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Maternal-placental-fetal drug metabolism is altered by late gestation undernutrition in the pregnant ewe

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Running title: Late gestation undernutrition in sheep alters maternal-placental-fetal drug metabolism

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HIGHLIGHTS

1. Late gestation undernutrition (LGUN) did not change hepatic- or placental-specific CYP activity
2. LGUN reduced CYP1A2 activity in the maternal compartment of the maternal-placental-fetal unit
3. Fetal glucose infusion in the presence of LGUN did not rescue any observed changes
4. These responses to LGUN may have consequences for fetal outcomes

ABSTRACT

Background: Maternal undernutrition during pregnancy disrupts both fetal growth and development with perturbations to certain physiological processes within the maternal-fetal-placental unit, including metabolic function. However, it is unknown if hypoglycemia during pregnancy alters maternal-fetal-placental drug metabolism as mediated by cytochrome P450 (CYP) enzymes. Despite this, hypoglycemia reduces CYP enzyme activity in non-pregnant animals. We therefore hypothesised that in a sheep model of hypoglycemia induced by late gestation undernutrition (LGUN), maternal-fetal-placental CYP activity would be reduced, and that fetal glucose infusion (LGUN+G) would rescue reduced CYP activity.

Methods: At 115d gestation (term, 150d), ewes were allocated to control (100% metabolic energy requirement (MER); n=11), LGUN (50% MER; n=7) or LGUN+G (50% MER + fetal glucose infusion; n=6) and maintained on their diets until post-mortem. Maternal-fetal-placental CYP activity assays were performed at 131-133d gestation. Microsomes were isolated from placenta and fetal liver collected at 139-142d gestation and incubated with CYP-specific probe drugs. Metabolite concentrations were measured using Liquid Chromatography – tandem mass spectrometry (LC-MS/MS).

Results: CYP2C19 and CYP3A were undetectable in placenta or fetal liver, and CYP1A2 was undetectable in the fetal liver. Placental-specific CYP1A2 and CYP2D6 activity and hepatic-specific CYP2D6 activity were unaffected by LGUN. Maternal-fetal-placental CYP1A2 activity was reduced in response to LGUN in the maternal compartment only.

Conclusions: Reduced maternal-fetal-placental CYP1A2 activity, but not placental-specific CYP1A2 activity, may lead to the developing fetus being exposed to increased concentrations of CYP1A2-specific substrates and suggests further consideration of drug dosing is required in instances of late gestation maternal undernutrition.

KEYWORDS: maternal undernutrition; cytochrome P450; microsomes; drug metabolism; fetal development

INTRODUCTION

Appropriate intrauterine growth throughout gestation is required to ensure normal perinatal outcomes. However, restrictions to *in utero* substrate supply hinder the fetuses normal growth trajectory, culminating in intrauterine growth restriction (IUGR); a condition associated with an increased risk of perinatal morbidities and mortality (Pallotto & Kilbride, 2006; Sharma *et al.*, 2016). The aetiology of IUGR is often a result of placental dysfunction (Morrison, 2008; Clifton, 2010; Zhang *et al.*, 2015; Darby *et al.*, 2020). Placental dysfunction can result in reduced oxygen and nutrient delivery to the feto-placental unit, leading to reduced intrauterine fetal growth, hypoxemia, and hypoglycemia in several preclinical models (Krishna & Bhalerao, 2011; Burton & Fowden, 2015; Dimasuay *et al.*, 2016; Gaccioli & Lager, 2016; Zhang *et al.*, 2016) and disrupted fetal organ development (Morrison *et al.*, 2007; Morrison, 2008; Darby *et al.*, 2020). Consequently, these perturbations to fetal growth and development may disrupt physiological processes within the feto-placental unit.

Fetal metabolic function is mediated via the biosynthesis and degradation of proteins, carbohydrates, and lipids within the maternal and fetal liver, as well as fetal extrahepatic tissue including the placenta (Bristow *et al.*, 1983; Garnica & Chan, 1996). Appropriate maternal-placental-fetal metabolic function is also necessary for the metabolism and clearance of endogenous and exogenous chemicals including steroids, drugs and other xenobiotics (Corless & Middleton, 1983; McDonnell & Dang, 2013; Griffiths & Campbell, 2014). This process initially occurs via the tightly regulated action of cytochrome P450 (CYP) enzymes (Zanger *et al.*, 2008; McBride *et al.*, 2020b), of which in humans there are 57 distinct isoforms. Despite this diversity, the majority of clinically prescribed drugs are metabolised by three distinct CYP isoenzymes: CYP3A, CYP1A2 and CYP2D6. Although the mRNA expression of *CYP1A2*, *CYP2D6* and *CYP3A* have been reported in early- and late-gestation placentae of humans and other eutherian mammals and in the late gestation human fetal liver (Hakkola *et al.*, 1996;

Hakkola *et al.*, 2001; Li *et al.*, 2008; Soo *et al.*, 2018; Robinson *et al.*, 2020), the functional activity of these enzymes has not been fully investigated.

Several factors contribute to dysfunction of CYP activity including altered concentrations of steroid hormones, increased reactive oxygen species (ROS) production, and abnormal levels of inflammatory cytokines (Zanger & Schwab, 2013). Interestingly, IUGR is associated with the dysregulation of these factors and may result in altered feto-placental metabolic function. In fact, our group recently identified that the IUGR lamb at postnatal day 21 had significantly reduced hepatic CYP3A activity (Soo *et al.*, 2018). More recently we reported no functional activity of CYP3A in the fetal liver irrespective of fetal growth outcomes (McBride *et al.*, 2021). However, CYP3A activity was reduced in the entire IUGR feto-placental unit, suggesting that extrahepatic CYP3A metabolism is reduced in response to IUGR. The mechanisms that contribute to reduced *in vivo* CYP activity in the IUGR feto-placental unit remains unclear but may be driven by changes in oxygen and/or nutrient supply. Given the intrinsic link between feto-placental hypoxemia and hypoglycemia in the pathophysiology of IUGR, it is unknown whether a reduction in feto-placental oxygen or nutrient supply contribute to metabolic dysfunction, and whether this drives the dysregulation of CYP activity.

Studies in sheep have shown that maternal undernutrition increased uteroplacental glucose uptake, but reduced fetal glucose uptake when compared with uteroplacental and fetal tissue glucose uptake in control animals (Carver & Hay, 1995). This redistribution of glucose consumption within the feto-placental unit may alter tissue-specific metabolic function. For example, reduced glucose availability increases CYP3A activity in primary human hepatocytes (Davidson *et al.*, 2016), whereas increased glucose consumption reduced hepatic CYP1A2 activity (Stewart & Strother, 1999). Whether similar changes in CYP function within the feto-placental unit occurs remains unclear. Therefore, in the current study we have used a sheep

model of late gestation undernutrition (LGUN) to examine the effect of hypoglycemia on maternal-placental-fetal CYP activity. In addition, we have explored whether increased fetal glucose availability by way of a constant fetal glucose infusion (LGUN+G) rescues any perturbations of CYP function observed in the LGUN maternal-placental-fetal unit.

METHODS

All procedures were performed with approval from the South Australian Health and Medical Research Institute's Animal Ethics Committee (SAM351) and complied with the Australian Code for the Care and Use of Animals for Scientific Research. The experimental design was informed by the ARRIVE guidelines (Kilkenny *et al.*, 2010) and the 3Rs (Tannenbaum & Bennett, 2015), and met the Australian Code for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, 2013).

Animals and Surgery

Pregnant Merino ewes carrying singleton fetuses (n=24) were sourced from the SAHMRI farm (Burra, SA). Ewes were housed in individual pens in animal holding rooms maintained at a constant ambient temperature of 20-22°C and a 12 h:12 h light/dark cycle. Surgery was performed on ewes between 109 to 112 d gestational age (GA; term=150 days) under aseptic conditions as previously described (Danielson *et al.*, 2005; Morrison *et al.*, 2005b; Dyer *et al.*, 2009). General anesthesia was induced by an intravenous injection of diazepam (0.3 mg/kg) and ketamine (5 mg/kg) and maintained with 1.5-2.5% isoflurane (Lyppards, South Australia, Australia) in 100% oxygen. Vascular catheters were implanted into a fetal femoral artery and vein and a maternal jugular vein as well as the amniotic cavity as previously described (Morrison *et al.*, 2005b; Dyer *et al.*, 2009) and exteriorized through an incision in the ewe's flank. During surgery, the fetus received subcutaneous injections of antibiotics: 1ml Duplocillin (procaine penicillin 150 mg/ml, benzathine penicillin 125 mg/ml, Intervet, Bendigo, Victoria, Australia) and 1 ml dihydrostreptomycin (125 mg/ml, Sigma) in sterile saline (Lyppards, South Australia, Australia). An intramuscular injection of antibiotics was administered daily to ewes (3.5 ml duplocillin and 2 ml of 125 mg/ml dihydrostreptomycin (Sigma)) for 3 days following the surgery. All ewes were fasted and received meloxicam (0.5 mg/kg, SC) one day before surgery and 24 h later (Varcoe *et al.*, 2019). Each fetus received a

daily dose of sodium ampicillin (500 mg, Clifford Hallam Healthcare, South Australia, Australia) intra-amniotically for 4 days post-surgery.

Nutritional management

At 110-113 d GA, ewes were allocated by minimization (maternal weight, maternal fasting blood glucose and then fetal sex) to control (n=11; female=5, male=6), late gestation maternal undernutrition (LGUN, n=7; female=3, male=4) or late gestation maternal undernutrition + fetal glucose infusion (LGUN+G, n=6; female=1, male=5) and maintained on their respective diet until post-mortem (25 ± 1 d later). The control diet consisted of Lucerne Chaff (15 g/kg) and Ewe and Lamb nuts (4.5 g/kg), containing cereal hay, lucerne hay, barley, oats, almond shells, lupins, oat bran, lime, and molasses (Johnsons & Sons, Kapunda, South Australia, Australia) with a total metabolizable energy intake of 0.17 ± 0.01 MJ/kg/day, which is defined as 100% nutritional requirement (Lie *et al.*, 2013; Nicholas *et al.*, 2013; Lock *et al.*, 2017). The Lucerne Chaff provided 80% of the total energy requirements (8.3 MJ/kg metabolizable energy, 193 g/kg of crude protein, and 85% dry matter) and 20% of the energy requirements were obtained from the Ewe and Lamb nuts (8.0 MJ/kg metabolizable energy, 110 g/kg of crude protein, and 90% dry matter) (Lie *et al.*, 2014). The LGUN group received 50% reduction of the control allowance (0.085 ± 0.01 MJ/kg/day) (Edwards & McMillen, 2001; Lie *et al.*, 2013; Darby *et al.*, 2018). The LGUN+G group received the same amount of metabolizable energy intake as the LGUN group, with continuous glucose infusion through the fetal femoral vein (0.57 ± 0.01 mmol/h dextrose; Baxter Healthcare, South Australia, Australia) (Ren *et al.*, 2021). All the dietary components were reduced by an equal amount in the restricted diet. The feed allowance of all ewes was proportionately increased by 15% every 10 days over the experimental period to meet the increasing substrate demands of the growing fetus.

Blood sampling

After the catherization surgery, fetal blood samples were collected daily from the fetal femoral artery to monitor fetal health by measuring partial pressure of oxygen (PaO_2), partial pressure of carbon dioxide ($PaCO_2$), pH, haemoglobin, haematocrit, oxygen saturation (SaO_2), base excess and lactate with temperature corrected to 39°C (RAPIDPOINT 500, Siemens Healthineers, Erlangen, Germany). Fetal arterial blood samples were also collected into heparinized tubes and centrifuged (3000 *g* at 4°C for 10 min). Plasma was then aliquoted and stored at -20°C for subsequent analysis. Fetal and maternal plasma glucose concentrations were determined as previously described (Dimasi *et al.*, 2021; Lazniewska *et al.*, 2021).

Maternal-placental-fetal CYP activity assay

The *in vivo* activity of specific CYPs was assessed by metabolite formation from specific substrates as previously described (Soo *et al.*, 2020; McBride *et al.*, 2021). At 131-133 d gestation, a bolus subtherapeutic dose of substrate cocktail (0.014 mg/kg midazolam, CYP3A probe (Sandoz); 0.105 mg/kg omeprazole, CYP2C19 probe (Sandoz); 1.35 mg/kg caffeine, CYP1A2 probe (Sigma Aldrich)) was administered intravenously to the ewe and blood was collected from the maternal and fetal compartments at 0, 5, 30, 60, 120 and 240 mins. Whole blood was centrifuged (3000 *g* at 4°C for 10 min) and the plasma was removed, aliquoted and stored at -30 °C until further analysis.

CYP substrate and metabolite quantification

CYP substrates and metabolites were quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described (McBride *et al.*, 2021). Briefly, internal standard containing Midazolam-d6, caffeine-d3, paraxanthine-d3, omeprazole-d3 and 5'-hydroxyomeprazole-d3 in acetonitrile (12.5 µl; Toronto Research Chemicals, Toronto, Canada) was added to 100 µl plasma. Protein was precipitated via the addition of acetonitrile followed by vortex mixing and centrifugation at 12,000 *g* for 10 minutes. The supernatant was

removed and evaporated to dryness under N_2. Samples were reconstituted in 150 µl 20% acetonitrile in water.

Compounds were analysed using a Shimadzu NexeraX2 LC-30AD (Shimadzu, Japan) liquid chromatograph (LC) and Triple Quad 6500^+ MS/MS (AB SCIEX, Switzerland) system to determine the concentration of substrates (midazolam, caffeine, and omeprazole) and metabolites (1'-hydroxymidazolam, paraxanthine and 5'-hydroxyomeprazole, respectively) in plasma (Soo *et al.*, 2018; McBride *et al.*, 2021). Compounds were separated using a reverse phase LC method with 10 µl of sample injected onto a Waters Acquity BEH C18 1.7 µm column (2.1 X 100 mm; Waters, Milford, MA). Mobile phase A was 0.05% formic acid in water and mobile phase B was 100% acetonitrile. The flow rate was 0.3 mL/min, and mobile phase B was initially 20%, which was increased linearly to 100% over 4 min. This was held for 30 seconds, returned to 20% over 30 seconds, and then held for 2 minutes prior to injection of the next sample. Compound quantification was performed as previously described (McBride *et al.*, 2021).

Post-mortem tissue collection

At 139-142d GA, pregnant ewes were killed with an intravenous overdose of sodium pentobarbitone (Virbac, Peakhurst, New South Wales, Australia) and fetuses were delivered by hysterectomy. Fetal body weight was measured and samples from major organs, including the liver, were dissected and weighed. The placenta was weighed and individual placentomes classified by type. A sample of the dorsal lobe of the fetal liver as well as each placentome type was then frozen in liquid nitrogen and stored at -80°C for subsequent molecular analyses.

Microsome extraction

Placenta and liver microsomes were extracted using differential centrifugation as previously described (Soo *et al.*, 2018; McBride *et al.*, 2020a). Briefly, frozen placenta and liver samples of ~100 mg were cut and homogenised (Tissue Lyser, Qiagen, Switzerland) in

500 µl of homogenising buffer (1.15% KCl, 1mM EDTA, pH 7.4). The homogenate was centrifuged at 9000 *g* for 20 min at 4°C. The supernatant was then transferred and centrifuged (10,600 *g* at 4°C for 60 min). Supernatant was discarded and the microsomal pellet was resuspended in buffer (100mM potassium phosphate, 20% glycerol, pH 7.4). The amount of protein in each extraction was determined using a Micro Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce, Thermo Fisher Scientific Inc, Rockford, USA) with bovine serum albumin (2 mg/ml) to generate a standard curve. Microsomal extractions were stored at -80°C until enzymatic assays.

***In vitro* CYP activity assay**

To determine the activity of CYP3A, CYP2C19, CYP2D6 and CYP1A2, the rate of midazolam to 1-hydroxymidazolam, omeprazole to 5-hydroxyomeprazole, dextromethorphan to dextrorphan, and phenacetin to paracetamol was determined by adding either 1 µM midazolam, 25 µM omeprazole, 1 µM dextromethorphan, or 5 µM Phenacetin to 40-250 µg of microsomal protein, 10 mM NADPH, and assay buffer (50 mM phosphate buffer, 2 mM magnesium chloride, pH 7.4) to a total reaction volume of 100 µl. Initial inhibition studies were performed by co-incubating CYP-specific probes with CYP-specific inhibitors (CYP3cide for CYP3A; ticlopidine for CYP2C19; quinidine for CYP2D6; and furafylline for CYP1A2) in isolated fetal liver and placental microsomes and showed no specific activity of CYP1A2, CYP2C19, or CYP3A in fetal liver samples, and no specific activity of CYP2C19 or CYP3A in placental samples. Liver samples were incubated at 37°C for 10 min (CYP2D6), whereas placental samples were incubated at 37°C for 1 hr (CYP1A2 and CYP2D6). Incubation times were chosen within the linear phase of hydroxylation based on preliminary 24 hr time course experiments. For CYP1A2, CYP2D6 and CYP3A, reactions were stopped via addition of 200 µl of 1:1 methanol and 0.2M zinc sulphate containing 100 ng/ml internal standards (midazolam d6 and dextrorphan d3, Toronto Research Chemicals, Toronto, Canada) and centrifuged

(16,000 *g* at 4°C for 10 min). Supernatant was removed and immediately analysed via LC-MS/MS.

Liquid chromatograph-tandem mass spectrometry (LC-MS/MS) for determination of *in vitro* CYP activity

Compounds were separated using a reverse phase LC method using a Shimadzu NexeraX2 LC-30AD (Shimadzu, Japan) whereby 10 µl of sample was injected on a Waters Acquity BEH C18 1.7 µm column (2.1 X 100 mm; Waters, Milford, MA). Mobile phase A was 0.05% formic acid in water and mobile phase B was 100% acetonitrile. The flow rate was 0.3 ml/min, and mobile phase B was initially 20% and was increased linearly to 100% over 4 min. Compounds were detected using MS/MS (SCIEX 4500 Triple-Quad (SCIEX, US) operated in positive ion mode. *In vitro* CYP activity was determined based on the amount of metabolite produced from the probe drug (pmol) per milligram of microsomal protein (µg) per minute of incubation time (i.e. pmol/µg/min) as previously described (McBride *et al.*, 2021; Wiese *et al.*, 2021).

Statistical Analysis

All statistical analyses were performed using SPSS v27 (IBM, USA). Initial normality testing was conducted on CYP activity data within each study group. For data where at least one of the groups was not normally distributed (Shapiro-Wilk *P*-value <0.05), non-parametric tests including Mann-Whitney U test or Kruskal-Wallis with Dunn's multiple comparisons post-hoc analysis was performed. For data where all groups were normally distributed (Shapiro-Wilk *P*-value >0.05), one-way ANOVA with Tukey's post-hoc analysis was performed. Graphs were generated using GraphPad Prism 8 (GraphPad Software, Inc. USA). Data are presented as mean ±SEM, unless stated otherwise. The alpha level was 0.05.

RESULTS

Mean gestational fetal blood gas measures and fetal and maternal plasma glucose concentrations are altered between study groups

There was no significant change in the mean gestation maternal glucose concentrations between the study groups (Table 1). Mean gestational fetal glucose concentration varied between study groups ($P < 0.0001$) and was significantly decreased in the LGUN and LGUN+G groups, when compared with controls (Table 1). Mean gestational fetal pH was significantly different between groups ($P = 0.032$), and post-hoc analysis identified a significantly higher mean gestational fetal pH in the LGUN+G group when compared with controls (Table 1). No other fetal blood gas measures were significantly different between the groups (Table 1).

Table 1. Mean gestational fetal blood gases and fetal and maternal plasma glucose concentrations

	Control	LGUN	LGUN + G	*p*
Fetal PaO_2 (mmHg)	18.7 ± 0.5	19.3 ± 0.7	19.0 ± 0.3	0.701
Fetal SaO_2 (%)	57.2 ± 2.8	61.7 ± 2.2	61.0 ± 1.7	0.381
Fetal $PaCO_2$ (mmHg)	49.9 ± 0.9	49.8 ± 0.7	50.8 ± 0.6	0.656
Fetal pH	7.367 ± 0.005^{a}	7.378 ± 0.004^{ab}	7.388 ± 0.005^{b}	**0.037**
Fetal haemoglobin (g/dL)	107.1 ± 6.7	95.8 ± 2.3	100.1 ± 1.8	0.407
Fetal lactate	1.14 ± 0.06	1.26 ± 0.09	1.39 ± 0.11	0.136
Fetal glucose (mmol/l)	1.14 ± 0.04^{a}	0.78 ± 0.03^{b}	0.95 ± 0.06^{b}	**0.000**
Maternal glucose (mmol/l)	1.99 ± 0.08	1.70 ± 0.06	2.00 ± 0.19	0.129

LGUN, late gestational undernutrition; LGUN + G, LGUN with fetal glucose infusion. Data is presented as mean ± SEM. Statistical analyses: one-way ANOVA with Tukey's post-hoc analysis or Kruskal-Wallis test with Dunn's post-hoc analysis

Maternal and fetal physical characteristics

There was no significant difference in maternal weight prior to study allocation or at day of post-mortem; however, maternal weight gain during the study period varied such that ewes allocated to the LGUN+G study group had significantly less weight gain (-2.08kg ± 0.70kg) compared with the control ewes (3.91kg ± 0.92kg; Table 2). There were no significant differences in fetal measures between study groups (Table 2).

Table 2. Maternal and fetal characteristics

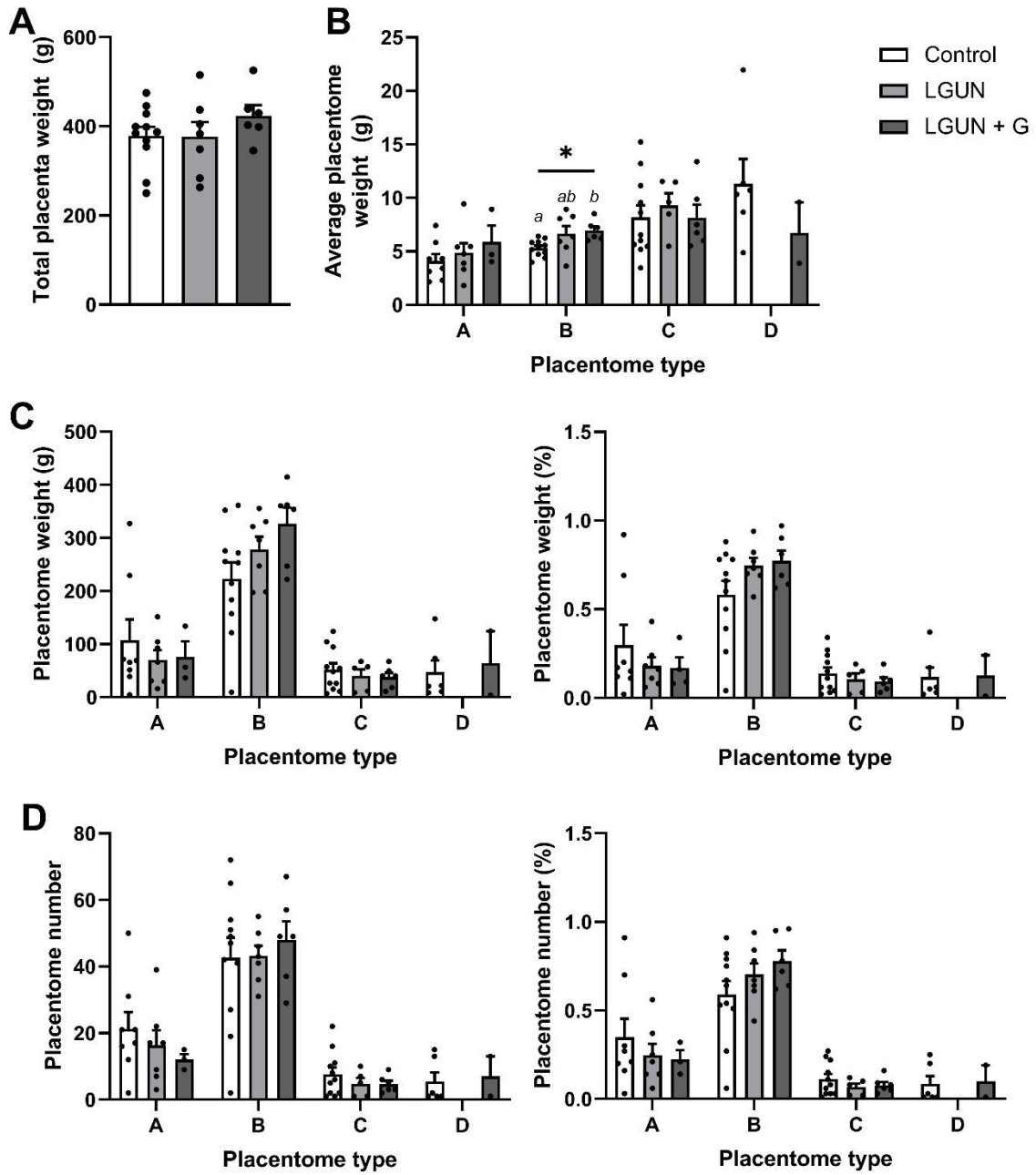
		Control	LGUN	LGUN + G	*p*
Maternal					
	Maternal weight at allocation (kg)	63.2 ± 1.7	63.1 ± 2.3	66.8 ± 2.1	0.319
	Maternal weight at post-mortem (kg)	67.1 ± 1.4	64.2 ± 1.9	64.8 ± 1.5	0.362
	Maternal weight change (kg)	3.9 ± 0.9^{a}	1.1 ± 1.0^{ab}	-2.1 ± 0.7^{b}	**0.001**
Fetal					
	Fetal weight (kg)	4.96 ± 0.18	4.48 ± 0.26	4.92 ± 0.23	0.280
	Fetal / maternal weight	0.07 ± 0.00	0.07 ± 0.00	0.08 ± 0.00	0.507
	Crown-rump length (cm)	57.1 ± 0.9	55.1 ± 1.5	55.3 ± 1.3	0.408
	Abdominal circumference (cm)	38.0 ± 0.9	37.5 ± 1.2	37.8 ± 0.6	0.921
	Liver (g)	121.12 ± 6.40	116.73 ± 11.49	120.12 ± 7.36	0.927
	Liver weight / fetal weight (g/kg)	24.58 ± 1.25	26.29 ± 2.71	24.43 ± 1.06	0.734

LGUN, late gestational undernutrition; LGUN + G, LGUN with fetal glucose infusion. Data is presented as mean ± SEM. Statistical analyses: one-way ANOVA with Tukey's post-hoc analysis or Kruskal-Wallis test with Dunn's post-hoc analysis.

Placental characteristics

Total placentome weight did not change between study groups (Figure 1A), but the average weight of type B placentomes varied significantly between the study groups, with post hoc analysis identifying a significantly higher weight of type B placentomes in the LGUN+G group compared to the control group (Figure 1B). There was no significant difference in the absolute or relative weight of placentome types between study groups (Figure 1C), nor were there any significant differences in the absolute or relative number of placentome types between study groups (Figure 1D).

There was an overall positive relationship between placenta weight and fetal weight (R^2 = 0.3944, P = 0.001; Data not shown). However, when regressions were performed within each study group, this relationship was only observed in the control animals (R^2 = 0.6244, P = 0.0038; Data not shown).



297

298 **Figure 1. Placental characteristics. A)** Total placentome weight between study groups. **B)**
299 Average placentome weight (g) between study groups. **C)** Placentome type weight (g) and
300 relative placentome type weight, and **D)** placentome number and relative placentome number
301 between study groups. Relative placentome values were normalised to the total value per
302 animal. Statistical analyses: one-way ANOVA with Tukey's post-hoc analysis or Kruskal-
303 Wallis test with Dunn's post-hoc analysis.; statistical significance: * <0.05.

Relationship between fetal blood gas measures, fetal and maternal plasma glucose concentrations, and feto-placental measurements

There was no relationship between mean gestation fetal glucose concentration and total placental weight or total type B placentome weight, irrespective of study group (Table 2). In LGUN animals only, mean gestational fetal glucose concentration was positively correlated with total placental weight (R^2 = 0.6253, P = 0.0342; Table 2) and total type B placentome weight (R^2 = 0.5875, P = 0.0444; Table 2). Additional correlations between mean gestational fetal glucose concentrations and both maternal and fetal measurements are reported in Table 2.

312 **Table 2. Correlations between mean gestational fetal glucose concentrations and maternal and feto-placental measurements**

	Mean gestation fetal glucose concentration							
	Combined		**Control**		**LGUN**		**LGUN+G**	
Maternal	***R***	***P***	***R***	***P***	***R***	***P***	***R***	***P***
Maternal glucose (mmol/l)	0.386	0.069	-0.307	0.389	-0.296	0.519	**0.831**	**0.040**
Maternal weight at post-mortem (kg)	0.460	0.024	0.405	0.217	0.580	0.172	0.210	0.690
Fetal								
Fetal weight (kg)	0.220	0.302	-0.320	0.337	**0.802**	**0.030**	-0.190	0.718
Fetal PaO_2 (mmHg)	-0.088	0.690	-0.252	0.482	0.679	0.094	0.435	0.389
Fetal SaO_2 (%)	-0.081	0.715	0.095	0.795	0.669	0.100	0.362	0.481
Fetal $PaCO_2$ (mmHg)	-0.027	0.903	0.016	0.964	-0.512	0.240	0.237	0.651
Fetal pH	-0.161	0.463	0.189	0.602	0.520	0.232	0.036	0.945
Fetal haemoglobin (g/dL)	0.090	0.683	-0.271	0.449	-0.218	0.639	-0.726	0.102
Fetal lactate	-0.094	0.668	0.629	0.051	-0.387	0.391	-0.002	0.997
Crown-rump length (cm)	0.141	0.510	-0.383	0.246	**0.769**	**0.043**	-0.460	0.359
Abdominal circumference (cm)	0.014	0.947	-0.388	0.238	0.640	0.122	-0.078	0.883
Liver (g)	0.179	0.403	0.350	0.291	0.600	0.154	-0.706	0.117
Liver weight / fetal weight (g/kg)	0.025	0.906	0.599	0.052	0.212	0.648	**-0.816**	**0.048**

Placental								
Total placenta weight (g)	-0.018	0.934	-0.167	0.624	**0.799**	**0.031**	-0.508	0.304
Total placentome A weight (g)	0.274	0.271	0.281	0.501	0.045	0.923	0.156	0.900
Total placentome B weight (g)	-0.352	0.092	-0.439	0.177	**0.777**	**0.040**	-0.230	0.662
Total placentome C weight (g)	0.179	0.426	0.009	0.980	0.140	0.822	-0.090	0.553
Total placentome D weight (g)	0.076	0.857	0.517	0.293	-	-	-	-

313 Statistical analysis: Pearson's Correlation.

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Placental or fetal hepatic CYP activity is not altered between study groups

Consistent with previous findings (McBride *et al.*, 2021), CYP1A2 and CYP3A activity was undetectable in fetal liver (Data not shown). Preliminary optimisation studies identified *in vitro* activity of CYP2C19 in the fetal liver (Data not shown); however, subsequent inhibition studies identified that the specificity of omeprazole hydroxylation was not CYP2C19-mediated in the fetal liver but was in the maternal liver (Supplementary Figure 1).

In the placenta, CYP2C19 and CYP3A activity was undetectable (Data not shown), whereas CYP1A2 and CYP2D6 activity did not vary significantly between study groups (Figure 2A). Liver CYP2D6 activity did not vary between study groups (Figure 2B), but its activity was significantly increased compared with placental CYP2D6 activity, but only in control animals (Figure 2C).

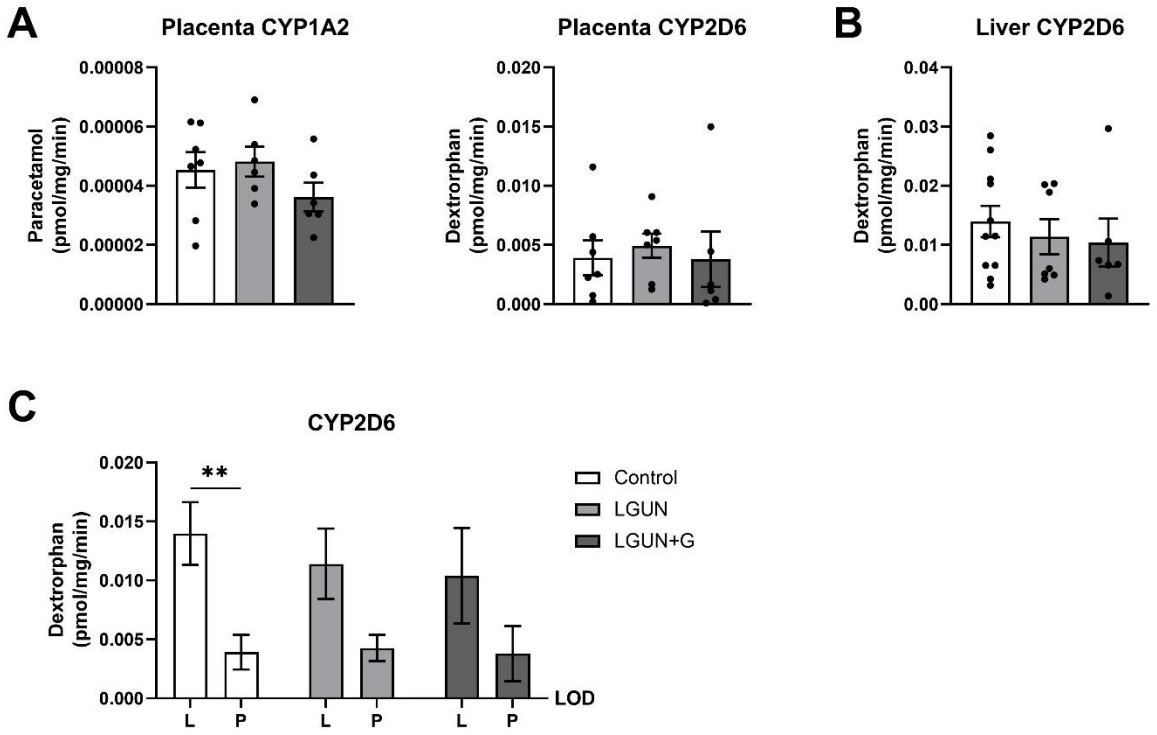


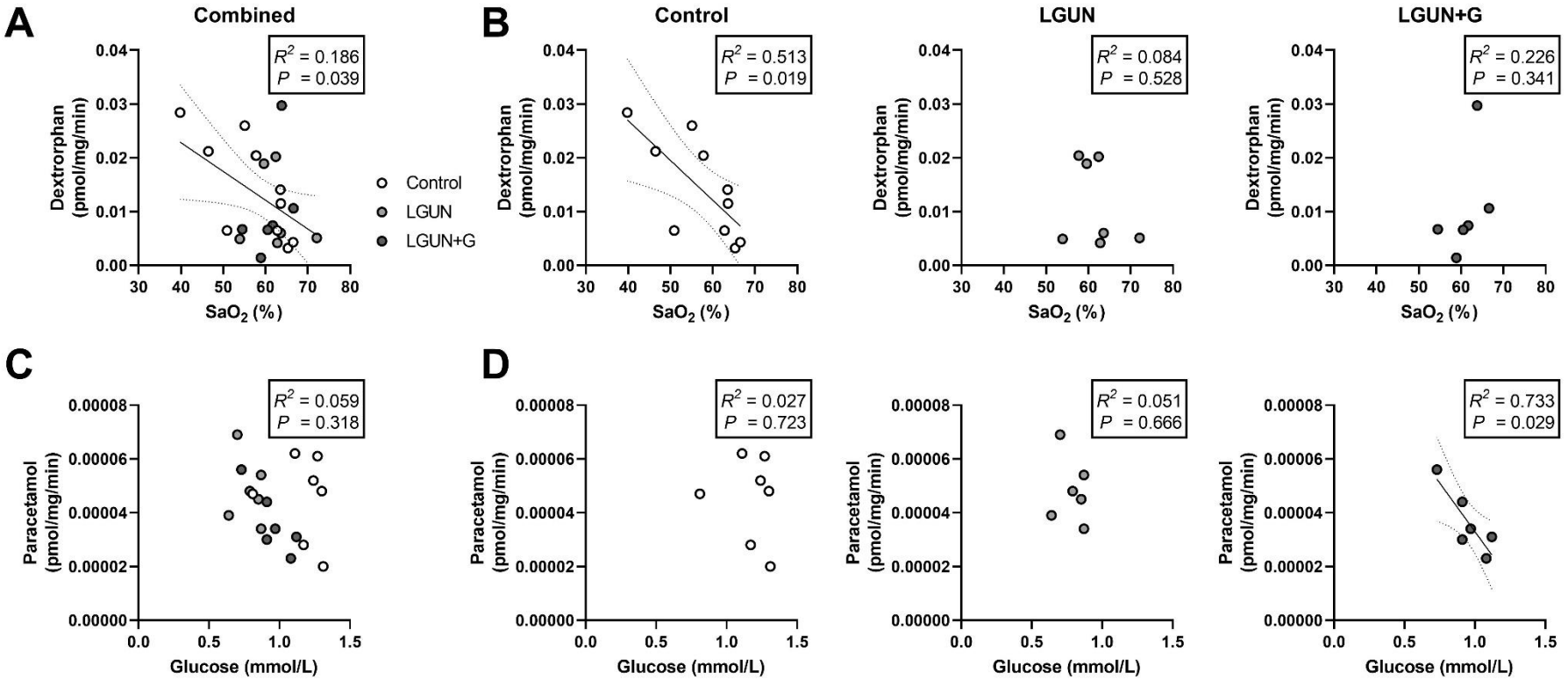
Figure 2. Placental- and hepatic-specific CYP1A2 and CYP2D6 activity do not vary between study groups. A) CYP1A2 and CYP2D6 activity in the sheep placenta, and **B)** CYP2D6 activity in the fetal liver. **C)** Comparison of CYP2D6 activity in liver (L) and placenta

(P). CYP activity is presented as the concentration (pmol) of metabolite measured per mg of tissue used per min of incubation time (pmol/mg/min). Statistical analyses: one-way ANOVA with Tukey's post-hoc analysis, Kruskal-Wallis test with Dunn's post-hoc analysis, or Mann-Whitney U test. Statistical significance: * <0.05, ** <0.01.

Feto-placental CYP activity is correlated with feto-placental and maternal measurements

A negative relationship was observed between fetal CYP2D6 and mean gestational fetal SaO_2 (R^2 = 0.1858, P = 0.040; Figure 3A), independent of study groups; however, when split by study group, this negative relationship was only observed in control animals (R^2 = 0.5127, P = 0.020; Figure 3B). No other feto-placental or maternal measurements were associated with fetal CYP2D6 activity independent of study groups. In LGUN+G animals, fetal CYP2D6 activity was positively correlated with mean gestation maternal glucose concentration (R^2 = 0.8987, P = 0.004; Data not shown).

Placental CYP2D6 was positively correlated with fetal weight independent of study groups only (R^2 = 0.2062; P = 0.039; Data not shown). There was no relationship observed between placental CYP1A2 activity and mean gestation fetal glucose concentrations independent of study groups (Figure 3C). However, in LGUN+G animals, placental CYP1A2 activity was negatively correlated with mean gestation fetal glucose concentrations (R^2 = 0.7621; P = 0.023; Figure 3D). No other correlations were observed between any placental CYP activity and feto-placental or maternal measurements of physiological significance independent of, or within, study groups.



351

352 **Figure 3. Mean gestational fetal oxygen saturation and glucose concentration is associated with placental CYP1A2 and fetal CYP2D6**

353 **activity. A)** Mean gestation fetal oxygen saturation (SaO_2, %) is negatively correlated with fetal CYP2D6 activity independent of study group. **B)**

354 Mean gestation fetal SaO_2 is negatively correlated with fetal CYP2D6 in control animals only. **C)** No relationship between mean gestation glucose

concentration and placental CYP1A2 activity, independent of study group. **D)** Mean gestation glucose concentration is negatively correlated with placental CYP1A2 activity in LGUN+G animals only.

Maternal-placental-fetal CYP activity at 132d gestation

CYP1A2 metabolite-to-substrate ratio in the maternal compartment was lower in LGUN compared to controls (Figure 4A). There was no significant difference in CYP2C19 or CYP3A metabolite-to-substrate ratio in the maternal or fetal compartments (Figure 4B and C).

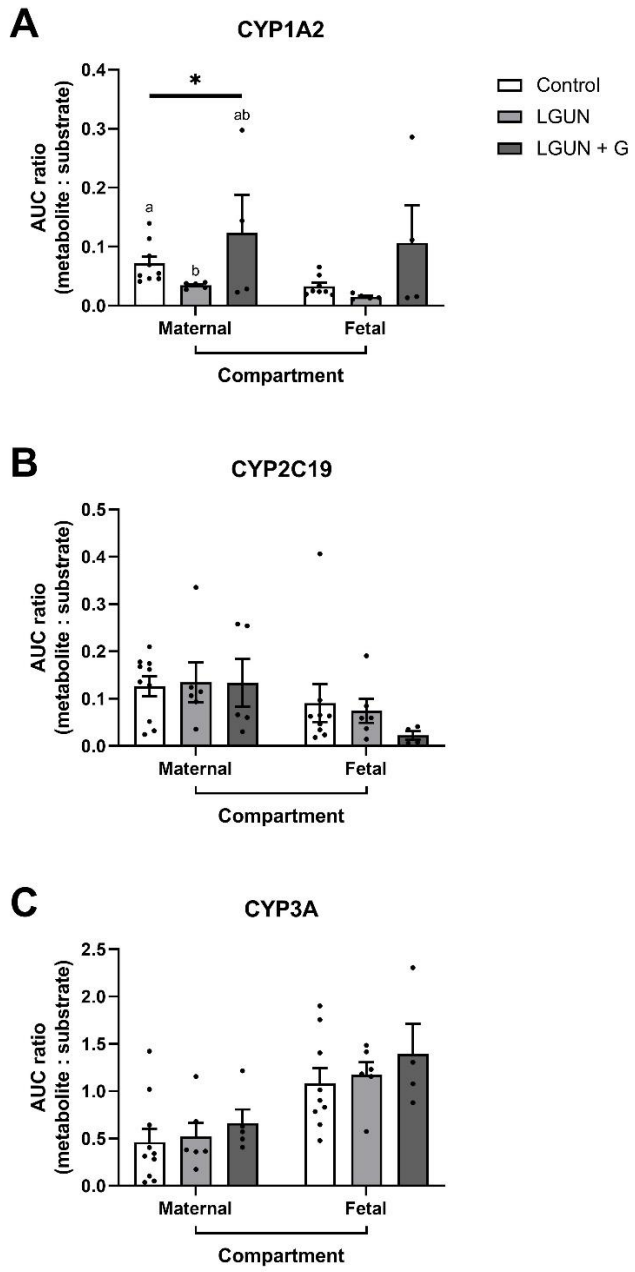


Figure 4. Maternal-placental-fetal CYP activity in plasma samples collected from the maternal and fetal compartment. A) CYP1A2 activity expressed as a ratio of paraxanthine to caffeine; **B)** CYP2C19 activity expressed as a ratio of 5'-hydroxyomeprazole to omeprazole; **C)** CYP3A activity expressed as a ratio of 1'hydroxymidazolam to midazolam. AUCs over 6 hours; data is presented as mean ± SEM. Statistical analyses: one-way ANOVA with Tukey's post-hoc analysis or Kruskal-Wallis test with Dunn's post-hoc analysis; statistical significance:

368 * <0.05.. Maternal: control n=10, LGUN n=6, LGUN+G n=5; fetal: control n=9, LGUN n=6,

369 LGUN+G n=4.

DISCUSSION

Although previous studies have found hypoglycemia to alter CYP enzyme activity, the present study showed that the physiological response to LGUN does not change the activity of CYP1A2 or CYP2D6 in placenta or fetal liver; however, there was a reduction in maternal-placental-fetal CYP1A2 activity in the maternal compartment. Importantly, our study is the first to quantify CYP1A2 and CYP2D6 activity in the sheep placenta and provides a validated experimental workflow that will enable future studies to investigate the activity of other CYP isoenzymes in the feto-placental unit and to assess how LGUN, as well as other pregnancy complications, may impact their function.

There is currently limited understanding about the activity of placental CYPs. In fact, to the best of our knowledge, this is the first study to detect activity of CYP1A2 in the placenta. The physiological relevance of placental CYP1A2 is unclear; however, given its activity was detected in the placenta but not fetal liver, indicates that placental CYP1A2 could play a role as a metabolising-barrier for the fetus against drugs, toxins, and endogenous chemicals throughout gestation. For example, paracetamol and ondansetron are CYP1A2-metabolised drugs that are taken during pregnancy for the management and relief of pain and nausea, respectively. Despite this, research has shown increased risks for adverse neonatal outcomes in response to fetal exposure to these drugs (Shaheen *et al.*, 2005; Anderka *et al.*, 2012; Koren, 2012). Within the context of the current study, we found a significant reduction in CYP1A2 activity in the maternal compartment of the maternal-placental-fetal unit in response to LGUN. This reduction in maternal compartment CYP1A2 activity, but no change in placental-specific CYP1A2 activity, suggests that placental drug metabolising efficacy is more resistant to changes in the maternal-placental-fetal environment.

Although detection of CYP2D6 activity in the fetal sheep liver has previously been reported (Soo *et al.*, 2018), our study is the first to report placental-specific activity of this CYP

isoenzyme. CYP2D6 is involved in the metabolism of at least 20% of clinically relevant drugs, some of which are likely to be prescribed during pregnancy including dextromethorphan, selective serotonin-reuptake inhibitors (SSRIs; fluoxetine, duloxetine, paroxetine) and opioid analgesics (e.g., codeine, oxycodone, and tramadol). While dextromethorphan, a cough suppressant found in over-the-counter cold medications, is not associated with any increased risk of major malformations (Einarson *et al.*, 2001; Werler, 2006), use of SSRIs during pregnancy can impact fetal development (for review, see Morrison *et al.* (2005a)) and is also associated with adverse neonatal and childhood outcomes (Grzeskowiak *et al.*, 2011; El Marroun *et al.*, 2014). Furthermore, use of opioid analgesics during pregnancy increases the risk of adverse neonatal outcomes including preterm delivery, both small and large for gestational age, birth defects and neonatal abstinence syndrome (Kelly *et al.*, 2011; Bloor *et al.*, 2012; Källén *et al.*, 2013; Smith *et al.*, 2015; Yazdy *et al.*, 2015). Despite studies demonstrating adverse neonatal outcomes in response to certain CYP2D6-metabolised drugs during pregnancy, our current work indicates that LGUN does not alter CYP2D6 activity in the feto-placental unit. However, we did find that the degree of difference in tissue-specific CYP2D6 activity was reduced in LGUN and LGUN+G animals only, but the relevance of this finding requires further investigation. Regardless, given CYP2D6 activity was comparable between groups in both the fetal liver and placenta, the associated risks of CYP2D6-specific drugs would be comparable to uncomplicated pregnancies during late gestation. While we did not detect a significant change in the activity of CYPs in the fetal liver or placenta between study groups, we did identify interactions of importance between CYP1A2 and CYP2D6 activity and fetal oxygen saturation or glucose concentrations. These interactions highlight changes in oxygen and/or glucose availability may drive changes in feto-placental CYP activity. We identified that fetal oxygen saturation was negatively associated with fetal liver CYP2D6 activity independent of study group. These findings contrast previous work that

identified a hypoxic environment increased CYP2D1 (a CYP2D6 ortholog) expression (Duan *et al.*, 2020) and activity (Li *et al.*, 2014) in adult rats. Comparatively, the observed negative interaction between glucose concentrations and placental CYP1A2 in the LGUN+G study group supports previous work demonstrating hyperglycemia decreased CYP1A2 activity in hepatic microsomes isolated from rats fed a high-glucose diet (Stewart & Strother, 1999). Indeed, given the activity of CYP1A2 in the placenta could function as the last metabolising barrier before substrates enter the fetal circulation, the efficacy of placental CYP1A2 activity may be perturbed in states of fetal hyperglycemia but not hypoglycemia, resulting in potential adverse fetal developmental outcomes; however, further studies are required to investigate these observed associations.

An explanation for our finding of no change in CYP1A2 or CYP2D6 activity in the feto-placental unit was that we used a model of late gestation undernutrition. Previous studies examining dietary interventions on hepatic CYP activity have primarily been carried out in non-pregnant rodent models (Patoine *et al.*, 2013; Abdussalam *et al.*, 2017; Zhang *et al.*, 2019; Al Nebaihi *et al.*, 2020; Wiese *et al.*, 2021); there are limited studies that have examined the effect of maternal undernutrition on maternal-placental-fetal drug metabolism and transport. Previous work in guinea pigs identified maternal undernutrition significantly reduced expression of placental P-glycoprotein (P-gp), an important drug efflux transporter, indicating that chronic maternal undernutrition can perturb the placental drug metabolising and transporter barrier (Soo *et al.*, 2012). However, unlike previous work by Soo *et al.* (2012), our current study investigated the effect of an acute nutritional deficit in late gestation on maternal-placental-fetal CYP activity. It is well understood that the timing, duration, and severity of a suboptimal intrauterine environment can differentially impact feto-placental physiological outcomes (Morrison, 2008; Darby *et al.*, 2020). Given we observed interactions of significance between fetal oxygen saturation and glucose concentrations and feto-placental CYP activity, it

445 is plausible that within the context of the current study the timing and duration of undernutrition
446 was not sufficient to induce a significant change in feto-placental CYP1A2 or CYP2D6
447 activity. Therefore, early gestation *in utero* insults, a critical window within which placentation
448 and subsequent feto-placental development occurs, may result in a greater perturbation of
449 functional CYP activity in the feto-placental unit. Thus, future studies would benefit from
450 investigating the effect of chronic maternal undernutrition beginning earlier in gestation on
451 maternal-placental-fetal CYP activity, as these conditions may more accurately reflect those
452 that are observed in human populations.

453 Although the current study furthers our understanding of maternal-placental-fetal drug
454 metabolism, and how acute perturbations during pregnancy impact this system, there are
455 certain limitations that should be considered. Despite being able to measure *in vivo* activity of
456 CYP1A2, CYP2C19 and CYP3A, we were limited to studying hepatic-specific CYP activity
457 solely within the fetal system as maternal tissue was not collected from this study. Previous
458 work from our group identified different responses to fetal growth restriction in the maternal
459 system at either the hepatic-specific *in vitro* or whole-system *in vivo* level (McBride *et al.*,
460 2021). These previous discrepancies may indicate that the maternal responses observed in the
461 present study may not be a true reflection of maternal hepatic-specific CYP function. Indeed,
462 drug metabolism during pregnancy is complex and involves both the maternal, placental, and
463 fetal compartments; drugs are transferred in a bidirectional manner between maternal and fetal
464 circulations and are metabolised by both hepatic and extrahepatic sites of metabolism.
465 Therefore, the current *in vivo* findings are likely reflective of both hepatic and extrahepatic
466 drug metabolism in both the maternal and fetal compartments. In addition, while our maternal-
467 placental-fetal CYP activity assays are limited to CYP1A2, CYP2C19 and CYP3A, our *in vitro*
468 assays are validated for maternal hepatic CYP1A2, CYP2C19, CYP2D6, and CYP3A. Our
469 ability to quantify *in vitro* activity of four hepatic CYPs in the pregnant ewe and her fetus

equips us with the tools to provide comprehensive overviews of how certain pregnancy complications associated with dysregulated maternal and/or fetal physiology impacts maternal-placental-fetal physiological function.

CONCLUSION

Collectively, our data has shown that the maternal-placental-fetal physiological response to LGUN does not affect placental- or fetal hepatic-specific activity of certain CYPs. However, CYP1A2 activity in the maternal compartment of the maternal-placental-fetal unit, which considers both hepatic and extrahepatic sites of metabolism, was reduced in response to LGUN. This reduction of CYP1A2 activity in the maternal compartment, but not in the placenta, may indicate a level of retained function in placental-specific metabolism in response to LGUN. Despite this, an overall reduction in maternal CYP1A2 activity in response to LGUN may expose the developing fetus to increased concentrations of CYP1A2-specific substrates and consideration of drug dosing may be required in instances of late gestation maternal undernutrition.

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DISCLOSURES

The authors have no conflict of interest.

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