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Version Post-print/Accepted Manuscript


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A mouse model of antepartum stillbirth

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Conflict of Interest Statement: The authors report no conflict of interest.
Financial Support: Funded by Canadian Institutes of Health Research Grant MOP130403.

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Abstract Word Count: 387
Main Text Word Count: 3,880
Condensation: In a mouse model of antepartum stillbirth, deficits in placental function result in fetal growth restriction and hypoxic organ injury.
Abstract

**Background:** Many stillbirths of normally-formed fetuses in the third trimester could be prevented via delivery if reliable means to anticipate this outcome existed. However, since the etiology of these stillbirths is often unexplained and, although the underlying mechanism is presumed to be hypoxia from “placental insufficiency”, the placentas often appear normal on histopathologic examination. Gestational age is a risk factor for antepartum stillbirth, with a rapid rise in stillbirth rates after 40 weeks gestation. We speculate that a common mechanism may explain antepartum stillbirth in both the late-term and post-term periods. Mice also show increasing rates of stillbirth when pregnancy is artificially prolonged. The model therefore affords an opportunity to characterize events that precede stillbirth.

**Objective:** To prolong gestation in mice and monitor fetal and placental growth and cardiovascular changes.

**Study Design:** From E15.5 to E18.5, pregnant CD-1 mice received daily progesterone injections to prolong pregnancy by an additional 24-hour period (to embryonic day 19.5). To characterize fetal and placental development, experimental assays were performed throughout late gestation (E15.5 to E19.5), including post-natal day 1 pups as controls. In addition to collecting fetal and placental weights, we monitored fetal blood flow using Doppler ultrasound and examined the feto-placental arterial vascular geometry using microcomputed tomography. Evidence of hypoxic organ injury in the fetus was assessed using magnetic resonance imaging and pimonidazole immunohistochemistry.
**Results:** At E19.5, mean fetal weights were reduced by 14% compared with control post-natal day 1 pups. Ultrasound biomicroscopy showed that fetal heart rate and umbilical artery flow continued to increase at E19.5. Despite this, the E19.5 fetuses had significant pimonidazole staining in both brain and liver tissue, indicating fetal hypoxia. Placental weights at E19.5 were 21% lower than at term (E18.5). Microcomputed tomography showed no change in quantitative morphology of the feto-placental arterial vasculature between E18.5 and E19.5.

**Conclusions:** Prolongation of pregnancy renders the murine fetus vulnerable to significant growth restriction and hypoxia due to differential loss of placental mass rather than any compromise in feto-placental blood flow. Our data are consistent with a hypoxic mechanism of antepartum fetal death in human term and post-term pregnancy and validates the inability of umbilical artery Doppler to safely monitor such fetuses. New tests of placental function are needed to identify the late-term fetus at risk of hypoxia in order to intervene by delivery to avoid antepartum stillbirth.

**Keywords:** antepartum stillbirth, fetal hypoxia, feto-placental circulation, intrauterine growth restriction, mouse, placenta, small for gestational age
Introduction

Stillbirth is the cause of death of 2.6 million babies annually and over 50% of these occur during the antepartum period (occurring prior to labor). Despite efforts to identify pregnancies at high risk of stillbirth during late gestation, the rate of antepartum stillbirth in developed countries have not changed significantly in recent years. In large part this lack of progress is due to our lack of understanding of the underlying etiology of antepartum stillbirth of the normally-formed fetus. A number of features including chronic meconium staining may support an asphyxia mechanism of fetal death; however, the underlying causal pathway is often difficult to establish, even following detailed autopsy. Low birthweight may suggest undiagnosed growth restriction from so-called “placental insufficiency” but establishing a causal relationship from autopsy data is challenging because the fetus loses weight during the interval between fetal death and delivery and the assessment of fetal or placental molecular signals is compromised. In addition, while the finding of specific histo-pathologic abnormalities such as placental infarction or chronic inflammation are common, these findings on their own do not imply a functional deficit in terms of oxygen delivery to the fetus.

There are many risk factors for unexplained antepartum stillbirth including advanced maternal age, multiparity, maternal smoking, obesity, exposure to environmental pollutants and inadequate antenatal care attendance. In addition, there is a strong association between antepartum stillbirth and gestational age. Pregnancies that remain undelivered at 40 weeks of gestation are at a subsequently higher risk of antepartum stillbirth. After 39 weeks
of gestation, the mortality risk of expectant management is higher than the risk of immediate delivery (i.e. at 39 weeks of gestation the calculated risk is 12.9 per 10,000 for expectant management compared with 8.8 per 10,000 for delivery). Moreover, the rate of unexplained fetal death increases at 41 weeks (1.24 per 1,000). To address this dilemma in obstetrical practice, several groups have explored whether induction of labor before term is justified; however, the results of these observational studies are heterogeneous. Attempts to address the issue by implementing “the 39-week rule” in the United States have shown conflicting results for the risk of stillbirth with induction at 39 weeks of gestation. Determination of optimal timing of delivery amongst specific high-risk populations such as twin pregnancies and pregnancies complicated by cholestasis has been attempted, yet the issue remains a matter of debate. To better inform management of pregnancies during late gestation, an understanding of the pathways by which the fetus is at risk of stillbirth is still required. A key observation of normal pregnancy after 36 weeks of gestation is a deceleration of fetal growth velocity, which is generally regarded as physiologic. However, this decline in fetal growth velocity towards the end of pregnancy may indicate that the growth potential of the fetus is not achieved due to suboptimal placental function. This may render the human term/post-term fetus vulnerable to stillbirth. To support this hypothesis, large population-based studies demonstrate that the risk of antepartum stillbirth at term is inversely associated with fetal growth. We speculate that the mechanisms that lead to stillbirth are similar for both the late-term and post-term period because both are characterized by reduced growth velocity.

Progesterone is an essential hormone for maintaining pregnancy and, in humans, functional withdrawal of progesterone activity at the uterus is associated with the onset of labor. The administration of progesterone is effective at prolonging human pregnancies at risk for
Several groups have shown in mice and rats that administration of progesterone during late gestation will prolong pregnancy, resulting in significant stillbirth rates after two days (>20%). The mouse reproduces many of the physiologic and molecular features of human pregnancy including that it develops a hemochorial placenta. Since the murine fetus and its placenta can now be interrogated during gestation with high-frequency ultrasound, the mouse pregnancy model has great potential to address the underlying pathophysiology of common pregnancy complications such as antepartum stillbirth. Moreover, we have developed a method for visualizing the micro-circulation of the mouse placenta using microcomputed tomography (micro-CT) and can characterize the growth of the feto-placental vascular tree across gestation. To study the pathways by which the fetus is at risk for stillbirth, we administered progesterone to mice during late gestation to prolong pregnancy and monitored the fetal and placental growth and cardiovascular changes that precede stillbirth.

**Materials and Methods**

*Study design.* The objective of the present study was to investigate the mechanisms that lead to antepartum fetal death using a mouse model of prolonged pregnancy. We artificially prolonged pregnancy by an additional 24-hour period by administration of exogenous progesterone. Full term for CD-1 mice is 18.5 days and therefore 24-hour prolongation increases the duration of pregnancy by 5%; equivalent to two weeks in humans. We studied the effect of this intervention on fetal and placental growth and cardiovascular development throughout late gestation (E15.5 to E19.5), including post-natal day 1 pups as controls. We monitored umbilical artery blood flow in utero using Doppler ultrasound and performed detailed analysis of feto-placental arterial vascular geometry using micro-CT. This was combined with assessment of asphyxia injury in target fetal organs using magnetic resonance imaging (MRI) and immunohistochemistry.
Animals. Adult CD-1 mice (6-9 weeks of age) from Charles River Laboratories (St Constant, QC, Canada) were used and mated in-house. The number of animals studied using each experimental assay is described in the subsections below. The morning that a vaginal plug was detected was designated as Embryonic Day 0.5 (E0.5). By convention, the age of postnatal mice is reported in days after birth (post-natal day 0) so that a P1 mouse pup is of equivalent post-conceptional age to an E19.5 mouse fetus. P1 mice were used for this reason as a control group for evaluating the E19.5 fetuses. All animal experiments were approved by the Animal Care Committee at the Toronto Centre for Phenogenomics and conducted in accordance with guidelines established by the Canadian Council on Animal Care.

Progesterone administration. From E15.5 to E18.5, pregnant mice were randomized to either receive daily subcutaneous injection of medroxyprogesterone acetate (Sigma-Aldrich) at 16 mg/kg in 0.4 mL sterile saline or to a control group that received no injections.

Ultrasound biomicroscopy. Pregnant mice at E15.5 (8 fetuses from 4 litters), E17.5 (8 fetuses from 4 litters), E18.5 (8 fetuses from 4 litters) and E19.5 (8 fetuses from 3 litters) were imaged using a high frequency ultrasound system (Vevo 2100; VisualSonics, Toronto, ON, Canada) with a 40 MHz linear array transducer as previously described. Two to three fetuses per dam with the most favourable spatial orientation (either right or left lateral position) in the lower abdomen were chosen for imaging. The dams were anesthetized with 1.5% isoflurane in 21% oxygen and the maternal body temperature was maintained at 36-37 °C. The blood flow in the umbilical artery was calculated using Doppler and M-mode recordings for velocity and diameter measurements respectively. The pulsed wave Doppler sample volume was adjusted to cover the entire vascular lumen and the angle of insonation was kept as small as possible (< 60°) with angle correction applied for analysis. M-mode recordings were made with the ultrasound beam.
perpendicular to the artery and at the same location as the Doppler spectrum. The flow was calculated by multiplying the velocity-time integral by the vessel area and the heart rate. All parameters were averaged over three cardiac cycles. The pulsatility index was calculated as the difference between peak systolic and end diastolic velocities, divided by the mean velocity over the cardiac cycle.

**Magnetic resonance imaging.** Pregnant mice at E17.5 (9 fetuses from 3 litters) and E19.5 (6 fetuses from 3 litters) were sacrificed by cervical dislocation. Fetuses for imaging were chosen randomly from the left and right side of the maternal abdomen. The fetuses were dissected from the uterus and fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and 2mM ProHance. The P1 pups (6 pups from 3 litters) were sacrificed and processed as described above. An anatomical MRI scan was performed using a custom-built 16-coil array and a T2-weighted, 3D fast-spin echo sequence (TR = 350 ms, echo train length = 6, TE_{eff} = 12 ms, field-of-view = 20x20x25 mm, matrix size = 504x504x630, isotropic resolution = 40 μm). Due to their larger size, the E19.5 and P1 specimens were scanned in a 7-coil millipede array. An anatomical T1-weighted, 3D gradient echo sequence was performed (TR = 50 ms, echo train length = 6, TE = 2.5 ms, field-of-view = 38x23x23, matrix = 280x168x168, isotropic resolution = 136 μm). The brain, lungs and liver were manually segmented in 3D on each of the MR images.

**Micro-CT imaging.** In a separate cohort, pregnant mice at E15.5 (5 placentas from 2 litters), E17.5 (8 placentas from 5 litters) and E19.5 (9 placentas from 7 litters) were prepared for micro-CT imaging as described previously. Briefly, the dams were sacrificed by cervical dislocation. The embryo and placenta were bathed in warm PBS to resume cardiac function, a cannula was inserted into the umbilical artery and heparinized saline, followed by the contrast agent (MV-122 Microfil, Flow Technology, Carver, MA) were manually infused. All placentas
for which the fetal heart was restarted were perfused. The placentas were fixed in 10% formalin and imaged using micro-CT (Skyscan 1272 scanner, Skyscan, Belgium). Three-dimensional datasets were acquired with each specimen rotated 360° and a resolution of 7.1 μm. The vascular structure was automatically identified using a previously described segmentation algorithm. Detection of vessels with a diameter smaller than 35 μm was unreliable and therefore the terminal segments of the vascular tree were pruned to 35 μm to improve data consistency. Feto-placental arterial span and depth were measured directly from surface renderings via digital calipers using the Amira software package (Visage Imaging, San Diego, CA).

**Histology.** Fetal tissue hypoxia was assessed using pimonidazole hydrochloride (Hypoxyprobe, Burlington, MA, USA). A subset of dams were injected with Hypoxyprobe (60 mg/kg) and fetuses at E18.5 (8 fetuses from 5 litters) and E19.5 (6 fetuses from 4 litters) were collected 2 hours following injection and fixed in 4% PFA for 3 days. The tissue was paraffin-embedded and cut into 5 μm sections for staining with an anti-pimonidazole monoclonal antibody (MAb1, Burlington, MA, USA). Staining quantification (percentage area of brown staining) was conducted using ImageJ software.

**Statistical analysis.** All statistical tests were conducted using the R statistical software (www.r-project.org). Data from each gestational age are reported as mean ± standard error of the mean (SEM). For the staining quantification, multiple sections per fetus were analyzed and therefore a linear mixed effects model was used with fetus as the random variable. The rest of the data was analyzed using two-way ANOVA to evaluate the effects of gestational age and litter. Litter was a significant factor in placental weights and feto-placental weight ratios thus litter means were used for statistical analysis. If the ANOVA was significant, Tukey post hoc tests were performed. A value of \( p < 0.05 \) was taken to be significant.
Results

To optimize the prolonged pregnancy mouse model, we sacrificed dams one and two days post-term (where term is E18.5 in CD-1 mice). The dams at E20.5 (n=3) had lost weight compared to their weight at E19.5 and suffered a high rate of fetal demise. While we observed no fetal loss at E19.5, there were at least 12 stillbirths in the cohort of dams at E20.5. We concluded that one day post-term was the most clinically relevant time point for studying the pathways by which the fetus is at risk of stillbirth. To characterize the fetal and placental development in the mouse model of prolonged pregnancy, experimental assays were performed throughout late gestation (E15.5 to E19.5), including P1 pups as controls where appropriate.

Prolonged pregnancy results in fetal growth restriction and decreased placental weights.

Litter size did not significantly change with gestational age (12 ± 1 fetuses/litter). Maternal and fetal body weight increased during the normal period of gestation (Figure 1a,b) and maternal weight continued to increase at one day post-term. At E19.5 the fetuses weighed 14% less than the control P1 pups, indicating that the post-term fetuses fail to reach their postnatal growth potential. Placental weights remained similar between E15.5 and E18.5, whereas the placental weights decreased by 21% between E18.5 and E19.5 (Figure 1c). Another measure of fetal health is the feto-placental weight ratio,

$$\frac{\text{fetal weight}}{\text{placental weight}}$$

when the feto-placental weight ratio is high, the demand of the fetus may exceed the capacity of the placenta to provide oxygen and nutrients. The feto-placental weight ratio was significantly increased at E19.5 compared to E18.5
such that, post-term, the placenta had to support more fetus per gram than during the normal period of gestation.

Umbilical artery blood flow was unaffected by prolonged pregnancy.

Representative umbilical artery Doppler waveforms in late gestation are shown in Figure 2a. There was a progressive increase in umbilical artery blood flow, umbilical artery diameter and fetal heart rate with gestational age (Figure 2b-d). Our group has previously described a rapid increase in heart rate in CD-1 mice during the embryonic and neonatal period. The reported fetal heart rates at E17.5 are similar between Zhou et al. and this study; however, the E19.5 heart rate is 1.6-fold higher than the P1 controls. The umbilical artery pulsatility index, a clinical metric of placental vascular resistance, decreased with gestational age; however, there was no difference between E18.5 and E19.5 (Figure 2e).

Fetal organ volume growth with prolonged pregnancy.

The brain, lung and liver volumes for E17.5 compared to post-term (E19.5 and P1) are summarized in Table 1. The absolute brain volume increased significantly between E17.5 and E19.5; however, there was no difference between E19.5 and P1 pups, indicating protection of brain growth. When normalized to fetal body weight, the difference in brain volume was no longer significant between E17.5, E19.5 and P1. The lung volume (both absolute and normalized) increased significantly after birth compared to both E17.5 and E19.5 while the liver showed a significant loss in volume.

Feto-placental arterial vascular morphology did not continue to remodel with prolonged pregnancy.
The geometry of the feto-placental arterial tree is summarized in Table 2. The depth and span of the tree did not change significantly between E15.5 and E19.5. Compared to the arterial tree at E15.5, there was a significant increase in the vascular volume at E17.5 (3.1-fold) and at E19.5 (2.6-fold). From E15.5 to E17.5, there was a significant increase in the number of vessel segments across all levels of the feto-placental arterial tree (arterioles (35-75 μm), intraplacental arteries (75-150 μm) and chorionic plate arteries (> 200μm)). However, there were no significant changes from E17.5 to E19.5 (Figure 3). These data indicate no further capacity of the feto-placental arterial tree to expand in post-term pregnancy in mice.

*Prolonged pregnancy causes hypoxia in the fetal brain.*

Increased feto-placental weight ratio suggested that the E19.5 fetuses would be hypoxic. To test this hypothesis, we used the immunohistochemical tissue hypoxia marker pimonidazole hydrochloride (Figure 4). There was strong immunoreactivity in the brains and livers of E19.5 fetuses whereas E18.5 fetuses only had faint staining in the liver. Semi-quantification of the hypoxic cells revealed a significant increase of staining in the E19.5 fetuses compared to term fetuses in both the brain (p=0.04) and liver (p=0.006).

**Comment**

*Principal findings of the study.*

Inadequate placental function causes stillbirth mediated by growth restriction and hypoxic injury

Prolonging mouse pregnancy by 24 hours, analogous to approximately two weeks in humans, resulted in fetal growth restriction and fetal hypoxia that we attribute to a progressive deficit in placental function. The feto-placental weight ratio provides a measure of the ongoing capacity of the placenta to meet the demands of the growing fetus. Between E15.5 and E17.5 the feto-placental weight ratio doubled, illustrating that the placental reserve steadily declines
towards term. The capacity to deliver oxygen and flow-dependent nutrients in the face of a declining feto-placental weight ratio was sustained via a doubling of the umbilical artery blood flow and an even greater increase in the feto-placental total vascular volume. In parallel, to support rapid fetal growth the uteroplacental blood flow is known to increase and the volume and surface area of the labyrinth zone, where most of the gas and nutrient exchange occurs, increases. Overall, these data indicate that by E18.5 the mouse placenta has evoked all of its compensatory mechanisms, such that any prolongation of pregnancy would become detrimental to fetal health especially in the face of further fetal growth. Consistent with this hypothesis, the E19.5 fetus demonstrated its ability to continue to grow although not at the same rate as term fetuses. This suboptimal additional fetal growth, in comparison with the initial post-natal period occurred in conjunction with a further increase in umbilical artery blood flow and thus illustrates the inherent vulnerability of the late-term fetal environment. Moreover, the umbilical artery pulsatility index did not continue to decrease post-term, suggesting the placental vessels are not able to further dilate to decrease the placental vascular resistance.

*Fetal compensation mechanisms are exhausted in late gestation*

The substantial increase in fetal heart rate at E19.5 is consistent with a fetal stress response and is analogous to the non-reactive fetal tachycardia on the human fetal non-stress test that would result in an emergency Cesarean delivery. More compelling evidence that the post-term placenta is unable to safely sustain the fetus between E18.5 and E19.5 is that the placental weight decreased. In addition, the feto-placental arterial vasculature did not continue to remodel. During the two-day time period between E15.5 and E17.5, there was a 3.1-fold increase in the placental vascular volume and a 1.9-fold increase in the number of vessel segments. We anticipated that the placenta would continue to expand post-term; however, during the two days
between E17.5 and E19.5, there is no further increase in vascular growth. It should be noted that only vessels greater than 35 μm are reliably visible by micro-CT imaging and therefore we are not able to determine if there were any differences in the number of capillaries of the post-term placenta, which is the principal site of diffusional gas exchange. Our immunohistochemical studies demonstrated that the brains and livers in the post-term fetuses were hypoxic, indicating that the progressive deficit in placental function led to inadequate oxygen delivery.

*No evidence of brain sparing physiology with prolonged pregnancy*

Despite the finding of hypoxic fetal organ injury, the brain and liver volumes continued to increase between E17.5 and E19.5. There was no significant difference between the brain volume of the post-term fetus and the P1 control. The significant decrease in liver volume after birth may be explained by the known loss of glycogen stores, but in addition the abrupt cessation of umbilical venous blood flow may contribute to this change. An important feature of acute fetal hypoxia is brain sparing, a redistribution of the oxygenated blood to the brain at the expense of other organs such as the lungs and liver. In humans this phenomenon is detected using middle cerebral artery Doppler and has been reported in post-term pregnancies. Here, the post-term fetuses do not show any evidence of brain sparing physiology (i.e. decrease in lung or liver volume:fetal weight ratio). To fully explore the dynamics of oxygen delivery to the fetal brain in the period E17.5-E19.5, we would need to perform non-invasive longitudinal studies using measurements such as Doppler ultrasound of the cerebral arteries or blood oxygen level-dependent MRI.

*Comparison with other studies.*

The finding that the feto-placental arterial vascular tree does not continue to expand post-term is consistent with a recent human study using three dimensional power Doppler which
reported that there was no difference in placental volume or vascularisation index between term and post-term pregnancies.\textsuperscript{64} Since the vascular tree is preserved, other cell types contributing to placental weight, especially trophoblast lineages, may therefore be lost, via apoptosis, during this time period. Both the mouse\textsuperscript{65} and the human placenta\textsuperscript{66} have been shown to undergo trophoblast apoptosis with advancing gestational age. Post-term, we speculate that the ageing placenta has continued to break down. This is supported by work done in mice that lack the prostaglandin F receptor and, as a result, fail to deliver fetuses.\textsuperscript{67} Mu and co-workers report an increase in apoptosis in the placenta and decidual tissue in the post-term placentas of the FP-deficient mice compared to term. High rates of apoptosis and necrosis in the outer villous trophoblast layer have been reported in post-term human pregnancies.\textsuperscript{68,69}

\textit{Clinical significance.}

Antepartum stillbirth remains a definite risk, and is compounded by our inability by ultrasound to detect the subtle degrees of fetal growth restriction and/or placental damage which is found at autopsy in term and post-term stillbirths. Currently, several non-invasive tests of “fetal wellbeing” are used to determine the timing of delivery in late-term fetuses,\textsuperscript{70} including fetal heart rate tracing, the quantity of amniotic fluid\textsuperscript{71,72} and the overall fetal biophysical profile. The risk of fetal death in the subsequent 48 hours is low, approximately 1-2 per 1,000 when these tests are normal. However, these are all indirect tests of fetal oxygenation, and do not address the issue highlighted by our study, that despite normal feto-placental blood flow, both the human and mouse fetus outstrips the capacity of its placenta to sustain adequate oxygenation in the late-term period. Our data are thus consistent with human research that shows no utility of umbilical artery Doppler in the assessment of term and post-term pregnancies.\textsuperscript{73–77} New tests that directly assess the fetal response to mild hypoxia in term fetuses, such as middle cerebral artery
Doppler\textsuperscript{78–82} and cerebroplacental ratio,\textsuperscript{83} may have diagnostic utility, and therefore merit further investigation, including with this model.

\textit{Research significance.}

The mouse model of prolonged pregnancy presented in this study is easily implemented, provides experimental accessibility and reproduces several important features of stillbirth in human pregnancies. Moreover, this model could provide a basis for developing new diagnostic techniques to identify fetuses at elevated risk of stillbirth including ultrasound of the cerebral vessels to study brain sparing physiology and blood oxygen level-dependent MRI to measure changes in blood oxygen saturation. In addition, this model could be utilized to study interventions to promote fetal survival (for example maternal hyperoxygenation).

\textit{Strengths and limitations.}

The main strength of this study is the use of state-of-the-art imaging to interrogate the mechanisms that lead to fetal death in a reproducible mouse model of prolonged pregnancy. The ultrasound techniques are directly translated from clinical methods whereas the X-ray micro-CT provides insight into vascular morphology that at present are impossible to obtain in humans. A limitation of the current study is that the control group did not receive vehicle injections. Given that the focus of this paper is not on maternal physiology, we do not believe receiving vehicle injections would have had any effect on the chosen experimental assays.

\textit{Conclusions}

In summary, we present a mouse model of prolonged human pregnancy where hypoxic fetal organ injury occurs secondary to a progressive deficit in placental function. The underlying basis of inadequate oxygen and nutrient transfer is likely due to defects in cells outside the fetoplacental vascular system, most likely in the trophoblast lineages. This model will be useful for
evaluating a variety of diagnostic methods, including 3D ultrasound and fetal MRI, \(^{84,85}\) for measuring placental volume, detecting deficits in placental function and for the early identification of reversible fetal hypoxia.

References:

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Table 1. Organ volume measurements. Data are presented as mean ± SEM. * p < 0.05 when compared to E17.5; ‡ p < 0.05 when compared to E19.5

<table>
<thead>
<tr>
<th>Organ</th>
<th>E17.5 (n=9)</th>
<th>E19.5 (n=6)</th>
<th>P1 (n=6)</th>
<th>E17.5 (n=9)</th>
<th>E19.5 (n=6)</th>
<th>P1 (n=6)</th>
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<td>Volume (mm³)</td>
<td>73.1 ± 2.0</td>
<td>100.6 ± 3.4*</td>
<td>107.6 ± 3.5*</td>
<td>64.6 ± 1.9</td>
<td>63.4 ± 1.8</td>
<td>59.6 ± 3.9</td>
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<td>Volume:Fetal weight (mm³/g)</td>
<td>100.6 ± 3.4*</td>
<td>107.6 ± 3.5*</td>
<td>64.6 ± 1.9</td>
<td>63.4 ± 1.8</td>
<td>59.6 ± 3.9</td>
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</table>

Table 2. Measurements of the feto-placental arterial tree from micro-CT imaging. Data are presented as mean ± SEM. There were no differences in feto-placental arterial vascular morphology between E17.5 and E19.5. * p < 0.05 when compared to E15.5

<table>
<thead>
<tr>
<th>Vascular depth (mm)</th>
<th>E15.5 (n=5)</th>
<th>E17.5 (n=8)</th>
<th>E19.5 (n=9)</th>
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<td>1.95 ± 0.04</td>
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<td>Vascular span (mm)</td>
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<td>Vascular volume (mm³)</td>
<td>2.54 ± 0.15</td>
<td>7.94 ± 1.15*</td>
<td>6.55 ± 1.03*</td>
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<td>No. vessel segments</td>
<td>3845 ± 273</td>
<td>7441 ± 547*</td>
<td>6774 ± 521*</td>
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<tr>
<td>Total length of vasculature (mm)</td>
<td>581 ± 23</td>
<td>914 ± 47*</td>
<td>992 ± 56*</td>
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Figures:

Figure 1. Developmental changes in CD-1 mice from E15.5 to E19.5. A) Maternal weights at E15.5 (N=19 dams), E17.5 (N=19), E18.5 (N=13) and E19.5 (N=33). There was a progressive increase in maternal weight with gestational age (p<0.001, linear regression). B) Fetal weights at E15.5 (n=57 fetuses), E17.5 (n=195), E18.5 (n=52), E19.5 (n=127), P1 (n=77). The growth trajectory is slowed for the post-term fetuses (dashed line) compared to the term fetuses (solid line) (*p<0.001 post-term (E19.5) when compared to P1 (term)). The weights are significantly different between all pairs of gestational ages. C) Placental weights remained similar between E15.5 and E18.5 and decreased significantly post-term (p=0.005, one-way ANOVA followed by Tukey post hoc test). D) The feto-placental weight ratio was significantly different between all
pairs of gestational ages except for E17.5-E18.5. N refers to the number of dams. Data are shown as mean ± SEM.

**Figure 2.** A) Representative umbilical artery Doppler flow waveforms. B) Umbilical artery blood flow, C) umbilical artery diameter, D) fetal heart rate and E) umbilical artery pulsatility index at E15.5 (n=8 fetuses), E17.5 (n=8), E18.5 (n=8) and E19.5 (n=8). There was a significant progressive increase in umbilical artery blood flow (p<0.001, linear regression), umbilical artery diameter (p<0.001) and fetal heart rate (p<0.001) and a significant progressive decrease in pulsatility index (p=0.005) with gestational age. Data are shown as mean ± SEM.
Figure 3. A) Representative surface renderings of feto-placental arterial vascular trees from E15.5, E17.5 and E19.5. Scale bar = 1 mm. B) Total vascular volume (mm$^3$) and total number of vessel segments (> 35 μm) at E15.5 (n=5 placentas), E17.5 (n=8) and E19.5 (n=9). There was a significant increase in both total vascular volume and number of vessel segments between E15.5 and E17.5 but no differences between E17.5 and E19.5 (one-way ANOVA followed by Tukey post hoc test). Data are shown as mean ± SEM.
**Figure 4.** Fetal hypoxia detected using pimonidazole immunohistochemistry (brown stain) in the brain and liver at E18.5 (A,B) and E19.5 (C,D). Negative controls (E,F) were not injected with Hypoxyprobe. Pimonidazole staining was seen in both the nucleus and the cytoplasm (insets).
Percent area of brain (G) and liver (H) with tissue hypoxia staining. *p<0.05, differences between groups as determined by a linear mixed effects ANOVA model followed by Tukey post hoc tests. Data are shown as mean ± SEM, n refers to the number of fetuses. Scale bar = 1 mm (scale bar = 25 μm in insets).