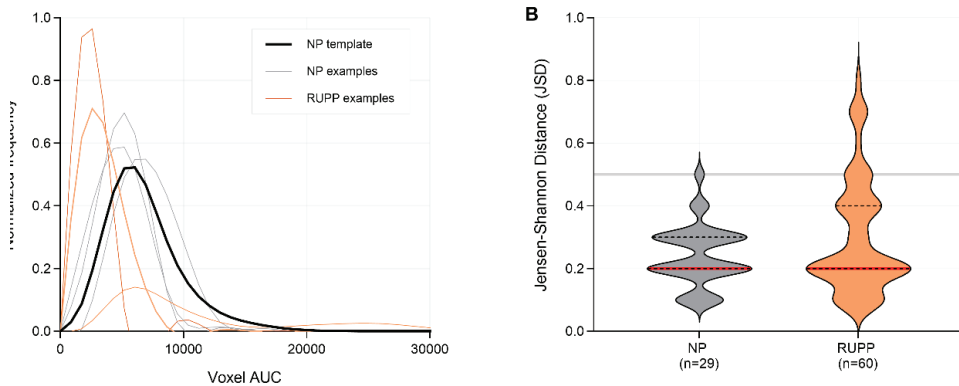


**Figure 4. Voxel-wise histogram analysis of placental perfusion.** (A) Normalized voxel-wise histograms of AUC values showing the NP template (black), representative NP examples (gray), and representative RUPP examples selected from placentas that deviated from the NP template (orange). (B) Violin plots of Jensen-Shannon distance (JSD) relative to the NP template. Median (red) and interquartile range (black) are indicated; the gray horizontal line marks the threshold (mean + 3 SD of NP).

## Discussion

This first application of longitudinal 3D DCE-MRI in the RUPP model demonstrated acute perfusion deficits after RUPP that resolved rapidly with advancing gestation, consistent with the adaptive placental responses to reduced uterine blood flow.

## Maternal and Fetal Outcomes

The observed variability in maternal weight gain and resorption rates highlights the heterogeneous impact of reduced uterine perfusion, consistent with previous reports [13]. Despite a clear early perfusion impairment in all RUPP animals, some RUPP dams maintained normal outcomes, underscoring that the absence of resorptions does not exclude placental ischemia.

Morphometric measures of placental and fetal weights were not different between groups, consistent with earlier work showing variable effects of RUPP on gross morphometry [18,19]. However, the higher brain-to-liver weight ratio observed at GD18 in RUPP fetuses suggests a compensatory brain-sparing response. Fetal brain sparing is a hallmark of placental insufficiency that has been described in both clinical and experimental settings [20,21]. This selective redistribution of growth supports the notion that, even in the absence of overt differences in fetal or placental mass, subtle adaptive responses can be detected in the RUPP model.

## Placental Perfusion Dynamics

The acute changes in perfusion parameters at GD14 indicate a coherent physiological response to RUPP. Delayed TTP, reduced ME, and reduced WO% reflect reduced inflow and impaired clearance of contrast, consistent with restricted uteroplacental conductance. At the same time, the increase in iAUC despite unchanged total AUC suggests slow vascular transit and prolonged contrast retention, rather than a reduction in overall perfusion volume.

By GD15, partial recovery was evident, with only TTP and WO% remaining abnormal and iAUC trending higher. The normalization of all parameters by GD16–GD18 suggests dynamic adaptation and recovery of perfusion. Together, these semiquantitative measures highlight that early placental ischemia induced by RUPP is transient and recovery involves restoration of flow dynamics without sustained loss of total perfusion capacity. This adaptive response is consistent with laser Doppler findings of reduced uterine blood flow immediately after surgery with partial recovery within 4 days [22]. These findings extend our previous work, where no perfusion differences were observed at GD19 [12], by demonstrating the transient nature of perfusion impairment and subsequent adaptation. The recovery observed here should be interpreted in the context that short-duration DCE acquisitions primarily reflect maternal perfusion as fetal uptake of gadolinium contrast in murine has been reported only after  $\geq 26$  min postinjection [23,24]. Thus, although our results indicate restoration of maternal blood supply, they do not exclude persistent fetal-side compromise. This interpretation is consistent with our earlier T2\* MRI study, which revealed reduced placental oxygenation despite unaltered perfusion, suggesting that maternal–fetal exchange rather than maternal inflow may remain impaired [12].

## Semiquantitative DCE-MRI: Strengths and Limitations

Semiquantitative DCE-MRI analysis provides a physiologically relevant approach to measure perfusion independently of invasive or model-based pharmacokinetic assumption [25]. Parameters like TTP, AUC, ME, and WO% reflect perfusion dynamics and efficiency but do not provide absolute physiological values. In this study, semiquantitative parameters were sufficient to capture acute alterations in perfusion after RUPP surgery and to follow their temporal evolution. This approach allowed relative comparisons between groups and across gestational stages, although it should be recognized that it cannot disentangle contributions of blood flow, blood volume, and permeability in the way quantitative modeling might.

Compared with fully quantitative analyses, which require advanced AIF modeling and can be challenging to apply in small animals with rapid circulation, semiquantitative analysis offers a practical compromise. Although it lacks absolute specificity, it enables robust relative assessment and longitudinal monitoring, which was the primary objective of this study.

## Voxel-Wise Histogram Analysis of Intralitter Variability

Placenta-level mean and median voxel-wise AUC did not demonstrate a consistent group-level shift at GD15, indicating that voxel-wise averaging alone does not separate NP from RUPP at this time point. Voxel-wise histogram analysis was therefore used as an exploratory approach to capture distributional differences and within-litter variability that may not be reflected by average AUC values.

Two NP placentas initially exceeded the JSD threshold and were excluded as both were associated with specific biological or procedural factors. Their exclusion ensured a robust NP reference template, while also highlighting the sensitivity of voxel-wise analysis to detect atypical perfusion patterns.

Within RUPP litters, many placentas maintained NP-like perfusion profiles, whereas only 20% showed clear deviations. This heterogeneity likely reflects differences in vascular remodeling or positional effects along the uterine horn. Although exploratory, this voxel-wise histogram may therefore help distinguish “resilient” from “compromised” placentas, underscoring its value as a complement to conventional ROI methods for characterizing within-litter variation.

## Implications for Timing of Assessment and Intervention

The longitudinal design of this study enabled a comprehensive evaluation of placental perfusion using repeated 3D DCE-MRI in the RUPP rat model. The impact of reduced uterine perfusion was strongly time dependent. The transient impairment and subsequent recovery underscore the importance of gestational timing when assessing placental function. The early window at GD14–GD15 appears most relevant for interventions targeting the acute perfusion deficit induced by RUPP. However, we do not exclude benefit from later interventions aimed at secondary injury cascades including the reduced placental oxygenation at GD19 in this model [12] and the higher brain-to-liver ratio we observed at GD18. Thus, although early measures may help preserve fetuses otherwise at risk of resorption, later therapies may still modify downstream pathology and growth trajectories. Finally, because RUPP imposes an abrupt reduction in uterine blood flow whereas human placental insufficiency typically evolves more gradually, these timing considerations should be factored into the design, interpretation, and translation of therapeutic studies using the RUPP model.

## Limitations

This study has several limitations that should be considered when interpreting the findings. First, the follow-up period was restricted to 3 days due to animal welfare considerations, which limited our ability to assess longer-term adaptations (e.g., at GD19–GD21). In addition, repeated anesthesia across sessions may have introduced confounding effects, although no overt

adverse outcomes were observed. A further methodological consideration is that, to minimize the impact of multiple contrast injections within the same day, group comparisons at GD14 were made between RUPP and NP animals rather than within the same animal presurgery and postsurgery. This approach constrained within-animal baseline assessments but still revealed a clear reduction in perfusion after surgery. Additionally, the voxel-wise analysis required highly time-consuming manual segmentation, limiting the number of placentas used for this analysis. This may be facilitated in future studies by the use of AI-based segmentation algorithms. Finally, the short acquisition window means that the results primarily reflect materno-placental perfusion and provide limited insight into the fetal circulation. Pairing DCE-MRI with oxygenation measures from T2\* MRI and photoacoustic imaging may provide a more complete picture of both maternal and fetal contributions to placental function.

## Conclusion

This study demonstrates that longitudinal 3D DCE-MRI can detect acute placental perfusion impairment immediately after RUPP surgery, followed by recovery at later gestational stages. These findings provide new insights into the temporal dynamics of placental adaptation to reduced uterine blood flow and identify an early window for potential therapeutic intervention.

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## Supplementary material

**Video S1.** 3D DCE-MRI of representative placentas from NP and RUPP dams at GD14 and GD16. At GD14, RUPP placentas show delayed and attenuated enhancement relative to NP; at GD16, enhancement is similar to NP, indicating recovery. For display, a single slice from the 3D volume is shown capturing most placentas. **Link:** [bit.ly/Supplementary\\_S1](https://bit.ly/Supplementary_S1)

# 6

## Chapter

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### Multimodality Imaging Assessment of Placental Function in a Rat Model of Reduced Uterine Perfusion Pressure

*In preparation*

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## Abstract

**Introduction:** Placental insufficiency underlies major pregnancy complications such as preeclampsia and fetal growth restriction, yet clinical surveillance relies on indirect ultrasound and Doppler surrogates rather than direct measures of placental perfusion and oxygenation. In the reduced uterine perfusion pressure (RUPP) rat model, placental remodeling deficits are established by mid-gestation, but functional imaging biomarkers remain incompletely characterized at this stage. We therefore performed a multimodality imaging study at gestational day (GD) 16 to assess placental oxygenation and perfusion in RUPP versus normal pregnant (NP) rats using MRI, contrast-enhanced ultrasound, and photoacoustic imaging (PAI).

**Methods:** Pregnant rats were allocated to RUPP and NP groups and imaged at GD16 using MRI (T2\* mapping and 3D dynamic contrast-enhanced MRI), contrast-enhanced ultrasound (CEUS), and PAI. Primary outcomes were placental oxygenation and perfusion metrics derived from each modality. Secondary outcomes included fetal brain and liver oxygenation assessed using T2\* MRI and PAI. Between-group comparisons were performed for modality-specific metrics, and exploratory correlations were evaluated between MRI, CEUS and PAI readouts.

**Results:** At GD16, placental oxygenation metrics (T2\* and PAI-derived sO<sub>2</sub>) and perfusion metrics (DCE-MRI and CEUS) did not differ between NP and RUPP. Exploratory cross-modality analyses identified significant correlations between CEUS metrics and MRI-derived R2\*: mTT correlated positively with placental fetal-side R2\* ( $r = 0.969$ ,  $p < 0.001$ ) and inversely with fetal liver R2\* ( $r = -0.900$ ,  $p < 0.001$ ), while rBV correlated positively with placental fetal-side R2\* ( $r = 0.800$ ,  $p = 0.005$ ) and inversely with fetal liver R2\* ( $r = -0.818$ ,  $p = 0.004$ ). In addition, fetal brain sO<sub>2</sub> correlated inversely with whole-placenta R2\* ( $r = -0.768$ ,  $p < 0.001$ ) and placental fetal-side R2\* ( $r = -0.666$ ,  $p = 0.005$ ), and positively with fetal liver R2\* ( $r = 0.502$ ,  $p = 0.048$ ).

**Conclusion:** At GD16, multimodality imaging did not reveal detectable NP–RUPP differences in placental perfusion or oxygenation, indicating preserved or heterogeneous functional status at this intermediate stage. These findings highlight gestational timing as a key determinant of detectability and support multimodality imaging as a framework to define sensitive windows and endpoints for mechanistic and therapeutic studies.

**Key words:** Placental perfusion, Placental oxygenation, DCE-MRI, CEUS, photoacoustic imaging, RUPP, Placental imaging

## Introduction

Placental insufficiency underlies major pregnancy complications such as preeclampsia (PE) and fetal growth restriction (FGR) and is a leading contributor to adverse perinatal outcome<sup>1,2</sup>. In current clinical care, management is largely based on surveillance with ultrasound-derived fetal biometry and uterine and umbilical artery Doppler indices, followed by timed delivery when fetal or maternal risk becomes unacceptable<sup>3</sup>. Although these approaches provide valuable information on fetoplacental status, they merely offer indirect insight into placental health and do not quantify key functional determinants of placental exchange capacity, particularly perfusion and oxygenation. This limitation constrains clinical decision-making: it hampers mechanistic interpretation of abnormal surveillance findings, limits the ability to identify pregnancies in which placental function is deteriorating before overt fetal compromise, and ultimately contributes to the central clinical dilemma of balancing the risks of prematurity against ongoing intrauterine hypoxia and stillbirth. These challenges have driven interest in non-invasive imaging biomarkers that can directly quantify placental function *in vivo*.

Animal models play a central role in placental insufficiency research, as they allow pathophysiological studies under controlled conditions. Within these models, noninvasive imaging can quantify placental function longitudinally *in vivo*, complementing conventional end-point measures such as fetal and placental weights and histology. However, despite this potential, functional imaging biomarkers remain poorly implemented in many commonly used pregnancy models, limiting their value for mechanistic studies and for generating clinically translatable biomarkers.

The reduced uterine perfusion pressure (RUPP) rat model is a well-established model that mimics placental insufficiency by mechanically restricting the uteroplacental blood supply<sup>4,5</sup>. We recently showed that, from mid to late gestation (GD16-19), RUPP placentas exhibit persistent abnormalities in key maternal–fetal interface processes including delayed uNK cell migration, reduced trophoblast invasion depth, and delayed spiral artery remodeling<sup>6</sup>. Noninvasive imaging biomarkers could complement these findings by enabling direct *in vivo* assessment of placental oxygenation and perfusion, offering a functional bridge between placental pathology and fetal outcome.

Magnetic resonance imaging offers several quantitative methods to assess placental function. T2\* mapping, which is sensitive to susceptibility effects driven by deoxyhemoglobin, has been used as a biomarker of placental oxygenation in clinical and preclinical studies<sup>7,8</sup>. Complementing this, dynamic contrast-enhanced (DCE) MRI assesses placental perfusion through analysis of contrast agent uptake and wash-out kinetics. DCE-MRI has been applied primarily in rodents and non-humans due to constraints on contrast administration during human pregnancy<sup>9</sup>.

Ultrasound (US) and photoacoustic imaging (PAI) offer additional readouts at high temporal resolution. Contrast-enhanced ultrasound (CEUS) quantifies placental perfusion by analyzing microbubble time–intensity curves and has been used in pregnant rats to derive perfusion-related parameters *in vivo*<sup>10</sup>. In parallel, PAI is an emerging hybrid modality that couples endogenous optical absorption contrast with acoustic detection enabling label-free mapping of hemoglobin oxygenation with high temporal resolution at biologically relevant depths in small animal organs. Integrated into standard preclinical ultrasound systems, PAI fits readily into existing workflows and is well-suited for repeated, non-invasive *in vivo* measurements. PAI estimates oxygen saturation (sO<sub>2</sub>) from the distinct absorption spectra of oxy- and deoxyhemoglobin and has been applied to the RUPP rat model. Using spectral PAI, Lawrence et al. found reduced placental oxygen saturation at GD16 with persistent differences at GD18<sup>11</sup>.

In view of these observations, we designed a multimodality imaging study with placental oxygenation and perfusion at GD16 as the primary outcomes. GD16 was selected as an intermediate stage in this RUPP protocol in which placental remodeling deficits are already established, and prior PAI work suggests that functional oxygenation differences may be detectable at this time point<sup>11</sup>. MRI (T2\* mapping and 3D DCE-MRI), CEUS, and PAI were combined to assess these primary placental readouts within the same experimental setting. As secondary, exploratory outcomes, we evaluated fetal brain and liver oxygenation using both T2\* MRI and PA. Our goal was to test whether modality-specific placental metrics differ between RUPP and normal pregnant (NP) group at GD16. In addition, we performed exploratory correlations between MRI, CEUS, and PAI readouts to evaluate how these readouts relate within this cohort.

## Material and Methods

All animal experiments were reviewed and approved by the Animal Ethics Committee of Amsterdam UMC. Procedures were performed and reported in line with the ARRIVE guidelines and in compliance with European legislation on the protection of animals used for scientific purposes (Directive 2010/63/EU). All animal experiments were reported in accordance with the ARRIVE guidelines wherever possible (**Table S1**).

### Animals

Timed-pregnant Sprague Dawley rats (12–13 weeks old; total *n* = 17) were obtained from Envigo (Envigo RMS B.V., Horst, the Netherlands) at GD8. Dams were pair-housed in conventional cages under controlled environmental conditions (12 h light/dark cycle, 22 °C, 50% humidity) with *ad libitum* access to food and water. Animals were allowed to acclimatize for five days before the start of experimental procedures.

## RUPP procedure

Pregnant dams were assigned unique animal IDs upon arrival and allocated by simple randomization using an ID draw without replacement. IDs were drawn at random and assigned to the RUPP group ( $n = 10$ ) or the NP control group ( $n = 7$ ). Group sizes were selected to be comparable to our previous RUPP MRI study, in which  $T2^*$  mapping was sensitive to detect differences in placental oxygenation<sup>12</sup>. The RUPP surgery was carried out as described previously<sup>12</sup>. Briefly, anesthesia was induced with 3% isoflurane and maintained at 2–2.5%. Following a midline laparotomy to expose the uterine horns, uterine perfusion was reduced by placement of vascular clips. A silver clip (AG000450, Goodfellow Cambridge, Pittsburgh, PA, USA, 15 mm length, 1.5 mm width, 0.203 mm inner diameter) was positioned around the abdominal aorta above the iliac bifurcation, and two additional clips (10 mm length, 1.5 mm width, 0.100 mm inner diameter) were applied to the left and right ovarian arteries. NP controls underwent anesthesia for a comparable duration but did not receive surgical intervention. These animals served as physiological controls for the imaging endpoints of this study. This choice is supported by evidence that the type of control group (normal pregnant versus sham-operated) does not significantly modify key RUPP phenotype outcomes such as maternal blood pressure, fetal weight and fetal reabsorptions<sup>5</sup>.

Peri-operative analgesia consisted of carprofen (5 mg/kg, subcutaneous), administered 30 min prior to surgery and then every 12 h for the first 24 h post-operatively. NP controls received the same carprofen dosing schedule to match peri-procedural handling. After surgery, dams were housed individually until scheduled euthanasia; the same individual housing was applied to NP controls for consistency. Blinding of animal care staff and investigators during the in vivo phase was not feasible due to visible surgical wounds. Image analysis was performed with investigators blinded to group allocation, except for the DCE-MRI datasets in which clip-related susceptibility artefacts could inadvertently reveal treatment status.

## Imaging protocols

Dams were imaged under isoflurane anesthesia (3% for induction, 1.5–2.5% for maintenance). To reduce order-dependent bias, the sequence of MRI and US/PAI was alternated between animals.

### *DCE-MRI*

MRI was conducted with a 7.0T preclinical system (MR Solutions Ltd., Guildford, UK) using a quadrature rat body volume coil. During scanning, respiration (40–50 breaths/min) and body temperature (36–37°C) were continuously monitored and regulated. Following a scout scan, 3D DCE-MRI was acquired using a 3D RF-spoiled gradient-echo sequence ( $TR/TE = 8.0/2.0$  ms, flip angle 25°, FOV  $60 \times 60 \times 60$  mm<sup>3</sup>, matrix  $128 \times 96 \times 96$ ). A 12 min 22 s acquisition

was performed, consisting of 1 min baseline imaging followed by 11 min after intravenous administration of Dotarem (0.025 mmol; 0.5 mM Gd; Guerbet) via a tail vein catheter at 3 ml/min using a syringe pump. To enable retrospective reconstruction at higher temporal resolution, k-space sampling used a pseudo-radial ky/kz trajectory with a golden-angle increment of 16.95°.

Data were processed using a publicly available in-house built MATLAB application<sup>13</sup> (<https://github.com/Moby1971/P2ROUD-v6.2>). The full acquisition was retrospectively binned into 60 temporal frames (12.367 s per frame) and reconstructed using the BART toolbox with compressed sensing<sup>14</sup>. Wavelet regularization ( $\lambda = 0.001$ ) and temporal total variation regularization ( $\lambda = 0.1$ ) were applied during reconstruction.

### *T2\*-mapping MRI*

A 2D multi-slice multi-gradient-echo sequence with respiratory gating was used to generate T2\* maps of the placenta and fetal organs. Imaging was performed in different planes with TR = 130 ms and eight echoes (TE range 2.2–17.6 ms, echo spacing 2.2 ms). The field of view was 60 × 60 mm<sup>2</sup> with a matrix size of 192 × 192 (in-plane resolution 0.31 × 0.31 mm<sup>2</sup>). Slice thickness was 1.2 mm with a 0.2 mm interslice gap. A flip angle of 30° and 8 signal averages were used, resulting in a total acquisition time of approximately 3–5 min per scan. Multiple T2\* scans were obtained per animal to cover multiple placentas and fetal organs.

### *CEUS*

Abdominal hair was removed with depilatory cream, and dams were positioned supine on a heated imaging platform (FUJIFILM VisualSonics, Toronto, Canada). CEUS scans were performed using a Vevo 3100 imaging system (FUJIFILM VisualSonics) equipped with an MX201 linear-array transducer. The ultrasound contrast agent, Vevo MicroMarker (FUJIFILM VisualSonics), was reconstituted according to the manufacturer's instructions. For each animal, a 250 µL bolus was injected through a tail vein catheter at 3 ml/min, immediately followed by a continuous infusion of 1750 µL at 0.250 ml/min. Imaging was continued for 1 min to allow the circulating microbubble levels to stabilize. Subsequently two or three placentas were imaged sequentially per animal using a destruction–replenishment protocol consisting of a high mechanical index burst followed by replenishment recording.

### *PAI*

For photoacoustic imaging, we used Vevo 3100 imaging system integrated with a Vevo LAZR-X (FUJIFILM VisualSonics). A generous amount of ultrasound gel was applied to ensure acoustic and optical coupling, and the probe with fiber-optic jacket was positioned approximately 7–10 mm above the skin, within the optical focus. After optimization of photoacoustic gain and time-gain compensation at a single wavelength, multiwavelength imaging in Oxy-Hemo mode was performed at near-infrared wavelengths (750 and 850 nm) to generate hemoglobin

oxygen saturation (sO<sub>2</sub>) maps. For each placenta, a short 3D scan of approximately 30 frames was acquired. For fetal liver and fetal brain, 3D scans of approximately 10 frames were acquired.

Due to practical constraints, it was not feasible to image all placentas within each dam. Specifically, the number of acquisitions was limited by total scan time under anesthesia and by variable acoustic access through the maternal abdominal wall, as some placentas were positioned deeper in the abdomen or had reduced image quality due to suboptimal windows (e.g., bowel gas or shadowing). Therefore, per animal, two to four placentas were imaged, selecting the most superficial placentas with a stable imaging window through the anterior abdominal wall. In addition, two fetal livers and two fetal brains were imaged per animal; in two animals, only one fetal brain was assessed.

### Conceptus measurements

Maternal weight was recorded before surgery (GD14) and before imaging (GD16). Dams were euthanized by isoflurane over-dose (5%) after imaging. Viable and reabsorbed fetuses were counted and placental and fetal weights were measured for each viable fetus. Following the measurements, all fetuses were euthanized by decapitation.

### Exclusion criteria

Exclusion criteria for data analysis were pre-specified before data collection to reduce heterogeneity. Animals or datasets were excluded for: total fetal resorption and substantial artefacts precluding reliable analysis.

### Image analysis

#### *DCE-MRI*

Regions of interests (ROIs) were manually drawn to delineate each placenta on a sagittal plane using 3D Slicer (<http://www.slicer.org>)<sup>15</sup> (**Figure 1A**). Signal intensity-time curves were extracted from these ROIs and normalized to the baseline signal. These DCE curves were fitted using nonlinear least-squares optimization (MATLAB, lsqnonlin). Because the DCE profiles varied between placentas, three heuristically selected parameter models were evaluated for each curve (linear rise with exponential decay, double-sigmoid, and generalized gamma). Signal- and time-based weighting was incorporated in the cost function to emphasize the enhancement peak, and the model with the lowest root-mean-square error was retained for subsequent analysis.

From the fitted curves, several semi-quantitative perfusion parameters were derived: time to peak (TTP), initial area under the curve until the peak (iAUC), total area under the curve (AUC), maximum enhancement (ME), and wash-out percentage (WO%). DCE-MRI acquisitions were successfully obtained in 5 NP and 8 RUPP dams; the remaining dams were not

scanned due to technical issues. A total of 51 NP and 42 RUPP placentas were segmented. Of these, 11 NP and 4 RUPP placentas were excluded due to substantial motion artefacts and/or association with clearly non-viable fetuses, resulting in 40 NP and 38 RUPP placentas included in the final DCE-MRI analysis.

### *T2\*-mapping*

T2\* mapping was successfully acquired in 7 NP and 9 RUPP dams. T2\* maps were generated using publicly available in-house software (<https://github.com/Moby1971/T2mapping-v4.0>). Each pixel was fitted with a mono-exponential decay function. T2\* data were acquired as 2D multi-slice imaging; because each slice could intersect more than one placenta, multiple placentas were often visible within a given imaging plane. The number of slices acquired per dam therefore varied and was adjusted during imaging to provide adequate in-plane coverage of the uterine horns while balancing total scan time.

During acquisition, we targeted 3–4 placentas per dam with clear in-plane delineation. For these placentas, ROIs were manually drawn to cover the whole placenta on the sagittal plane, as well as two visually delineated compartments with relatively higher and lower signal intensity. Subsequently, R2\* ( $1/T2^*$ ) values were extracted from these placentas (28 NP and 32 RUPP placentas). Additional ROIs were drawn in fetal brain and fetal liver, and R2\* values were obtained for 2–4 fetuses per dam (27 NP and 32 RUPP fetal livers; 27 NP and 29 RUPP fetal brains) (**Figure 2B**).

### *CEUS*

CEUS acquisitions with a successful destruction–replenishment protocol were obtained in 4 NP and 7 RUPP dams. At least two placentas were recorded per animal. However, only placentas showing a clear destruction–replenishment response were included for analysis, resulting in 1–3 placentas per dam (9 NP and 14 RUPP placentas). Data were processed using an in-house built MATLAB application (<https://github.com/Moby1971/CEUS-v1.1>). For each placenta, a whole-placenta ROI was manually segmented (**Figure 3**), and time intensity curves were generated. From these curves, the following perfusion parameters were derived: mean transit time (mTT), relative blood volume (rBV), and relative blood flow (rBF).

### *PAI*

PAI was successfully acquired in all dams (7 NP and 10 RUPP). In total, 24 NP and 28 RUPP placentas, 15 NP and 17 RUPP fetal livers, and 15 NP and 15 RUPP fetal brains were included in the analysis. Photoacoustic data with co-registered B-mode images were processed using the manufacturer's software (Vevo LAB, FUJIFILM VisualSonics). Regions of interest were manually delineated for the whole placenta, fetal liver and fetal brain. For placental analysis,

the mean  $sO_2$  value was calculated from four frames of the 3D sequence, whereas fetal liver and fetal brain analyses were based on three frames (first, middle, and last), thereby incorporating information from multiple slices (**Figure 4**).

## Statistical analysis

Statistical analyses were performed in GraphPad Prism (version 9.1.0, GraphPad Software, San Diego, CA, USA). Continuous variables were assessed for normality using Shapiro–Wilk test. Differences between RUPP and NP dams were analyzed using unpaired Student’s t-test in case of normality, otherwise, Mann-Whitney test was used. A nested t-test was performed when the feto-placental units were analyzed per dam. Exploratory cross-modality correlations between imaging metrics were computed on dam-averaged values, using Pearson’s correlation coefficient ( $r$ ). A two-sided p-value of  $< 0.05$  was considered statistically significant.

## Results

### Maternal and fetal outcomes

RUPP dams showed a higher resorption rate between GD14 and GD16 than NP dams ( $p < 0.001$ ). Maternal body weight change from GD14 to GD16 was lower in the RUPP group ( $p < 0.05$ ). Fetal weight, placental weight and the placental-to-fetal weight ratio did not differ significantly between NP and RUPP pregnancies (**Table 1**).

| Outcome                            | NP (n = 7)     | RUPP (n = 10)     |
|------------------------------------|----------------|-------------------|
| Resorptions GD14–16 (%)            | 0.0 [0.0–25.0] | 69.2 [59.6–76.6]* |
| Maternal weight change GD14–16 (g) | 9.2 ± 13.9     | 9.2 ± 13.9*       |
| Placental weight (mg)              | 426.4 ± 153.8  | 426.4 ± 153.8     |
| Fetal weight (mg)                  | 569 ± 167      | 542 ± 96          |
| PW:FW ratio                        | 1.40 ± 0.41    | 1.50 ± 0.18       |

**Table 1. Maternal and fetal outcomes in NP and RUPP groups at GD16.** Data are presented as mean ± SD or median [IQR]. NP, normal pregnant; RUPP, reduced uterine perfusion pressure; GD, gestational day; PW, placental weight; FW, fetal weight. Asterisks indicate statistically significant difference.

## Imaging outcomes

### *Perfusion metrics*

Semi-quantitative DCE-MRI parameters, including TTP, ME, iAUC, AUC or WO, did not differ between NP and RUPP groups (**Figure 1**). Similarly, mean CEUS-derived perfusion parameters did not differ between groups, with no differences in mTT, rBV and rBF between NP and RUPP placentas (**Figure 3**). Notably, both DCE-MRI and CEUS metrics exhibited wide dispersion within groups.

### *Oxygenation metrics*

For T2\*-mapping MRI, mean whole-placenta R2\* was similar between NP and RUPP dams. No between-group differences were observed for placental fetal-side R2\* or placental maternal-side R2\*. Fetal organ oxygenation assessed with T2\* MRI was also comparable between groups, with no differences in fetal liver R2\* or fetal brain R2\* (**Figure 2**). In agreement with the MRI-derived oxygenation readouts, PAI-derived oxygen saturation measures were similar between NP and RUPP pregnancies, with no group differences in placental sO<sub>2</sub>, fetal liver sO<sub>2</sub> or fetal brain sO<sub>2</sub> (**Figure 4**).

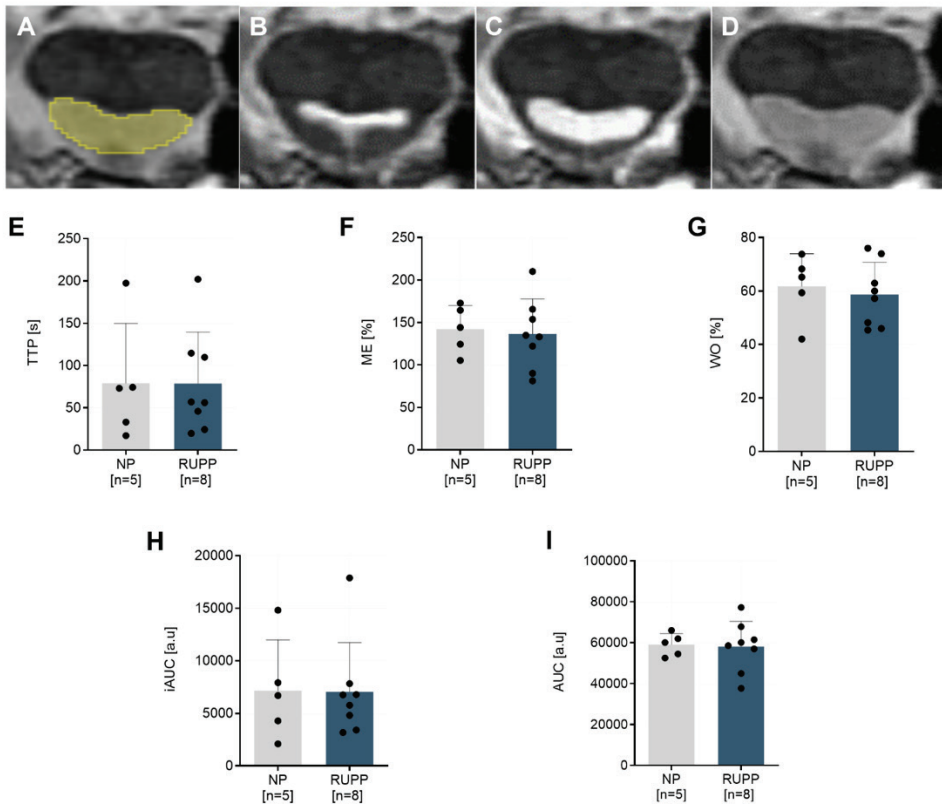


**Figure 1. Dynamic contrast-enhanced MRI of placental perfusion at GD16.** (A–D) Representative coronal DCE-MRI images of a single placenta from a RUPP dam showing (A) whole placenta region of interest (yellow), (B) early enhancement at contrast arrival, (C) peak enhancement, and (D) late wash-out phase. (E–I) Group comparisons of semi-quantitative perfusion parameters derived from fitted signal–time curves in NP (grey) and RUPP (blue) placentas: (E) time to peak (TTP), (F) maximal enhancement (ME), (G) wash-out percentage (WO), (H) initial area under the curve until the peak (iAUC), and (I) total area under the curve (AUC). Bars represent mean  $\pm$  SD with individual dams overlaid.

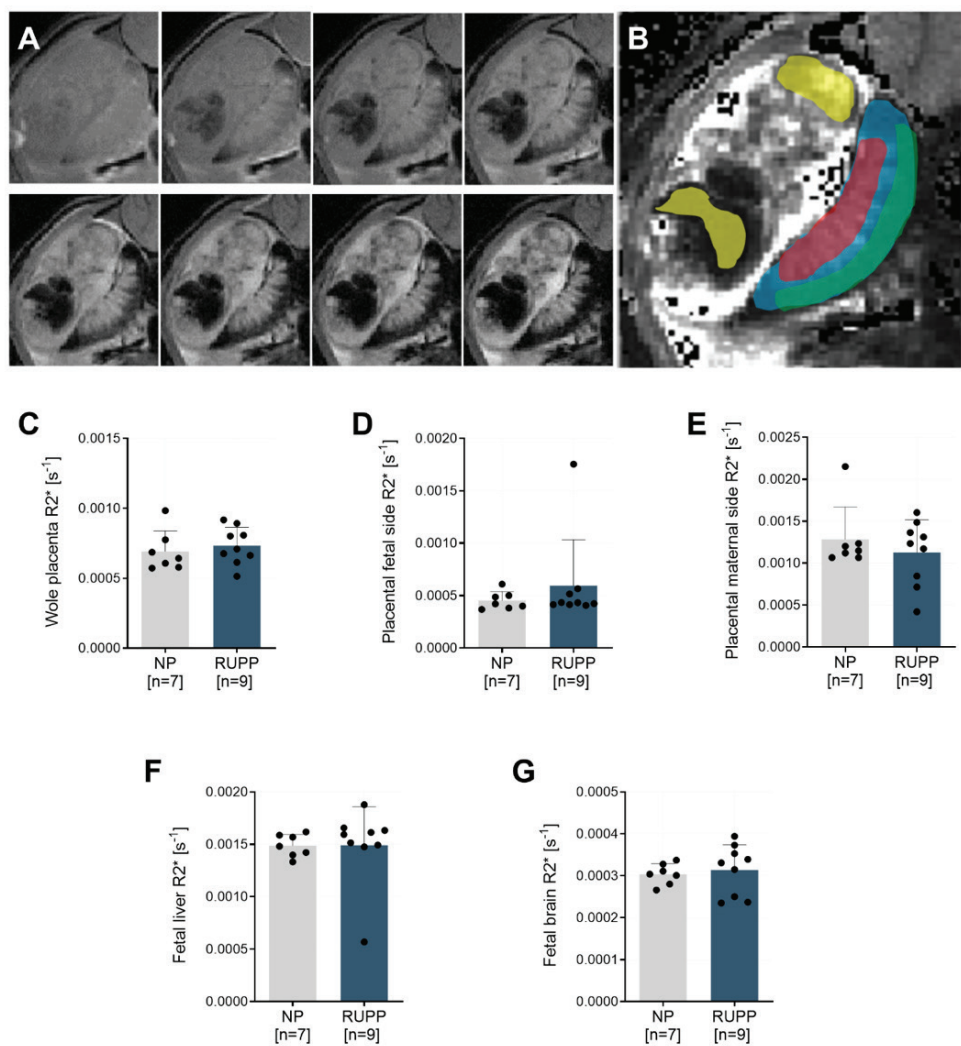


**Figure 2. T2\*-weighted MRI and R2\* mapping of placenta and fetal organs at GD16.** (A) Representative T2\*-weighted multi-echo images in a normal pregnant (NP) dam illustrating placental and fetal anatomy across 8 echo times. (B) Example T2\* map with regions of interest drawn for whole placenta (blue), placental fetal side (red), placental maternal side (green), fetal liver and fetal brain (yellow). (C–G) Group comparisons of R2\* (1/T2\*) between NP and RUPP pregnancies for (C) whole placenta, (D) placental fetal side, (E) placental maternal side, (F) fetal liver, and (G) fetal brain. Bars show mean ± SD.

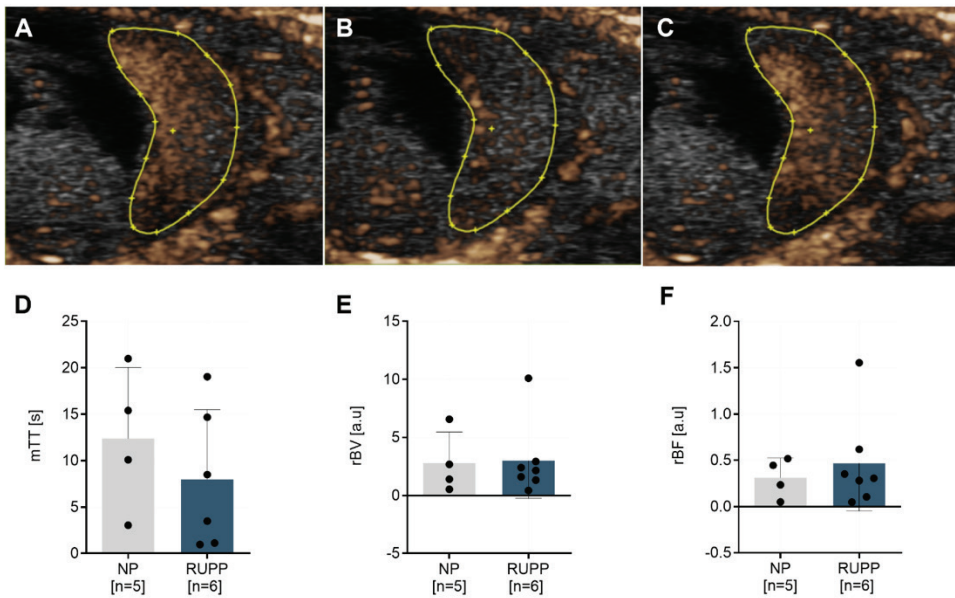


**Figure 3. Contrast-enhanced ultrasound (CEUS) assessment of placental perfusion at GD16.** (A–C) Representative CEUS images from a destruction–replenishment sequence of a single placenta: (A) baseline enhancement before the high–mechanical index burst, (B) immediately after microbubble destruction, and (C) 5 s after contrast replenishment. The whole placenta region of interest is outlined in yellow. (D–F) Group comparisons of CEUS-derived perfusion metrics between normal pregnant (NP) and RUPP dams: (D) mean transit time (mTT), (E) relative blood volume (rBV), and (F) relative blood flow (rBF). Bars show mean  $\pm$  SD.

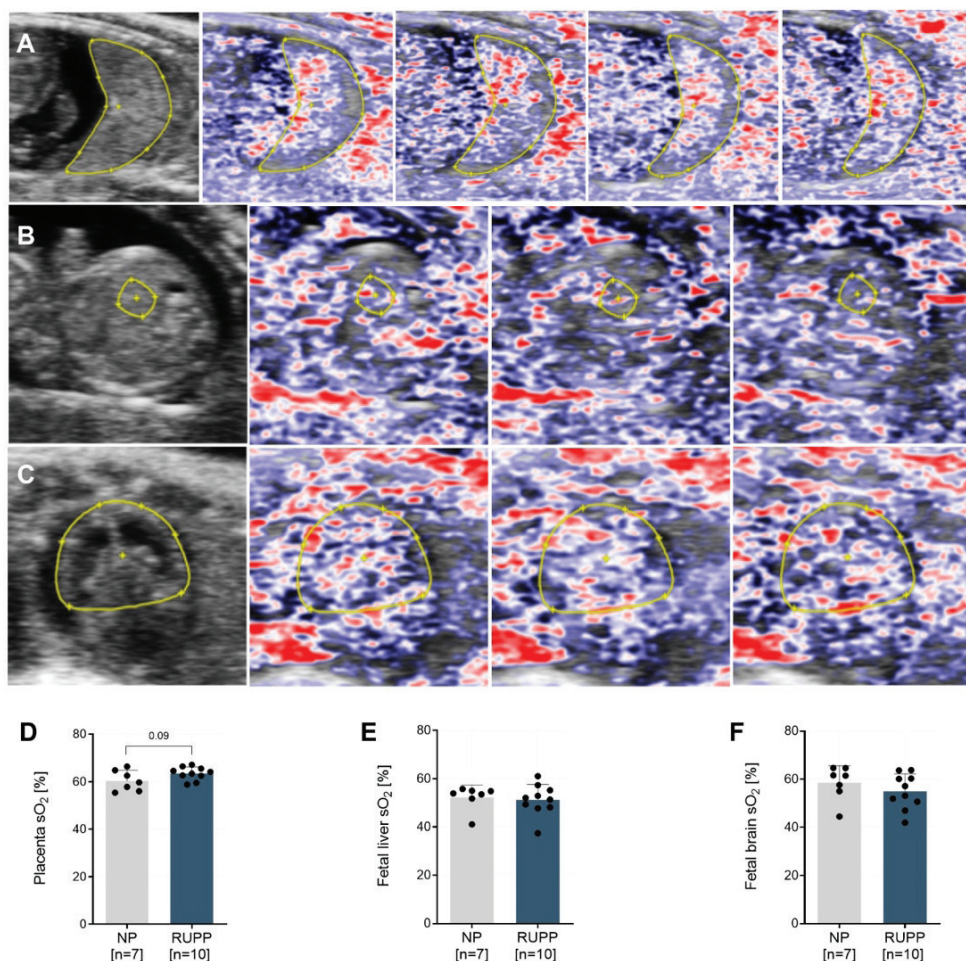


**Figure 4. Photoacoustic imaging of placental and fetal organ oxygenation at GD16.** (A–C) Representative B-mode ultrasound images (left) and corresponding oxy-heme photoacoustic maps (different frames) for (A) placenta (frame 1, 10, 20, 30), (B) fetal liver (frame 1, 5, 10), and (C) fetal brain (frame 1, 5, 10). Yellow contours indicate the regions of interest used for quantification. (D–F) Group comparisons of oxygen saturation (sO<sub>2</sub>) between normal pregnant (NP) and RUPP pregnancies for (D) placenta, (E) fetal liver, and (F) fetal brain. Bars represent mean ± SD.

## Correlations between MRI and CEUS, PAI

Significant cross-modality correlations were observed for specific MRI and CEUS derived measures (Table S2). CEUS mTT correlated positively with placental fetal-side R2\* ( $r = 0.97$ ,  $p < 0.001$ ) and inversely with fetal liver R2\* ( $r = -0.90$ ,  $p < 0.001$ ). CEUS relative blood volume (rBV) also correlated with placental fetal side R2\* ( $r = 0.80$ ,  $p = 0.005$ ) and inversely with fetal liver R2\* ( $r = -0.82$ ,  $p = 0.004$ ). In addition, fetal brain sO<sub>2</sub> measured by PAI correlated

inversely with whole-placenta  $R2^*$  ( $r = -0.77$ ,  $p = <0.001$ ) and with placental fetal side  $R2^*$  ( $r = -0.67$ ,  $p = 0.005$ ) and correlated positively with fetal liver  $R2^*$  ( $r = 0.50$ ,  $p = 0.047$ ). Other cross-modality pairs were not significant.

## Discussion

In this multimodality imaging study, placental oxygenation and perfusion metrics at GD16 did not differ between RUPP and normal pregnant groups. Placental  $T2^*$  values and PAI-derived  $sO_2$  were similar between groups, and perfusion parameters from both DCE-MRI and CEUS likewise showed no between-group differences. Fetal brain and liver oxygenation assessed with  $T2^*$ -mapping MRI and PAI also did not differ at this time point, indicating concordant group-level findings across modalities.

### Perfusion

The absence of perfusion differences at GD16 in both DCE-MRI and CEUS is consistent with our earlier longitudinal DCE-MRI findings, in which alterations emerged early after the insult (GD14) and persisted for a subset of parameters at GD15, but were no longer detectable at GD16 and GD18<sup>16</sup>. This time course supports an early post-surgical perfusion impairment followed by a gradual hemodynamic adaptation over the ensuing days, such that group-level differences are attenuated by GD16.

An important aspect of the present work is that two complementary perfusion techniques were applied in the same cohort. DCE-MRI characterizes contrast-agent kinetics within the utero-placental volume, whereas CEUS destruction–replenishment analysis interrogates microbubble wash-in at the level of individual placentas. Despite these different contrast mechanisms and acquisitions, both methods led to concordant conclusions, at GD16 differences in perfusion metrics were not detectable. This cross-modality agreement indicates that any difference in global perfusion between NP and RUPP at this stage is small and may fall below the detection sensitivity of these methods. From a design perspective, these observations strengthen the view that the early post-surgical window (around GD14–15) is likely to be more informative for detecting perfusion impairment or testing perfusion-targeted interventions in this RUPP protocol than imaging at GD16.

### Oxygenation

At GD16, placental oxygenation indices did not differ between NP and RUPP dams.  $T2^*$ -based measures were comparable between groups for whole placenta as well as for the fetal- and maternal-sides, and fetal oxygenation was likewise similar when assessed in fetal liver and brain using both  $T2^*$  MRI and PAI. The agreement between modalities with distinct contrast

mechanisms supports the conclusion that, under the conditions of this experiment, neither placental nor fetal organ oxygenation shows measurable group-level differences at this intermediate gestational stage. By comparison, in our late-gestation cross-sectional study, placental  $T2^*$  differences were readily detectable at GD19<sup>12</sup>, indicating that oxygenation abnormalities become more pronounced as the phenotype evolves.

Our PAI findings differ from those reported by Lawrence et al., who observed lower placental  $sO_2$  in RUPP compared with sham rats at GD16 with persistence to GD18 using spectral PAI. This discrepancy is most probably driven by differences in spectral PAI acquisition and  $sO_2$  processing (including wavelength selection and spectral unmixing), which directly affect the derived oxygenation estimates. This highlights that PAI-derived  $sO_2$  is method-dependent and that absolute values and effect sizes are not directly comparable across studies without harmonized acquisition and processing. It could also be influenced by practical, partially operator-dependent factors including placental depth, imaging window quality, and ROI definition. In our protocol, we prioritized the most superficial placentas with a stable window to ensure sufficient photoacoustic signal and robust  $sO_2$  estimation given depth-dependent optical attenuation. Differences in sampling and ROI placement strategies, as well as animal positioning and window quality, could therefore contribute to between-study differences in measured  $sO_2$ . Within the present protocol, however, the agreement of  $T2^*$  MRI and PAI towards similar findings indicates that any RUPP-related changes in placental and fetal oxygenation at GD16 are limited. In combination with the GD19 MRI data, these results support a gestation-dependent pattern in which oxygenation measures become more discriminative later in pregnancy than at this intermediate time point.

### **Imaging at GD16 in the context of RUPP placental remodeling**

GD16 represents an intermediate stage within this RUPP protocol in which placentation-related abnormalities are established, while gross placental structure and fetal growth can remain relatively preserved. In our recent time-course pathological characterization, remodeling deficits were already present from GD16 onward, including persistent disruption of uNK cell dynamics, shallow trophoblast invasion, and delayed spiral artery remodeling, despite maintenance of overall placental macrostructure<sup>6</sup>. These processes directly influence the development of a low-resistance uteroplacental circulation and the capacity of the maternal-fetal interface to accommodate increasing late-gestation demands. At GD16, two days after the RUPP intervention, these abnormalities may therefore reflect a placenta in transition: structural and cellular pathways linked to vascular adaptation are impaired, but the integrated functional state may still be maintained within a physiological range under baseline conditions.

An additional consideration at this stage is potential selection bias related to fetal loss in the RUPP group. Because imaging analyses focus on placentas associated with viable fetuses, an

increased resorption rate may enrich the analyzed RUPP dataset for pregnancies and placentas that have maintained functional compensation at GD16. Such a survivor effect would reduce between-group separation in oxygenation and perfusion metrics, even when remodeling deficits are already present at the tissue level. In this context, the absence of detectable group differences in placental oxygenation and perfusion at GD16 is consistent with limited functional differences among the viable placentas available for analysis at this time point.

Although differences were not detected at GD16, cross-modality correlations were observed between CEUS metrics and placental/fetal  $R2^*$ , as well as between fetal brain  $sO_2$  and placental and fetal  $R2^*$  (Table S1). These secondary analyses should be regarded as exploratory, but they support internal consistency across modalities within this dataset, even in the absence of clear NP–RUPP separation at this gestational stage.

### Strength and limitations

A major strength of this study is the integration of MRI, PAI, and CEUS within the same experimental setting, providing a rare multimodality assessment of placental perfusion and oxygenation in the same RUPP cohort under matched physiological conditions. This design reduces between-study and between-cohort variability and allows a coherent interpretation of modality-specific readouts. In addition, the inclusion of fetal organ measurements adds an additional layer, allowing evaluation of potential downstream effects on fetal liver and brain oxygenation.

Several limitations should be acknowledged. First, imaging was performed at a single gestational time point, which limits inference on the temporal evolution of oxygenation and perfusion changes after the RUPP insult. Second, CEUS destruction-replenishment analysis is sensitive to multiple sources of variability, including burst effectiveness (incomplete or non-uniform microbubble destruction across placentas and depths) and respiratory motion which can alter the time-intensity curves and increase within-group variance. In addition, because multiple placentas were imaged sequentially during a single contrast administration, temporal variation in circulating microbubble concentration or baseline signal may have occurred over the imaging session, which could influence rBV and rBF estimates. Finally, quantitative PAI-derived  $sO_2$  remains sensitive to imaging depth and light attenuation, and although averaging three representative frames per 3D acquisition provides a practical summary measure, it does not capture the full within-scan variability across frames.

## Conclusion

Placental oxygenation and perfusion metrics derived from T2\* MRI, PAI, DCE-MRI, and CEUS were indistinguishable between RUPP and normal pregnant rats at GD16, and fetal brain and liver oxygenation measures likewise did not differ between groups. The close agreement across these independent imaging modalities indicates that, at this gestational age, functional indices of placental and fetal oxygenation and blood flow are preserved despite reported placental remodeling deficits in the RUPP model. Importantly, this study supports multimodality imaging as a valuable framework for placental health assessment: the absence of group separation at GD16 is itself informative, defining the temporal limits of detectability and guiding selection of the most sensitive windows and endpoints for mechanistic studies and therapeutic evaluation.

These findings emphasize gestational timing as a critical determinant of when functional imaging readouts can resolve group differences. Defining the optimal time frame will require longitudinal multimodality studies, alongside standardized acquisition/analysis and integration with ex vivo outcomes to identify which readouts best predict clinically relevant endpoints. Translationally, while routine multimodality imaging is not currently practical for broad clinical screening, placental perfusion and oxygenation remain highly relevant to PE and FGR care. Continued development and validation of clinically feasible functional placental imaging (e.g., advanced ultrasound-based approaches and MRI sequences that avoid contrast agents) may ultimately improve interpretation of placental status and support decision-making around surveillance intensity and timing of delivery.

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## Supplementary material



### The ARRIVE guidelines 2.0: author checklist

#### The ARRIVE Essential 10

These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

| Item                                      | Recommendation                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               | Section/line number, or reason for not reporting                                                 |
|-------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|
| <b>Study design</b> 1                     | For each experiment, provide brief details of study design including: <ol style="list-style-type: none"> <li>The groups being compared, including control groups. If no control group has been used, the rationale should be stated.</li> <li>The experimental unit (e.g. a single animal, litter, or cage of animals).</li> </ol>                                                                                                                                                                                                                                                           | Materials and Methods<br>Materials and Methods                                                   |
| <b>Sample size</b> 2                      | <ol style="list-style-type: none"> <li>Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used.</li> <li>Explain how the sample size was decided. Provide details of any <i>a priori</i> sample size calculation, if done.</li> </ol>                                                                                                                                                                                                                                                | Materials and Methods, Results<br>Not reported                                                   |
| <b>Inclusion and exclusion criteria</b> 3 | <ol style="list-style-type: none"> <li>Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established <i>a priori</i>. If no criteria were set, state this explicitly.</li> <li>For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so.</li> <li>For each analysis, report the exact value of <i>n</i> in each experimental group.</li> </ol> | Materials and Methods<br>Results<br>Materials and Methods, Results                               |
| <b>Randomisation</b> 4                    | <ol style="list-style-type: none"> <li>State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence.</li> <li>Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.</li> </ol>                                                                                                                                                  | Materials and Methods. Simple randomization<br>Materials and Methods                             |
| <b>Blinding</b> 5                         | Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).                                                                                                                                                                                                                                                                                                                                                                                              | Materials and Methods                                                                            |
| <b>Outcome measures</b> 6                 | <ol style="list-style-type: none"> <li>Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes).</li> <li>For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.</li> </ol>                                                                                                                                                                                                                                                                              | Materials and Methods<br>Not reported                                                            |
| <b>Statistical methods</b> 7              | <ol style="list-style-type: none"> <li>Provide details of the statistical methods used for each analysis, including software used.</li> <li>Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.</li> </ol>                                                                                                                                                                                                                                                                              | Materials and Methods<br>Materials and Methods                                                   |
| <b>Experimental animals</b> 8             | <ol style="list-style-type: none"> <li>Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight.</li> <li>Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.</li> </ol>                                                                                                                                                                                                         | Materials and Methods<br>Materials and Methods                                                   |
| <b>Experimental procedures</b> 9          | For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including: <ol style="list-style-type: none"> <li>What was done, how it was done and what was used.</li> <li>When and how often.</li> <li>Where (including detail of any acclimatisation periods).</li> <li>Why (provide rationale for procedures).</li> </ol>                                                                                                                                                                                                  | Materials and Methods<br>Materials and Methods<br>Materials and Methods<br>Materials and Methods |
| <b>Results</b> 10                         | For each experiment conducted, including independent replications, report: <ol style="list-style-type: none"> <li>Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range).</li> <li>If applicable, the effect size with a confidence interval.</li> </ol>                                                                                                                                                                                                                                         | Results<br>Not reported                                                                          |

Table S1. ARRIVE guidelines

| Metric 1                    | Metric 2                | r      | p value |
|-----------------------------|-------------------------|--------|---------|
| mTT                         | placenta fetal side R2* | 0.969  | <0.001  |
| mTT                         | fetal liver R2*         | −0.900 | <0.001  |
| rBV                         | placenta fetal side R2* | 0.800  | 0.005   |
| rBV                         | fetal liver R2*         | −0.818 | 0.004   |
| fetal brain sO <sub>2</sub> | whole placenta R2*      | −0.768 | <0.001  |
| fetal brain sO <sub>2</sub> | placenta fetal side R2* | −0.666 | 0.005   |
| fetal brain sO <sub>2</sub> | fetal liver R2*         | 0.502  | 0.048   |

**Table S2. Significant correlations between MRI and CEUS/PAI metrics.** mTT, mean transit time; rBV, relative blood volume; sO<sub>2</sub>, oxygen saturation; R2\* = 1/T2\*

# 7

## Chapter

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General discussion and future perspectives

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## Summary of the main findings

Placental insufficiency plays a key upstream role in preeclampsia (PE) and fetal growth restriction (FGR). Despite its impact, assessing placental function directly *in vivo* remains limited. Clinically, this challenge is compounded by the lack of therapies that restore placental function. As a result, current management strategies relies on indirect maternal and fetal surveillance and, ultimately, decisions about timing of delivery rather than targeted treatment of the placenta itself. This monitoring and therapy gap motivates the development of functional biomarkers that can interrogate placental perfusion and oxygenation across gestation. Within this clinical landscape, this thesis investigated how reduced uterine perfusion reshapes placental structure, perfusion and oxygenation over gestation in the RUPP rat model, using an integrated approach that combined advanced MRI, ultrasound- and photoacoustic imaging (PAI) with histology of the maternal–fetal interface. Across these studies, placental insufficiency emerged as a temporally evolving trajectory in which hemodynamic, structural and oxygenation changes are only partially aligned in time (**Figure 1**).

Immediately after mechanical reduction of uterine blood flow, placental perfusion was severely disturbed. Dynamic contrast-enhanced (DCE) MRI revealed delayed contrast arrival, blunted peak enhancement and impeded wash-out, indicating marked compromise of maternal inflow and transit through the placental bed in the acute post-surgical phase. These perfusion abnormalities diminished rapidly and, in surviving fetoplacental units, group-level perfusion metrics approximated those of normal pregnancies by mid-gestation, indicating substantial hemodynamic adaptation. At the histological level, however, this apparent normalization of perfusion occurred in a placenta that had already diverged structurally from normal development (**Chapters 3 & 4**).

Histological analyses performed in this thesis showed that RUPP induced persistent defects in the processes that normally convert the uterine vasculature into a high-capacitance, low-resistance system. From mid-gestation onwards, the maternal–fetal interface was characterized by sustained retention of uterine natural killer (uNK) cells in the mesometrial triangle, shallower trophoblast invasion and a shift towards unremodeled or only partially remodeled spiral arteries. Gross placental architecture remained intact, but the vascular bed supplying the exchange region was systematically less transformed. These changes represent a durable structural substrate for functional compromise, even when global perfusion appears restored (**Chapter 4**).

Late-gestation T2\* mapping demonstrated that this structurally abnormal placenta is chronically hypoxic (**Chapter 2**). At gestational day (GD) 19, placental T2\* was reduced in RUPP placentas on both maternal and fetal sides of the interface, while 3D DCE-MRI showed no between-group differences in semi-quantitative perfusion parameters. In contrast, fetal brain and liver T2\* values remained comparable between groups, consistent with preserved fetal organ

oxygenation in the setting of placental hypoxia. An intermediate multimodal imaging study at GD 16, combining T2\*-MRI, DCE-MRI, CEUS and PAI, did not detect group-level differences in perfusion or oxygenation, but revealed coherent correlations between modality-specific readouts within the cohort, suggesting tightly coupled but compensated placental–fetal oxygenation at that stage (**Chapter 5**).

Taken together, these findings delineate a sequence in the RUPP model in which abrupt uterine perfusion reduction causes an early but transient perfusion deficit, is followed by structural vascular maladaptation that does not spontaneously normalize, and results in late-gestation placental hypoxia despite restored perfusion. Perfusion and oxygenation measures therefore capture distinct, temporally shifted facets of the same disease process, and the placenta’s ability to normalize perfusion metrics masks an underlying chronic vulnerability that only becomes apparent when oxygenation and vascular remodeling are investigated.

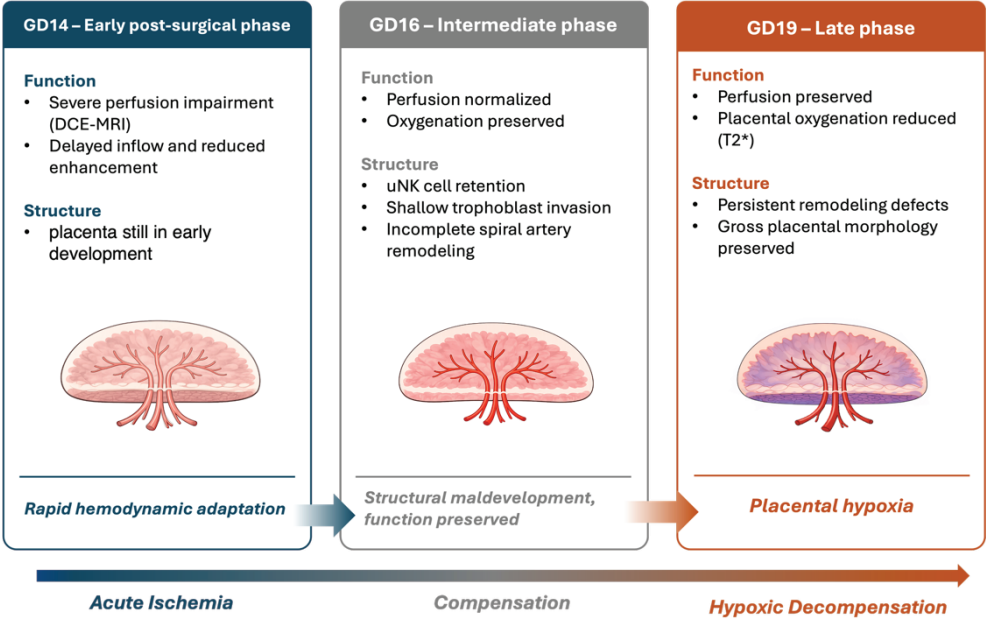


**Figure 1. Temporal trajectory of placental insufficiency in the RUPP rat model.** Immediately after surgical reduction of uterine perfusion (GD14), placental function is dominated by an acute ischemic insult characterized by severe perfusion impairment, while structural remodeling is still incomplete in both groups. By mid-gestation (GD16), perfusion and oxygenation are functionally compensated despite established structural abnormalities. In late gestation (GD19), perfusion remains preserved, but placental oxygenation is reduced, consistent with chronic hypoxia arising from a structurally compromised maternal–fetal exchange interface.

## General Discussion

### Placental insufficiency as a temporally evolving syndrome

The RUPP intervention imposes an abrupt reduction of uterine perfusion at a defined time point, allowing the early phase of this trajectory to be captured under controlled conditions. What emerges is not a stable “low-perfusion state”, but a sequence of phases that differ in which components of placental physiology are most perturbed.

In the immediate post-surgical period, maternal inflow is the dominant constraint. The perfusion deficits observed with DCE-MRI in this phase represent a direct hemodynamic consequence of the clips placed on the aorta and ovarian arteries. The fact that these differences are most pronounced shortly after surgery and already attenuated one day later indicates that the uteroplacental circulation is highly plastic functionally. Within a narrow time window, compensatory hemodynamic adjustments such as redistribution of flow and possibly local vasomotor responses may be sufficient to re-establish bulk perfusion to a level that, on average, is indistinguishable from normal. At this point, the placenta remains structurally immature and vulnerable, but its global inflow has recovered to near-physiological values (**Chapter 4**).

As gestation progresses, the balance of constraints shifts. Perfusion metrics at the uterine level no longer differ, yet histology reveals that the vascular tree supplying the placenta has not undergone the full extent of remodeling seen in normal pregnancy. The system has thus moved from an inflow-limited to an architecture-limited state: blood arrives in adequate quantity, but through a vascular network that has reduced reserve capacity and altered flow distribution. By late gestation, this architectural limitation manifests as a deficit in oxygenation rather than in perfusion metrics. Reduced placental T2\* at GD19 reflects this chronic state of compromised oxygen delivery and diffusion within the exchange region, even while DCE-MRI parameters remain within the control range. The placenta has adapted sufficiently to mask the effects of the initial ischemic phase at the level of perfusion, but not at the level of oxygenation (**Chapters 2 & 4**).

The absence of detectable differences in perfusion and oxygenation at GD16 marks a transient interval of apparent compensation between these two extremes. By that time, the early perfusion deficit has largely resolved, and chronic hypoxia has not yet fully emerged at the group level. Yet the histological abnormalities are already present, indicating that the groundwork for later dysfunction has been laid. This triphasic pattern of acute ischemia, mid-gestation compensation and late hypoxic decompensation provides a coherent conceptual framework that links the individual experimental findings into a single pathophysiological narrative of placental insufficiency in the RUPP model (**Chapters 3-5**).

## Dissociation between perfusion and oxygenation

A central observation emerging from this thesis is the clear dissociation between perfusion and oxygenation in late gestation. In a purely hemodynamic model, improved or normalized perfusion would be expected to restore oxygenation, provided maternal oxygen content remains constant. The observation that RUPP placentas at GD19 remain hypoxic despite normal perfusion parameters indicates that this expectation does not fully capture the complexity of placental physiology. Oxygenation is determined not only by how much blood arrives, but by its distribution within the labyrinthine structures, the time it remains within the exchange region, the distance over which oxygen must diffuse to reach fetal capillaries, and the integrity of the exchange surface.

The reduced T2\* values in the whole placenta suggest that oxygen extraction and gradients across the interface are altered. The preserved fetal brain and liver T2\* values imply that fetal adaptive mechanisms, such as redistribution of cardiac output and metabolic adjustments,<sup>[1]</sup> successfully maintain organ oxygenation within a normal range under baseline conditions, even when the placenta supplying them operates in a hypoxic state. This divergence between placental and fetal organ oxygenation reinforces the notion that placental imaging provides information that is not reflected in fetal outcome measures and may precede overt fetal compromise (**Chapter 2**).

The multimodality study at mid-gestation further illustrates that perfusion and oxygenation can be tightly coupled within the physiological range without yielding between-group differences. At GD16, CEUS-derived metrics of microvascular perfusion correlate with MRI-based R2\* and with PAI-derived oxygen saturation, both in the placenta and in fetal organs, indicating that the expected physiological relationships between perfusion and oxygenation hold. Yet, at the level of group means, neither perfusion nor oxygenation differ. This implies that in the compensated phase, the relationship between perfusion and oxygenation remains qualitatively intact, but the system operates closer to its functional limits, close to the threshold at which group-level differences would appear if progression of disease was imposed (**Chapter 5**). To translate these insights into clinically useful readouts, it will be important to move beyond single time-point averages and test whether gestation-specific perfusion–oxygenation relationships, spatial heterogeneity, and/or oxygenation responses to standardized maternal stimuli (e.g., an oxygen challenge) improve risk stratification and longitudinal monitoring of pregnancies at risk for PE/FGR.

## Structural vascular maladaptation as a persistent substrate

The durability of the structural changes in the uterine vasculature is a crucial pillar of the overall interpretation. The persistent presence of uNK cells in the mesometrial triangle, shallow trophoblast invasion and an over-representation of unremodeled or partially remodeled

spiral arteries demonstrate that the RUPP insult permanently alters the trajectory of vascular adaptation, rather than only inducing a transient hemodynamic disturbance. These histological abnormalities offer a mechanistic explanation for the functional profile observed in this RUPP model (**Chapter 3**).

It is particularly notable that these structural abnormalities coexist with apparently normal gross placental morphology. Standard measurements such as placental weight and overall architecture are not dramatically different between groups, yet the microanatomy of the maternal vascular supply is. This discrepancy underlines the limitations of relying solely on gross anatomical or morphometric indicators of placental health and argues strongly for functionally oriented assessments. It also helps explain why perfusion imaging at later time points may underestimate the severity of underlying disease: an incompletely remodeled vascular bed can sustain near-normal flow under baseline conditions, but at the cost of reduced capacity to accommodate further increases in demand or to buffer against systemic perturbations.

This structural–functional disjunction suggests that the placenta in the RUPP model reaches a compensated but vulnerable state late in gestation. It is neither clearly ischemic nor robustly normal. Its macrovascular supply has adapted, but on an impaired microvascular and remodeling scaffold. In such state, oxygenation-sensitive imaging may be more informative than perfusion-based techniques, as it can detect impaired oxygen availability that persists despite normal macroscopic perfusion, reflecting the consequences of a structurally compromised exchange interface.

### **Intra-uterine heterogeneity and unit-specific vulnerability**

Another important dimension that emerges from the imaging and histological data is intra-litter heterogeneity. Not all placentas within a RUPP uterus respond identically to the imposed reduction in uterine perfusion. Voxel-wise DCE-MRI histograms analysis reveal that, even when litter-averaged perfusion metrics have normalized, a subset of placentas retains perfusion distributions that diverge significantly from the normal template. Similarly, conceptus-level outcomes such as resorption suggest that some fetoplacental units are more vulnerable than others to the same maternal insult (**Chapter 4**).

This heterogeneity has several implications. Biologically, it suggests that local factors such as uterine position, implantation site, local vascular branching patterns or random variation in early trophoblast behavior may modulate how individual units experience and adapt to reduced uterine perfusion. In the present work, we recorded left versus right uterine horn and within-horn position at harvest for fetal weight, placental weight, and resorption. However, exploratory inspection of these data did not reveal a consistent relationship with outcome, and we therefore did not pursue further analysis. A more definitive evaluation likely requires

prospective, standardized positional annotation and sufficient power to detect potentially subtle spatial effects.

A practical challenge is that, *in vivo*, the gravid uterine horns are highly compliant and mobile rather than straight, fixed structures. As they expand to accommodate multiple conceptuses, they curve and may partially fold or coil within the abdominal cavity, and their configuration shifts with posture and physiological motion. This changing geometry complicates reliable tracking of individual conceptuses and consistent assignment of an exact implantation position across imaging sessions. Future studies could address this limitation by introducing MRI/ultrasound-visible fiducial markers to provide a reproducible coordinate frame that supports consistent within-horn localization across sessions. Using the same principle by which multimodality fiducials are used to enable reliable long-term tracking and target re-localization over time<sup>[2,3]</sup>.

Methodologically, these observations caution against overreliance on dam-level averages or pooled metrics to characterize placental status. They support placenta-specific and voxel-wise analyses and motivate future work that links imaging signatures of individual placentas to downstream fetal outcomes, because variability between fetoplacental units indicates that placental insufficiency is not uniformly expressed within a single uterus.

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## Implications for imaging biomarkers

Viewed together, the findings of this thesis argue for a nuanced, stage-specific deployment of imaging biomarkers. Perfusion-sensitive methods, such as DCE-MRI, are particularly powerful for detecting acute or early reductions in uteroplacental perfusion. They excel in the phase immediately following a hemodynamic insult, when differences in inflow and transit are large. However, as structural adaptation progresses, perfusion metrics may normalize even while the placenta remains structurally and functionally compromised. Interpreting normal perfusion in isolation, particularly in later gestation, thus risks underestimating ongoing disease.

Oxygenation-sensitive techniques, including T2\*-weighted MRI and PAI, appear more suited to capturing the chronic consequences of impaired vascular remodeling and microstructural alteration. Reduced placental T2\* in late gestation, as observed in this work, likely reflects the cumulative effects of incomplete spiral artery remodeling and microvascular remodeling (**Chapters 2 & 3**). In human pregnancy, reductions in placental T2\* have also been reported in preeclampsia and fetal growth restriction,<sup>[4,5]</sup> indicating that lower placental T2\* is not unique to the RUPP model and may be a general feature of compromised placental function. Combining oxygenation-sensitive imaging with structural information derived from, for instance, diffusion-weighted MRI or, in the research setting, histology could further refine the interpretation of T2\* changes and help distinguish between different patterns of pathology.

PAI broadens this framework by providing an oxygen saturation estimate based on hemoglobin absorption, which can be interpreted alongside perfusion measures and T2\* MRI in both the placenta and fetal organs. This supports multimodal validation of oxygenation readouts across distinct contrast mechanisms and extends the physiological interpretation beyond a single modality (**Chapter 5**). Translationally, its appeal lies in the prospect of a hemoglobin-based oxygenation metric that is physiologically intuitive and, in principle, compatible with ultrasound-based workflows. placental PAI nonetheless remains largely investigational and has not yet been demonstrated for in vivo human placental imaging<sup>6</sup>. Key constraints include achieving sufficient light delivery to generate signal at clinically relevant and variable placental depths together with depth-related attenuation and variable acoustic access that depends on placental position and maternal anatomy. Finally, safety considerations require explicit attention as concerns have been raised regarding tissue damage and potential fetal risk from optical exposure, and any clinical translation will need rigorous demonstration of safety while operating within established laser exposure limits and obstetric safety guidelines<sup>[6]</sup>.

## Future perspectives and translational outlook

### Translational relevance and clinical pathway

The findings of this thesis support a stage specific view of placental insufficiency in which perfusion, microstructural remodeling, and oxygenation do not deteriorate synchronously. A translational next step is systematic alignment of imaging readouts with clinically meaningful endpoints in longitudinal human cohorts, such as gestational age at delivery, perinatal mortality and morbidity and birth weight, supported by standardization of acquisition and analysis and by defining gestation-specific reference ranges. This includes evaluating whether oxygenation-sensitive MRI metrics (e.g., placental T2\*) and non-invasive perfusion proxies can improve risk stratification in pregnancies at risk for PE/FGR, and determining how these markers relate to placental pathology at delivery. **Table 1** contrasts the present findings with the available evidence from human imaging studies and highlights gaps and follow-up questions.

### Value of the RUPP model informed by imaging

The RUPP model is particularly valuable because it introduces a placenta-centered reduction in uterine perfusion at a defined gestational time point, allowing temporal dynamics and intervention effects to be interrogated under controlled conditions<sup>[7,8]</sup>. Compared with models that primarily mimic downstream maternal features (e.g., anti-angiogenic or NO-inhibition models), RUPP preserves an upstream hemodynamic trigger and thereby supports mechanistic separation of how placental ischemia initiates the maternal syndrome and fetal growth restriction<sup>[9]</sup>.

At the same time, like any other animal model, RUPP is not a complete model of early placenta-tion failure: the insult is acute, surgically induced, and applied later in gestation, and phenotype magnitude can vary with technique and other experimental factors, underscoring the need for standardization and reproducibility<sup>[10]</sup>. A practical approach is to implement a predefined, cross-institutional reporting framework that targets the principal sources of phenotypic variability. Specifically, the surgical procedure should be operationalized using explicitly specified, reproducible parameters (e.g., clip/suture material and internal diameter, anatomical placement, procedural sequence, anesthesia/analgesia, thermal support, and operator experience) and reported in sufficient detail to enable replication and inter-study comparability. Importantly, the framework should also document facility biosecurity and husbandry stringency to facilitate interpretation of between-laboratory differences that may reflect environmental and handling related factors. In addition, the severity of the perfusion reduction should be verified with a brief peri-operative confirmation (e.g., a standardized hemodynamic measure or imaging/Doppler surrogate), so that “successful RUPP” is defined by objective procedural criteria rather than downstream phenotype alone. The polytocus nature of the rat uterus is also a double-edged feature, enabling analysis of unit-specific vulnerability under shared maternal conditions, but necessitating placenta-level analyses and caution when extrapolating to singleton human pregnancy. To strengthen cross-species comparability and reduce intra-uterine complexity, bridging studies in larger animal models with smaller litter sizes may be valuable, particularly for positional mapping, longitudinal tracking of individual placentas, and evaluation of imaging protocols under constraints closer to clinical translation.

## Methodological and protocol developments

Several methodological developments could increase sensitivity and interpretability in future studies. First, expanding T2\* MRI toward volumetric coverage (3D T2\* mapping) would reduce slice-selection bias and better capture within-placenta heterogeneity, which is particularly relevant in polytocus models and in conditions where pathology may be patchy. Achieving this will require optimization of coverage–resolution trade-offs and motion robustness, including golden-angle 3D radial sampling with prospective respiratory triggering and k-space trajectory correction<sup>[11]</sup>. Second, integrating oxygenation-sensitive metrics with structural information (e.g., diffusion-weighted MRI) and, where feasible, validated ex vivo endpoints could help differentiate whether changes in T2\* primarily reflect oxygenation shifts, microvascular architecture, or tissue composition.

**Table 1.** Translational comparison and future directions informed by this thesis

| Domain / readout                                              | Human PE/FGR: what is established                                                                                                                                                                                                                                                                                                                                                                                                                           | RUPP model: what this thesis adds                                                                                                                                                                                                                     | Translational gaps and next steps                                                                                                                                                                                                                                                                                                                                                                                        |
|---------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Spiral artery / uteroplacental remodeling defects (histology) | Defective deep placental—impaired spiral artery remodeling and associated vascular lesions—remains a core pathophysiological feature in PE/FGR, particularly early-onset disease. Evidence is mainly derived from placental bed biopsies at delivery (and selected early-gestation samples), with standardized lesion definitions increasingly guided by consensus frameworks for placental pathology (maternal vascular malperfusion) <sup>[12–14]</sup> . | Histology indicates persistent uteroplacental remodeling abnormalities after RUPP, supporting the concept that apparent recovery of bulk perfusion can coexist with a structurally maladapted maternal–fetal interface. ( <b>Chapters 2 &amp; 4</b> ) | <b>Human gap:</b> direct <i>in vivo</i> readouts of spiral artery remodeling remain limited; most confirmation comes late (delivery).<br><b>Next steps:</b> In RUPP, strengthen imaging–histology bridging using standardized lesion scoring, placenta-by-placenta linkage to imaging, and (where feasible) larger-animal models with more human-like placentalion like non-human primates for translational validation. |
| Perfusion and maternal inflow (ultrasound/DCE-MRI)            | In clinical care, uterine/umbilical Doppler provides indirect perfusion-related information; direct placental perfusion MRI is an active research area, mainly using non-contrast approaches (e.g., ASL/VS-ASL) because gadolinium contrast is generally avoided in pregnancy unless essential <sup>[15,16]</sup> .                                                                                                                                         | DCE-MRI detects a marked acute perfusion deficit shortly after RUPP (GD14), which attenuates by GD15, indicating rapid functional adaptation at the level of group averages ( <b>Chapter 4</b> ).                                                     | <b>Translation:</b> In humans, prioritize contrast-free placental perfusion methods (ASL/VS-ASL) <sup>[16,17]</sup> and standardized pipelines (motion correction, segmentation, reporting) to enable multi-center comparability.<br><b>Next step:</b> In RUPP, test whether early perfusion deficits predict later oxygenation impairment or adverse conceptus outcomes when analyzed at the unit level.                |

| Domain / readout                | Human PE/FGR: what is established                                                                                                                                                                                                                                                                                                                                                                                                                                                               | RUPP model: what this thesis adds                                                                                                                                                                                                                                                              | Translational gaps and next steps                                                                                                                                                                                                                                        |
|---------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Placental oxygenation (T2* MRI) | Placental T2* decreases with gestation and is frequently reported to be lower in PE/FGR/SGA compared with controls, supporting T2* as a broadly relevant marker of compromised placental function in humans <sup>[4,18,19]</sup> .                                                                                                                                                                                                                                                              | Despite recovery of group-level perfusion metrics, placental T2* indicates late-gestation hypoxia in RUPP, consistent with a temporally shifted emergence of oxygenation impairment ( <b>Chapters 2 &amp; 4</b> ).                                                                             | <b>Translation:</b> Move toward harmonized acquisition, robust motion handling, and gestation-specific reference curves to interpret “low T2*” relative to normal developmental trajectories.                                                                            |
| PAI                             | Clinical PAI exists in several fields, but placental PAI is largely investigational. Human studies to date are mainly ex vivo (and procedure-adjacent demonstrations), and key technical barriers include depth/light delivery, fluence uncertainty (quantitative sO <sub>2</sub> ), limited-view artefacts, motion sensitivity, and real-time constraints; translation also requires explicit adherence to optical exposure limits and careful fetal safety considerations <sup>[6,20]</sup> . | In the preclinical setting, PAI adds a hemoglobin-based oxygen saturation estimate that can be interpreted alongside perfusion measures and T2* MRI in placenta and fetal organs, enabling cross-modality corroboration of oxygenation trends without exogenous contrast ( <b>Chapter 5</b> ). | <b>Translation and human gap:</b> Treat PAI as a targeted research adjunct rather than a near-term obstetric tool, valuable for mechanistic studies of oxygenation, but requiring standardized workflows, and rigorous laser safety testing before obstetric deployment. |

| Domain / readout                              | Human PE/FGR: what is established                                                                                                                                                                                                       | RUPP model: what this thesis adds                                                                                                                                                                                                                | Translational gaps and next steps                                                                                                                                                                                                                                                                                                   |
|-----------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Heterogeneity and unit-specific vulnerability | Human pregnancy is typically singleton; however, placental disease can be regionally heterogeneous, and advanced imaging increasingly aims to quantify spatial heterogeneity rather than relying only on global means <sup>[21]</sup> . | RUPP reveals pronounced intra-litter heterogeneity: even when litter-averaged perfusion normalizes, a subset of placentas retains divergent perfusion distributions; resorption further indicates unit-level vulnerability ( <b>Chapter 4</b> ). | This is difference from humans, RUPP's litter structure can be leveraged to study vulnerability vs resilience under identical maternal conditions. <b>Next steps:</b> In RUPP, strengthen placenta-level linkage and implement practical localization strategies (e.g., fiducial marker) to test spatial effects more definitively. |

Legend: PE, preeclampsia; FGR, fetal growth restriction; RUPP, reduced uterine perfusion pressure; ASL, arterial spin labeling; VS-ASL, velocity-selective arterial spin labeling; DCE-MRI, dynamic contrast-enhanced MRI; GD, gestational day; SGA, small for gestational age; PAI, photoacoustic imaging; sO<sub>2</sub>, oxygen saturation.

## Concluding remarks

This thesis show that the placenta under reduced uterine perfusion is not simply a “low-perfusion organ”, but a dynamically adapting system that moves through phases of ischemia, compensation and chronic hypoxia. Perfusion and oxygenation behave as related yet temporally decoupled markers, reflecting different facets of the underlying vascular and structural changes. By demonstrating how these markers evolve and diverge across gestation, and by anchoring them in concrete histological abnormalities at the maternal–fetal interface, this thesis provides a coherent narrative of placental insufficiency in the RUPP model. This temporal characterization offers a rationale for selecting gestational windows and imaging readouts when designing future studies, including pathophysiological studies that interrogate vascular reactivity in the acute post-surgical window, prophylactic studies that aim to prevent maladaptive remodeling and should therefore be initiated before or at the time of perfusion reduction, and therapeutic studies that aim to restore exchange capacity later in gestation and may require oxygenation-sensitive readouts as primary outcomes. Ultimately, defining stage-appropriate, non-contrast imaging biomarkers is also an essential step toward clinical translation, where such markers could complement ultrasound surveillance to identify pregnancies in which placental function is deteriorating before overt fetal compromise.

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# Appendices

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English Summary

Nederlandse samenvatting

List of Publications

Acknowledgments

About the Author

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## English Summary

Placental insufficiency refers to a placenta-centered failure to establish or maintain sufficient maternal–fetal exchange. It underlies key obstetric complications, especially preeclampsia (PE) and fetal growth restriction (FGR). In clinical practice, assessment of placental function remains largely indirect, relying on fetal biometry and Doppler indices rather than direct measures of perfusion and oxygenation. In parallel, there is still no widely effective therapy that restores placental function once insufficiency is established. As a result, management frequently focuses on surveillance and timing of delivery. These limitations underscore the urgent need for functional biomarkers that can measure placental oxygenation and perfusion *in vivo*, across gestation.

This thesis aimed to advance a mechanistic, time-specific understanding of placental insufficiency in the reduced uterine perfusion pressure (RUPP) rat model by integrating *in vivo* functional imaging readouts of placental perfusion and oxygenation with biological readouts of uteroplacental remodeling. Across the experimental chapters, magnetic resonance imaging (MRI), including T2\* mapping and 3D dynamic contrast-enhanced MRI (3D DCE-MRI), was complemented by contrast-enhanced ultrasound (CEUS), photoacoustic imaging (PAI), and histology of the maternal–fetal interface to map how functional and structural components of placental insufficiency evolve over gestation.

**Chapter 2** provides the clinical and translational rationale by reviewing molecular and imaging biomarkers linked to placental hypoxia and insufficiency. The chapter highlights that molecular tissue biomarkers are typically available only after delivery, while circulating biomarkers in maternal blood are proxies rather than direct readouts of placental function. Non-invasive imaging, therefore, emerges as a promising method to monitor placental function longitudinally, and as a bridge between biology and clinically relevant biomarkers.

**Chapter 3** establishes quantitative late-gestation MRI readouts in the RUPP model at gestational day (GD) 19. T2\* mapping showed reduced placental T2\* in RUPP pregnancies in both the labyrinth and junctional zone, indicating reduced placental oxygenation, while fetal brain and liver T2\* values remained comparable between groups. In contrast, semi-quantitative 3D DCE-MRI parameters did not differ between RUPP and controls at this time point. Together, these results demonstrate a dissociation between oxygenation and perfusion readouts late in gestation and suggest that oxygenation sensitive measures may reveal placental compromise even when perfusion metrics appear preserved.

**Chapter 4** shows how RUPP influences key maternal–fetal interface processes over time (GD14, GD16, GD18, and GD19). Gross placental macro-architecture was preserved, but the processes that normally remodel the uterine vasculature into a high-capacitance, low-resistance system

were persistently impaired. Specifically, uNK cell migration from the mesometrial triangle was disrupted with sustained uNK cell presence, trophoblast invasion depth was reduced, and spiral artery remodeling was impaired, with a shift towards unremodeled or only partially remodeled arteries by late gestation. These findings identify a durable structural and immunovascular maladaptation that can constrain functional reserve.

**Chapter 5** resolves the temporal dynamics of perfusion impairment after RUPP using longitudinal 3D DCE-MRI from GD14 to GD18. Immediately after surgery (GD14), RUPP placentas showed delayed time-to-peak, reduced maximum enhancement, reduced wash-out, and elevated early AUC, consistent with restricted inflow and impeded transit. By GD15, abnormalities persisted for a subset of parameters, but by GD16–GD18 group-level differences in semi-quantitative perfusion metrics were no longer detectable, indicating substantial hemodynamic adaptation. Importantly, exploratory voxel-wise histogram analyses revealed marked intra-litter heterogeneity, with a subset of RUPP placentas deviating clearly from a normal pregnancy template. Together, these results identify GD14–GD15 as a critical window for detecting acute perfusion impairment and for evaluating interventions targeting early injury mechanisms, while also emphasizing the need to quantify heterogeneity beyond placenta-averaged metrics.

**Chapter 6** extends the functional characterization to a multimodality setting at mid-gestation (GD16) by combining MRI ( $T2^*$  mapping and 3D DCE-MRI) with CEUS and PAI. At this intermediate stage, neither placental perfusion nor oxygenation metrics differed between RUPP and controls across modalities, and fetal brain and liver oxygenation measures were likewise comparable. However, exploratory cross-modality analyses revealed coherent correlations between specific CEUS perfusion measures and MRI-derived  $R2^*$  readouts, suggesting that physiological coupling between perfusion and oxygenation remains detectable within the cohort even when group means are similar. This chapter therefore supports the view that GD16 represents a compensated phase in which detectable between-group differences may be small or heterogeneous, reinforcing the importance of gestational timing, sensitivity, and analytical strategies that capture dispersion and within-litter variability.

In **Chapter 7**, the thesis findings are integrated into a temporally evolving trajectory of placental insufficiency in the RUPP model. Immediately after mechanical reduction of uterine blood flow (GD14), placental function is dominated by an acute ischemic insult with pronounced perfusion impairment. By mid-gestation (around GD16), perfusion and oxygenation measures can appear functionally compensated despite established structural abnormalities at the maternal–fetal interface. By late gestation (GD19), perfusion metrics remain largely preserved, yet placental  $T2^*$  indicates chronic hypoxia, consistent with a state in which oxygenation is constrained by impaired uteroplacental remodeling rather than by insufficient inflow alone.

Overall, this thesis demonstrates that placental insufficiency in the RUPP model is not a static low-perfusion phenotype but a time-dependent sequence in which acute hemodynamic disruption, structural vascular maladaptation, and late oxygenation deficits are only partially aligned in time. These findings argue for time-specific, multimodal imaging biomarker strategies that integrate perfusion and oxygenation readouts and that explicitly address heterogeneity within litters and across placentas. Such temporally informed functional outcomes provide a platform for designing mechanistic studies and for testing therapies. They help align interventions with the window in which the targeted pathophysiology is most detectable and most modifiable.

## Nederlandse samenvatting

Placenta-insufficiëntie is het falen van voldoende maternale–foetale uitwisseling tot stand te brengen of te behouden. Het ligt ten grondslag aan belangrijke zwangerschapscomplicaties, met name pre-eclampsie (PE) en foetale groeirestrictie (FGR). In de klinische praktijk wordt de placentafunctie nog grotendeels indirect beoordeeld. Daarbij worden vooral foetale biometriemetingen en Doppler-indices gebruikt, in plaats van directe metingen van perfusie en oxygenatie van de placenta. Tegelijkertijd is er nog geen breed effectieve therapie die placentafunctie kan herstellen. De zorg richt zich daarom vaak op monitoring en het bepalen van het optimale moment van bevalling. Deze beperkingen onderstrepen de urgente behoefte aan functionele biomarkers die placentaire oxygenatie en perfusie in vivo kunnen meten gedurende de zwangerschap.

Het doel van dit proefschrift was om een beter mechanistisch en tijdsafhankelijk begrip te krijgen van placenta-insufficiëntie in het rattenmodel met verlaagde uteriene perfusiedruk (RUPP). Hiervoor zijn in vivo beeldvormende uitleesparameters van placentaire perfusie en oxygenatie geïntegreerd met biologische uitleesparameters van utero-placentaire remodellering. In de experimentele hoofdstukken stonden MRI-metingen centraal, waaronder T2\*-mapping en 3D dynamic contrast-enhanced MRI (3D DCE-MRI). Deze werden aangevuld met contrastversterkte echografie (CEUS), foto-akoestische beeldvorming (PAI) en histologie van de maternale–foetale interface. Met deze multimodale aanpak is onderzocht hoe functionele en structurele componenten van placenta-insufficiëntie zich gedurende de dracht ontwikkelen.

**Hoofdstuk 2** schetst de klinische en translationele onderbouwing via een overzicht van moleculaire en beeldvormende biomarkers die gekoppeld zijn aan placentaire hypoxie en insufficiëntie. Het hoofdstuk benadrukt dat moleculaire weefselbiomarkers meestal pas na de bevalling beschikbaar zijn. Biomarkers in maternale circulatie zijn waardevol, maar blijven vooral indirecte aanwijzingen voor placentafunctie. Niet-invasieve beeldvorming komt daardoor naar voren als een veelbelovende methode om placentafunctie longitudinaal te volgen en als brug tussen onderliggende biologie en klinisch relevante biomarkers.

**Hoofdstuk 3** beschrijft kwantitatieve MRI-readouts in de late dracht in het RUPP-model op gestatiedag (GD) 19. T2\*-mapping liet een verlaagde placentaire T2\* zien bij RUPP-zwangerschappen, zowel in de labyrinthzone als in de junctional zone. Dit wijst op verminderde placentaire oxygenatie. T2\*-waarden van foetale hersenen en lever bleven daarentegen vergelijkbaar tussen groepen. Semi-kwantitatieve 3D DCE-MRI-parameters verschilden op GD19 niet tussen RUPP en controles. Samen laten deze bevindingen zien dat oxygenatie- en perfusie uitleesparameters in de late dracht uiteen kunnen lopen. Ze suggereren dat oxygenatie-gevoelige metingen placentaire kwetsbaarheid kunnen aantonen, ook wanneer perfusiematen ogenschijnlijk behouden blijven.

**Hoofdstuk 4** laat zien hoe de RUPP ingreep processen aan de maternale–foetale interface beïnvloedt door de tijd (GD14, GD16, GD18 en GD19). De globale placentaire macro-architectuur bleef behouden. Toch waren de processen die normaal gesproken de uteriene vascularisatie omvormen tot een systeem met hoge capaciteit en lage weerstand persistent verstoord. Migratie van uteriene NK-cellen (uNK) vanuit de mesometriale driehoek was ontregeld, met aanhoudende uNK-aanwezigheid. De invasiediepte van trofoblast was verminderd. Ook spiraalarterie-remodellering was beperkt, met in de late dracht relatief meer ongeremodelleerde of slechts deels geremodelleerde arteriën. Deze resultaten wijzen op een duurzame structurele en immunovasculaire maladaptatie die de functionele reserve kan beperken.

**Hoofdstuk 5** brengt de tijdsdynamiek van perfusiestoornissen na RUPP in kaart met longitudinale 3D DCE-MRI van GD14 tot GD18. Direct na de operatie (GD14) vertoonden RUPP-placenta's een vertraagde time-to-peak, een lagere maximale enhancement, een verminderde wash-out en een verhoogde vroege area-under-the-curve (AUC). Dit patroon past bij beperkte instroom en belemmerde transit. Op GD15 bleven afwijkingen bestaan voor een deel van de parameters. Op GD16–GD18 waren groepsverschillen in semi-kwantitatieve perfusiematen niet langer detecteerbaar, wat wijst op substantiële hemodynamische adaptatie. Exploratieve voxel-gewijze histogramanalyses toonden echter duidelijke heterogeniteit binnen nesten. Een subset van RUPP-placenta's week duidelijk af van een normaal-zwangerschap template. Samen identificeren deze resultaten GD14–GD15 als een kritisch venster om acute perfusiestoornis te detecteren en interventies te evalueren die vroege schade-mechanismen beogen. Ze benadrukken ook dat heterogeniteit verder moet worden gekwantificeerd dan gemiddelden per placenta.

**Hoofdstuk 6** breidt de functionele karakterisering uit met een multimodale meting in de mid-gestatie (GD16) door MRI ( $T2^*$ -mapping en 3D DCE-MRI) te combineren met CEUS en PAI. Op dit tijdstip verschilden placentaire perfusie- en oxygenatiematen niet tussen RUPP en controles, en ook foetale hersen- en leveroxygenatiematen waren vergelijkbaar. Exploratieve cross-modality analyses lieten wel consistente correlaties zien tussen specifieke CEUS-perfusiematen en MRI-afgeleide  $R2^*$ -uitleesparameters. Dit suggereert dat fysiologische koppeling tussen perfusie en oxygenatie binnen het cohort detecteerbaar blijft, ook wanneer groeps-gemiddelden vergelijkbaar zijn. Dit ondersteunt het beeld dat GD16 een gecompenseerde fase kan representeren, waarin tussen-groepsverschillen klein of heterogeen zijn. Het onderstreept het belang van het moment van zwangerschapsduur ten tijde van meting, meetgevoeligheid en analytische strategieën die spreiding en variatie binnen nesten expliciet meenemen.

In **Hoofdstuk 7** worden de bevindingen samengebracht in een tijdsafhankelijk traject van placenta-insufficiëntie in het RUPP-model. Direct na de mechanische reductie van uteriene bloedstroom (GD14) wordt placenta-functie gedomineerd door een acute ischemische insult met uitgesproken perfusie-impairment. In ongeveer het eind-middelste deel van de dracht (ongeveer GD16) kunnen perfusie- en oxygenatiematen functioneel gecompenseerd lijken,

ondanks reeds aanwezige structurele afwijkingen aan de maternale–foetale interface. In de late dracht (GD19) blijven perfusiematen grotendeels behouden, terwijl placentaire T2\* wijst op chronische hypoxie. Dit past bij een toestand waarin oxygenatie beperkt wordt door verstoorde uteroplacentaire remodellering, en niet uitsluitend door onvoldoende instroom.

Samengevat laat dit proefschrift zien dat placenta-insufficiëntie in het RUPP-model geen statisch “laag-perfusie” fenotype is. Het is een tijdsafhankelijke sequentie waarin acute hemodynamische verstoring, structurele vasculaire maladaptatie en late oxygenatiedeficiënties slechts deels synchroon verlopen. De resultaten pleiten voor tijd-specifieke, multimodale beeldvormende biomarkerstrategieën die perfusie en oxygenatie combineren en heterogeniteit binnen nesten en tussen placenta’s expliciet adresseren. Dergelijke tijdsg geïnformeerde functionele uitkomstmaten vormen een platform voor mechanistische studies en voor therapietesten. Ze helpen interventies af te stemmen op het venster waarin de beoogde pathofysiologie het best meetbaar en het best beïnvloedbaar is.

## PhD Portfolio

| General courses                                                                                | Year      | ECTS |
|------------------------------------------------------------------------------------------------|-----------|------|
| The Amsterdam UMC World of Science                                                             | 2021      | 0.7  |
| Research Data Management                                                                       | 2021      | 0.2  |
| Talents in PhD                                                                                 | 2022      | 0.2  |
| Specific courses                                                                               |           |      |
| Laboratory Animal Science                                                                      | 2021      | 3.9  |
| 7th in Vivo NMR                                                                                | 2021      | 1.5  |
| Data analysis in MATLAB                                                                        | 2021      | 0.7  |
| Mouse Morphology, Function & Genetics                                                          | 2021      | 1.5  |
| Practical Biostatistics                                                                        | 2022      | 1.4  |
| Computing in R                                                                                 | 2022      | 0.7  |
| FUJIFILM VisualSonics PA Winter School                                                         | 2022      | 0.5  |
| Advanced Topics in Biostatistics                                                               | 2024      | 1.5  |
| Seminars, workshops and master classes                                                         | Year      | ECTS |
| MRM weekly meeting, attendance/ presentation                                                   | 2021-2025 | 2    |
| CVeng weekly meeting, attendance/ presentation                                                 | 2021-2025 | 2    |
| 2nd Dutch Network Meeting on PAUS, oral presentation                                           | 2022      | 0.5  |
| Nederlandse werkgroep preeclampsie (NedWep) meeting, oral presentation                         | 2023-2025 | 1    |
| Research retreat, Cardiovascular Research Institute, poster presentation                       | 2023      | 1    |
| 4th Meeting of the Benelux Network for Ultrasound and Photoacoustic Imaging, oral presentation | 2024      | 0.5  |
| The Amsterdam UMC PhD Science & Awards Day, Poster presentation                                | 2024      | 1    |

| <b>(Inter)national conferences</b>                                                                                                                | <b>Year</b> | <b>ECTS</b> |
|---------------------------------------------------------------------------------------------------------------------------------------------------|-------------|-------------|
| Joint Annual Meeting ISMRM-ESMRMB & ISMRT 31st Annual Meeting - London, UK - poster presentation                                                  | 2022        | 1.5         |
| ISMRM Benelux Chapter – Maastricht the Netherlands - oral presentation                                                                            | 2022        | 0.5         |
| 23rd World Congress of the International Society for the Study of Hypertension in Pregnancy (ISSHP) - Montpellier, France - oral presentation     | 2022        | 1           |
| 18th European Molecular Imaging Meeting - Salzburg, Austria - attendance                                                                          | 2023        | 1           |
| ISMRM Benelux Chapter - Brussels, Belgium - poster presentation                                                                                   | 2023        | 0.5         |
| 2024 ISMRM & ISMRT Annual Meeting & Exhibition - Singapore - poster presentation                                                                  | 2024        | 1.5         |
| 19th European Molecular Imaging Meeting - Porto, Portugal - poster presentation                                                                   | 2024        | 1           |
| European Society for Magnetic Resonance in Medicine and Biology (ESMRMB) 40th Annual Scientific Meeting - Barcelona, Spain - oral presentation    | 2024        | 1           |
| 20th European Molecular Imaging Meeting - Bilbao, Spain - poster presentation                                                                     | 2025        | 1           |
| European Society for Magnetic Resonance in Medicine and Biology (ESMRMB) 41st Annual Scientific Meeting - Marseille, France - poster presentation | 2025        | 1           |
| <b>Supervision</b>                                                                                                                                | <b>Year</b> | <b>ECTS</b> |
| Research practical- 2 weeks research project                                                                                                      | 2022/2023   |             |
| <b>Organizational activities</b>                                                                                                                  | <b>Year</b> | <b>ECTS</b> |
| CVeng weekly meeting                                                                                                                              | 2022-2023   |             |
| ISMRM Benelux organization committee member                                                                                                       | 2024        |             |

## List of Publications

**Al Darwish, F. M.**, Meijerink, L., Coolen, B. F., Strijkers, G. J., Bekker, M., Lely, T., & Terstappen, F. (2023). From molecules to imaging: Assessment of placental hypoxia biomarkers in placental insufficiency syndromes. *Cells*, 12(16), 2080. <https://doi.org/10.3390/cells12162080>

**Al Darwish, F. M.**, Coolen, B. F., van Kammen, C. M., Alles, L. K., de Vos, J., Schiffelers, R. M., Lely, T. A., Strijkers, G. J., & Terstappen, F. (2024). Assessment of feto-placental oxygenation and perfusion in a rat model of placental insufficiency using T2\* mapping and 3D dynamic contrast-enhanced MRI. *Placenta*, 151, 19–25. <https://doi.org/10.1016/j.placenta.2024.04.008>

**Al Darwish, F. M.**, van Kammen, C. M., Terstappen, F., Alles, L. K., Schiffelers, R. M., Lely, A. T., Strijkers, G. J., & Coolen, B. F. (2026). Longitudinal 3D-DCE MRI assessment of placental perfusion under reduced uterine perfusion pressure in pregnant rats. *NMR in Biomedicine*, 39(4), e70251. <https://doi.org/10.1002/nbm.70251>

Van Kammen, C. M\*, **Al Darwish, F. M\***, Strijkers, G. J., Coolen, B. F., Alles, L. K., Faas, M. M., Schiffelers, R., Lely, A. T., & Terstappen, F. (2026). Impaired placental vascular remodeling and persistent uNK cells in the RUPP model: A time-dependent perspective. *Placenta*. <https://doi.org/10.1016/j.placenta.2025.11.008>

Rabineau, J., **Al-Darwish, F.**, Mulder, E., Coolen, B. F., Hoffmann, F., Hautemann, D., Tank, J., Migeotte, P.-F., Strijkers, G. J., & Wüst, R. C. I. (2026). Apical hypercontractility mitigates impaired diastolic filling and lower intraventricular haemodynamic forces in human bed rest. *Experimental Physiology*, 1–18. <https://doi.org/10.1113/EP093671>

## Acknowledgments

Completing this thesis has taken more than science. Behind every experiment, every manuscript, and every late hour, there were people. To everyone who walked alongside me, in the lab, in the corridors, at conferences, and at home, this section is for you. I am deeply grateful.

Gustav, thank you for being a constant source of reassurance. Whenever doubt crept in about an experiment or the direction of the project, you were there. Your availability and calm confidence in me made all the difference. You also gave me the opportunity to attend conferences and present this work to the wider scientific community. Those experiences shaped me as a researcher in ways that go beyond the thesis itself.

Bram, your way of thinking never ceases to surprise me. You have a gift for seeing things from an angle no one else has considered. That perspective touched everything: how we designed experiments, how we approached manuscripts, how we refined ideas. Your hands-on help during experiments meant more than I can say.

Titia and Fieke, your contribution to this project was invaluable on multiple levels. Your clinical expertise ensured that this preclinical work never lost sight of the patients it is meant to serve. Your experience with the RUPP animal model was equally essential. You consistently brought us back to what matters, bridging the bench and the bedside in ways that shaped this work.

Fieke, you have a rare quality: the willingness to challenge existing ideas, not to dismiss them, but to make them better. Discussing ideas with you always brought clarity. Your calm and thoughtful way of engaging made even complex questions feel approachable.

I would like to express my sincere gratitude to the members of my doctoral committee for their time, expertise, and willingness to evaluate this work: Prof. dr. E.T. van Bavel, Prof. dr. ir. L. van der Weerd, Dr. M.R. de Vries, Prof. dr. M.N. Bekker, Prof. dr. L. Vogt, and Dr. J.W. Ganzevoort. I am honored to have my work assessed by such an esteemed group of scholars.

Lindy, thank you for being such a dependable presence in the lab. You helped me find my footing in so many practical aspects of experimental work. When things did not go as planned, your support kept me going. I am grateful for everything you taught me.

Caren, thank you for your invaluable contribution to this project. Your expertise with the animal model was essential. The surgeries and experimental support you provided were at the core of much of this work. Your involvement in planning experiments and the practical skills I learned from working alongside you shaped this research in meaningful ways.

Raymond, thank you for your role in the TRIPLET consortium, for the work you contributed to our publications, and for taking care of the annual progress reports that kept this project on track.

Judith and Luna, thank you for your help in the chemical lab. Judith, your contribution was particularly significant. From tissue preparation and sectioning to staining and microscopy scanning, your technical skill and patience made a real difference. Luna, your willingness to help with staining and scanning was always appreciated.

Carla, thank you for making so many things simply work. Conference travel, logistics, and all the behind-the-scenes coordination, you handled it all with such care. It made a bigger difference to my daily life as a PhD student than you might realize.

To my colleagues on M01, Nina, Meagan, Morgan, Daisy, Chrysa, Luuk, Shakira, Ellen, Susi, Erik, Federica, and Daphne, thank you for the lunches, the laughter, and the sense of community that made every day better. A special thanks to those of you who volunteered to help me during experiments: Nina, Shakira, Ellen, and especially Daphne, who gave more than she ever needed to. That kind of generosity is something I will not forget.

Daisy and Morgan, a special thank you is due. As my office mates, you were the people I saw every single day, and our little coffee corner became one of my favorite parts of the working day. And as my paranymphs, you will be by my side on one of the most important days of my PhD journey. I could not have chosen better.

To the Z0 group at the Department of Nuclear Imaging, being part of the weekly MRI research meetings gave me a broader perspective on my work. I always left those meetings with something to think about.

To my colleagues at the Department of Biomedical Engineering and Physics, the weekly CVEng meetings were a good exercise in communicating science beyond your own field. Presenting to an interdisciplinary audience teaches you to think about your work differently, and that is a skill I will carry forward.

Anas, I am glad our offices were once neighbors. There was always something special about crossing paths with you. The instant ease of speaking Arabic meant a great deal. Thank you for the warmth you brought to ordinary days.

Vanja and Michael, what started as organizing committee of the ISMRM Benelux conference turned into a friendship I truly treasure. Our regular sushi dinners became one of the things I looked forward to most. You are both brilliant and fun, and you made life abroad feel warm and like home.

To my friends back home, thank you for always making time to meet during my holidays. Those moments of warmth and familiarity were more restorative than you know.

To my family, you are the foundation of everything.

My husband, my biggest supporter. As I often say, I am lucky to have my own psychologist at home. You took on so much to give me the time and space I needed to work and to rest. This thesis carries your effort in it too.

To my son, smart, considerate, and endlessly surprising. Thank you for helping me prepare for presentations and for being patient with a mother whose mind was sometimes on MRI sequences at the dinner table.

To my parents, your support, your prayers, and your unwavering belief in me have been a quiet strength throughout this journey. Knowing you were rooting for me, even from afar, made the hard moments easier to carry.

To my siblings, thank you for your love and your check-ins. The conversations, the calls, and the simple act of asking how things were going were never small gestures. They were a reminder that I was never doing this alone.

To my in-laws, thank you for your support and your prayers throughout these years. Your warmth and encouragement were a steady presence behind the scenes, and knowing you were in our corner meant more than I can say.

## About the Author

Fatimah Al Darwish was born in 1991 in Dhahran, Saudi Arabia. She obtained her Bachelor of Science in Cardiovascular Technology from the University of Dammam in 2014. During and after her studies, she trained and volunteered as a Cardiac Technology Specialist at several hospitals in the Eastern Province of Saudi Arabia, where she gained hands-on experience in adult and pediatric echocardiography, ECG and Holter monitoring, and cardiac image analysis.

In 2018, she moved to the Netherlands to pursue a Master's degree in Cardiovascular Research at Vrije University Amsterdam, which she completed cum laude in 2020. Her Master's took her through two research internships: at the Department of Clinical Genetics at the Vrije Universiteit Medical Center, under the supervision of Dr. Natalija Bogunovic, she investigated the role of PRDM proteins in the development of abdominal aortic aneurysm; and at the Department of Biomedical Engineering and Physics at Amsterdam UMC, under the supervision of Prof. Gustav Strijkers and Prof. Rob Wüst, she contributed to a study on cardiac adaptations to prolonged bed rest. This research came together in a publication in *Experimental Physiology*.

In 2021, she began her PhD at the Department of Biomedical Engineering and Physics at Amsterdam University Medical Centers, University of Amsterdam under the supervision of Prof. Gustav Strijkers, Dr. Bram Coolen, Prof. Titia Lely, and Dr. Fieke Terstappen. Her doctoral research focused on placental insufficiency in the RUPP rat model, using a multimodality imaging approach, including T2\* mapping, 3D dynamic contrast-enhanced MRI, contrast-enhanced ultrasound, and photoacoustic imaging, to assess feto-placental oxygenation and perfusion, with longitudinal assessment of placental perfusion using 3D dynamic contrast-enhanced MRI, alongside evaluation of placental vascular remodeling over time.

Throughout her PhD, she presented her research at several national and international conferences, and her findings came together in publications in *NMR in Biomedicine*, *Cells*, and *Placenta*. Beyond her research, she also took part in various organizational activities during her studies, including the ISMRM Benelux organizing committee in 2023.

With her thesis complete, she is now looking for the next opportunity to apply her expertise.



