A novel intervention strategy to prevent intrauterine growth restriction

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Abstract

Intrauterine growth restriction (IUGR) increases risks of perinatal death >5-fold, and has lifelong adverse effects on health. There is currently no treatment to prevent or cure IUGR, although animal studies have shown promising effects of maternal growth hormone (GH) treatment on fetal and placental growth. The overall aim of this project was to test a novel approach to stimulate the mother's own production of GH, to promote placental function and fetal growth in normal and IUGR pregnancies, and hence develop a therapy to prevent IUGR which is acceptable to patients.

My first aim was to investigate whether the abundance or activation of the GH secratagogue, ghrelin, increased during pregnancy or in response to dietary supplementation with octanoic acid, which is required for activation (acylation) of ghrelin. Maternal circulating total and acyl-ghrelin and GH profiles were measured in late pregnant mice and in aged-matched non-pregnant females, fed a diet supplemented with octanoic or palmitic (control) acid. However, dietary supplementation did not increase the activation of ghrelin in female mice, whilst the pregnancy-associated increase in maternal GH secretion occurred without elevated circulating acyl-ghrelin concentrations.

The second aim was to develop a novel method to measure placental function, a major determinant of fetal growth, using non-radioactive tracers in mice. A dye containing fluorescently-labelled glucose was injected into pregnant dams near term, and its maternal, placental and fetal uptake were characterised. Although the results indicated that this tracer was not specific for placental glucose uptake, its uptake provided a good indication of overall placental function and fetal nutrient uptake.

Lastly, in order to test potential interventions, a murine model of IUGR in multiple pregnancies was established. Mouse pregnancies were generated by transferring

variable numbers of embryos into pseudo-pregnant mice, generating litters with varying degrees of maternal constraint near term. Fetuses from the largest litters were ~25% lighter as well as thinner and with evidence of brain-sparing, compared to fetuses from smaller litters. Interestingly, the relationship between viable litter size and fetal weight differed between sexes, such that fetal weights of males, but not females, correlated negatively with litter size. However, this sex difference was not explained by morphological and functional changes in the placenta. The results of this experiment suggested that male fetuses grew as fast as permitted by nutrient supply, whereas the female maintained placental reserve capacity. This strategy, likely reflecting sex-specific gene expression, would be expected to place the male fetus at greater risk of death in the face of any additional intrauterine stressors.

In conclusion, the dietary supplementation trialled in this project did not promote fetal growth in normal pregnancies or stimulate responses in the mother that would promote fetal growth in growth restricted pregnancies. However, this project has generated valuable new tools to help investigate and ultimately address the major clinical challenge of poor growth before birth. The development and optimisation of this IUGR model will now allow us to combine genetic models in mice with testing of intervention strategies to prevent IUGR due to chronic restriction.

Statement of originality and authenticity

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Table of Abbreviations

2-DG	2-deoxyglucose
ACTH	Adrenocorticotropic hormone
AEBSF	4-(2-aminoethyl) benzenesulphonyl fluoride
AGA	Appropriate for gestational age
AgRP	Agouti-related protein
AIB	Amino-isobutyric acid
ALS	Acid-labile subunit
ApEn	Approximate entropy
BM	Basal membrane
CB1	Cannabinoid receptor subtype 1
CSH	Chorionic somatomammotropin hormone
DAG	Diacylglycerol
EDTA	Ethylene diamine tetra acetic acid
EDV	End diastolic velocity
EFW	Estimated fetal weight
FABPpm	Fatty acid binding protein of the plasma membrane
FAT/CD36	Fatty acid translocase
FATP	Fatty acid transport proteins
FFA	Free fatty acid
FW:PW ratio	Fetal:placental weight ratio
GD	Gestational day
GH	Growth hormone
GHBP	Growth hormone binding protein
GHR	Growth hormone receptor

GHRH	Growth hormone releasing hormone
GHSR1-a	GH secretagogue receptor 1a
GH-V	Growth hormone variant
GLUT	Glucose transporter
GOAT	Ghrelin O-acyltransferase
hCG	Human Chorionic Gonadotropin
hFABP	Heart-type fatty acid binding protein
HPA axis	Hypothalamic pituitary axis
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor-binding protein
IGFR	Insulin-like growth factor receptor
IP_3	Inositol 1,4,5- trisphosphate
IR	Insulin receptor
IUGR	Intrauterine growth restriction
JAK/STAT	Janus Kinase/Signal Transducing Activators of Transcription
LAT	Large neutral amino acid transporter
LBW	Low birthweight
LC-PUFA	Long chain polyunsaturated fatty acid
MAPK	Mitogen activated protein kinase
MBOAT4	Membrane bound O-acyl transferase-4
MCFA	Medium-chain fatty acid
MCT	Medium chain triglyceride
MG	Methyl-D-glucose
mTOR	Mammalian target of rapamycin
MVM	Microvillous membrane
NICU	Neonatal intensive care unit

NPY	Neuropeptide-Y
OCT	Optimum cutting temperature
PB	Phosphate buffer
PC1/3	Prohormone convertase
PFA	Paraformaldehyde
PI	Pulsatility index
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5 bisphosphate
PKB/AKT	Protein kinase B
PL	Placental lactogens
PLC	Phospholipase C
PRL	Prolactin
PSV	Peak systolic velocity
RAAS	Renin-angiotensin-aldosterone system
RI	Resistance index
RIPA	Radioimmunoprecipitation assay
SGA	Small for gestational age
SHC2	Src homology 2
SIDS	Sudden infant death syndrome
SNAT	Sodium-coupled neutral amino acid transporter
SRIF	Somatostatin
SSTR	Somatostatin receptors
STRIDER	Sildenafil TheRapy In Dismal Prognosis Early-onset Fetal Growth Restriction
SUAL	Single umbilical artery ligation
TAV	Time-averaged velocity

WPC	Weeks post-conception
ZT	Zeitgeber

Manuscripts arising from this thesis

Published manuscripts

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Submitted manuscript

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Other papers published during PhD candidature

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Harleen Kaur, Hui Li, Pamela Su-Lin Sim, Rebecca L Wilson, Lili Huang, Chen Chen, Johannes D Veldhuis, Amanda Page, Beverly S Muhlhausler, Claire T Roberts, Kathryn L Gatford. A potential role for ghrelin as a driver of increased growth hormone during murine pregnancy. Fetal and Neonatal workshop of Australia and New Zealand, Queenstown, March 2018. *Oral presentation by Harleen Kaur*

Chapter 1 Literature review

1.1 Maternal adaptations to pregnancy

A female undergoes significant anatomical and physiological adaptations in order to support the developing conceptus while still being able to cope with the increased metabolic and physical demands of pregnancy. Changes in maternal physiology occur progressively throughout pregnancy, as the metabolic demands of the conceptus increase [1-3]. Most of the information below is derived from investigations in human pregnancies, however, similar maternal adaptations during pregnancy are also observed in rodents.

During early pregnancy, maternal systemic vascular resistance decreases by 25-30%, which in turn contributes to a decrease in maternal diastolic blood pressure during the first and second trimesters [4, 5]. In response, cardiac output increases by ~30-50% and the renin-angiotensin-aldosterone system (RAAS) is activated early in pregnancy, thereby promoting sodium and water retention and contributing to the progressive increase in plasma volume [6-8]. The resulting increase in total maternal blood volume, as well as cardiac output allows enhanced blood flow to the uterus and placental perfusion to promote its growth and development [5, 9, 10]. Besides adaptations to the cardiovascular system, a number of other maternal systems also undergo significant changes during pregnancy. These include mechanical alterations to the chest wall and diaphragm to accommodate the enlarging uterus and increased maternal ventilation to meet the significant increase in oxygen demand [11, 12]. Pregnancy-associated increases in circulating progesterone concentrations are associated with decreased gastric contractility and delayed gastrointestinal transit time, possibly to allow a longer time for absorption of nutrients [13-15].

In order to cope with the increased energy demands of pregnancy, significant alterations occur to the maternal metabolic and endocrine state. Hyperplasia of insulin-secreting pancreatic β-cells in early pregnancy results in increased maternal insulin secretion compared to the non-pregnant state. Maternal insulin resistance develops in skeletal, adipose and hepatic tissues during mid-late pregnancy by the actions of placental hormones like human placental lactogen, progesterone, cortisol and prolactin, and this ensures availability of maternal glucose to maintain an adequate fetal glucose supply [16, 17]. In addition to effects on glucose metabolism, early human pregnancy is associated with increased insulin sensitivity in white adipose tissue, giving rise to adipocyte hypertrophy, increased lipogenesis and lipid storage [16, 18, 19]. An average pregnant woman gains ~2.5 kg of fat mass [20], while abdominal fat mass doubles by late pregnancy in rats [21]. Hepatic glycogen storage also increases and hepatocytes undergo hypertrophy and hyperplasia to meet the demands of pregnancy in both humans and mice [22, 23].

The maternal insulin-resistant state also contributes to increased lipolysis and hepatic gluconeogenesis in the latter part of pregnancy. This in turn allows the mother to preferentially use fat for fuel, while preserving glucose and amino acids for the fetus [24]. Pregnancy-associated increases in maternal food intake and decreases in energy expenditure, observed in humans and rodents, allow maintenance of a positive energy balance and supply of nutrients to the fetus [3, 20, 25, 26]. Collectively, these adaptations minimise maternal protein catabolism to supply the nutrients required for accelerating fetal growth. The increase in maternal weight during pregnancy occurs predominantly during the second and third trimesters, and in addition to fat deposition and increase in maternal blood volume, reflects growth of the fetus, placenta and fetal membranes, amniotic fluid, as well as expansion of uterine tissue and mammary glands (Figure 1.1) [2, 3, 27]. In litter-bearing species, including mice, the gravid uterus accounts for approximately 30% of maternal weight at term. However, in species like

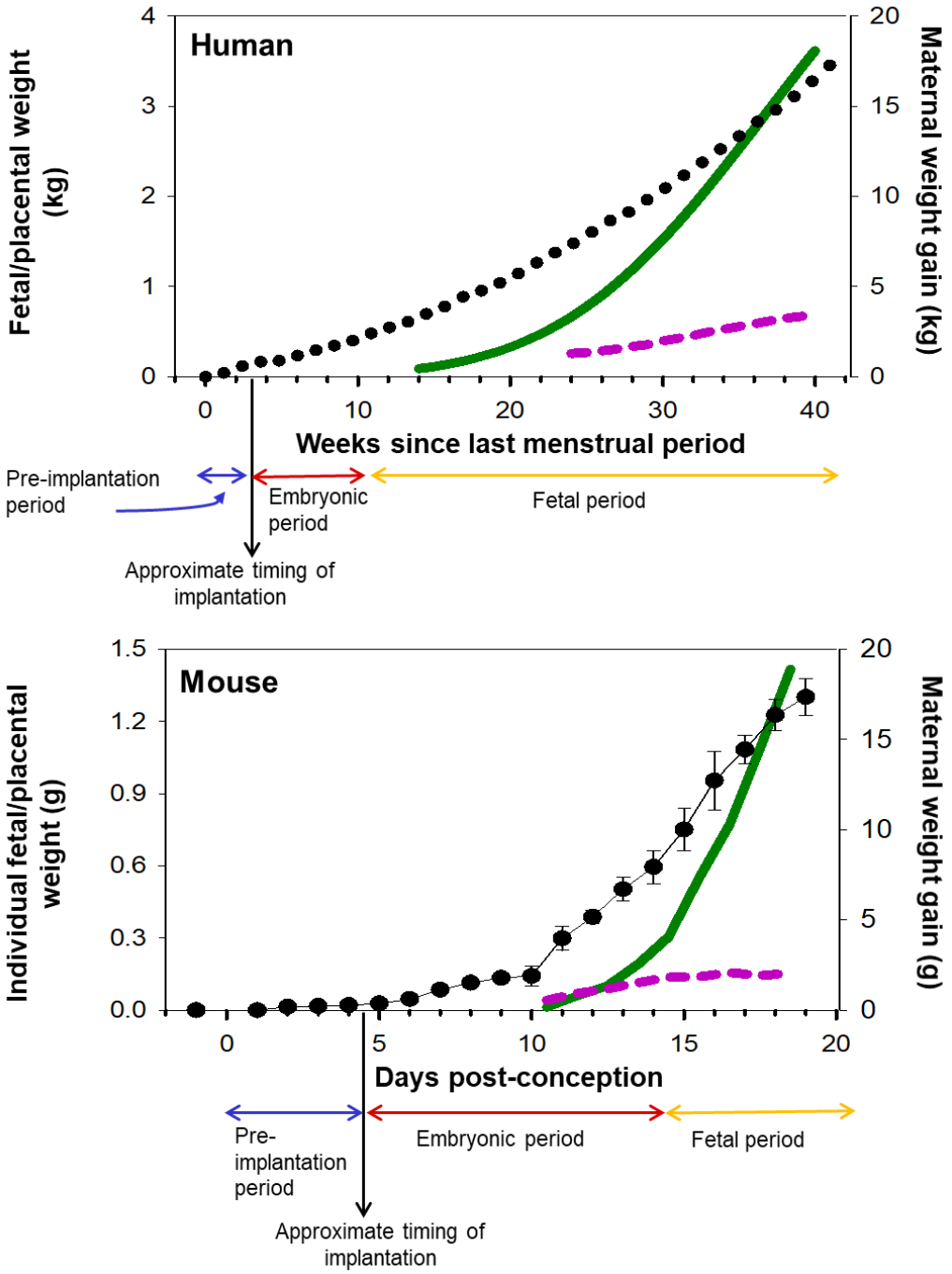


Figure 1.1 *Comparison of changes in fetal (green line), placental (purple dashes) and maternal weight (black dots) during pregnancy in humans (top figure) and mice (bottom graph) and periods of conceptus development. Term in humans is typically 37-41 weeks from the last menstrual period (LMP), implantation occurs around 6 days post-ovulation, and the embryonic period with developmental events such as gastrulation and organogenesis is completed by 10 weeks of gestation/ since LMP, or the end of the first trimester. Fetal growth and maturation then continues from 11 weeks' gestation until birth. In mice, gestation lasts for approximately 20 days post conception, implantation occurs around gestational day (GD)4.5, at the beginning of the embryonic period. Gastrulation occurs on ~GD6.5 and is shortly followed by organogenesis on ~GD8.5, and the fetal growth phase begins by GD14.5 and continues until birth.*

Maternal, fetal and placental weight data extracted from [3, 28-30];mouse fetal and placental weight data are from CD-1 mice [28]; mouse maternal weight data is from C57BL/6J mice [3].

humans and sheep, that typically only bare one fetus at a time, about 6-9% of maternal weight is attributable to the fully developed conceptus (Figure 1.1) [2].

1.2 Fetal growth

The process of growth and development within the uterus from fertilisation until birth can be categorised into three phases: the pre-implantation phase, the embryonic phase and the fetal phase (Figure 1.1). Prior to implantation, the fertilised zygote undergoes rapid cell cleavage without increasing in size to form a blastocyst, which implants into the maternal uterine decidua (endometrium) and where subsequent development takes place. Post-implantation, organ primordia form during the embryonic phase and undergo further extensive expansion and remodelling during the fetal phase (Figure 1.1). In humans, the pre-implantation and embryonic stages take place during weeks 1-2 and 2-8 weeks post conception (2-4 weeks and 4-10 weeks since the first day of the last menstrual period; LMP), respectively. The fetal period begins from the 9th week of development (11 weeks LMP) and continues until term (typically 37-41 weeks LMP) [31] although women are usually told their estimated date of delivery is 40 weeks' LMP.

Mice have a much shorter gestation than humans (typically 19-21 days post-conception, depending on the strain [32]). Therefore, stage of development is usually indicated by gestational age, with the night of mating designated as gestational day (GD)0, and the following morning, when the copulation plug is detected in the female, marked as GD0.5. Implantation in mice occurs around GD4.5, and gastrulation (~GD6.5) is shortly followed by organogenesis (~GD8.5), and by GD14.5, the fetal growth phase begins and continues until birth (Figure 1.1). Hence, mice spend approximately two-thirds of gestation (up to GD14.5) undergoing important development events such as gastrulation and organogenesis and spend a

comparatively shorter period of time *in utero* undergoing fetal maturation and growth. Consequently, a significantly greater proportion of these maturational events and growth continue into the postnatal period in mice. Conversely, humans only spend around one-fifth of the gestation in the embryonic phase and a significantly longer period is spent in the fetal period [33]. As a result, human infants are more developmentally mature than mouse pups at birth [34, 35].

1.2.1 *Fetal sex*

It is now well established in humans, sheep and rodents that males grow faster *in utero* and on average, are heavier than females at an equivalent gestational age [36-39]. Since fetal growth is largely dependent on the capability of the placenta to facilitate materno-fetal exchange, sex-specific developmental disparities are likely in part orchestrated by the placenta, which shares the same sex as the fetus [40-42]. The rapid growth observed in male fetuses is associated with a higher fetal to placental weight ratio than for females, and may mean that male fetuses have limited placental reserve capacity to draw upon in the event of perturbations experienced later in gestation [43]. This in turn, is believed to put them at a higher risk of inadequate nutrient supply and growth restriction, hence increasing their risk of developing adult-onset disorders like cardiovascular disease [44, 45]. In contrast, females appear to invest more in placental development, and have a better chance of survival in the face of suboptimal intrauterine conditions [46, 47]. As a result of their different growth strategies, the incidence of pregnancy complications differs between sexes; while intrauterine growth restriction (IUGR) is more prevalent in females [48], males have higher rates of severe placental dysfunction, spontaneous preterm birth and perinatal death if they experience an intrauterine perturbation [44, 49-53]. As an example, in the presence of maternal asthma, female fetuses slow their growth and are therefore protected from adverse fetal outcomes and stillbirth in the face of an exacerbation of

maternal asthma in late gestation [54]. In contrast, male fetuses of asthmatic women maintain normal growth across gestation, and are therefore at increased risk of intrauterine death, stillbirth and being born SGA [55].

There is now good evidence indicating that differences in fetal outcomes in response to the same intrauterine challenge are likely to be associated with sex-specific morphological and/or functional adaptations made by the placenta. RNA sequencing analyses of sex differences in placental gene expression indicate that almost half are X-linked [56, 57]. Placental global gene microarray conducted on placentas from non-asthmatic and asthmatic women revealed that the number of genes whose expression was significantly impacted by maternal asthma was 10-fold higher in female placentas compared to male placentas [58]. Furthermore, the changes in placental gene expression in females were associated with the suppression of immune and growth factor pathways, and would thus be expected to lower fetal growth rate [58]. Conversely, the relatively conserved placental gene expression in males may account for the maintenance of fetal growth in an adverse maternal environment [58]. Furthermore, genes of the VEGF signalling pathway, implicated in placental development and angiogenesis, also demonstrate general sex-bias [41]. Such sex-specific expression of genes involved in placental development pathways may influence key placental processes, such as establishment of the placental vascular architecture [41, 59].Consistent with this hypothesis, uterine artery Doppler measurements from uncomplicated singleton pregnancies show higher pulsatility index, and higher prevalence of notching, in women carrying a male fetus, reflecting higher utero-placental resistance [60].

As a result of sex differences in the placenta and fetal growth rates, the male fetus is more susceptible to suboptimal placentation and less capable of adapting to adverse conditions than the female fetus [41, 44, 61]. This has clinical implications, since it implies that male and female fetuses may respond differently to the same intervention.

Therefore, it is also crucial for studies evaluating mechanisms contributing to fetal growth or growth restriction, and testing interventions, to analyse feto-placental outcomes separately within each sex [40, 61].

1.3 Placental development

The placenta is the interface between the mother and fetus and performs a number of crucial functions that are essential for the maintenance of a successful pregnancy. It mediates exchange of nutrients and gases between the maternal and fetal compartments, prevents the semi-allogeneic fetus from being rejected by the mother's immune system and produces a range of peptide and steroid hormones that mediate the changes in maternal physiology and metabolism described above [62].

Both humans and mice have haemochorial placentas, where the tissue separating the maternal blood from fetal circulation consists of only fetal tissues (chorionic trophoblast and allantoic mesenchyme and vasculature) [63]. Additionally, both species have a haemochorial placental interface, as interactions between the materno-fetal blood circulations involves the direct contact of maternal blood and chorionic trophoblast [63]. However, the pattern of branching of these trophoblast layers to form the area of materno-fetal exchange differs between the two species.

In humans, chorionic villi form a complex, highly branched villous tree structure, and each villus is bound by a single syncytiotrophoblast layer in contact with maternal blood. Inside the villus is a layer of cytotrophoblast and the basement membrane, while the central part of the villus contains fetal mesenchyme and vessels. Maternal blood flows in from the decidual spiral arteries into the intervillous space and flows around these terminal villi. This requires the modification of uterine spiral arteries from tight spirals with high-resistance, low-flow, to wide conduits with low-resistance high-flow

blood vessels [64]. Spiral artery remodelling occurs during the first few weeks of pregnancy when maternal vascular endothelial and smooth muscle cells are lost from the spiral arteries and replaced by fetal trophoblast cells, resulting in at least 10-fold increase in their diameter [65]. This is also accompanied by a reduction in the responsiveness of the vessels to vasoconstrictors [65]. The exchange of gases and metabolic products between the materno-fetal circulations hence takes place across the chorionic membrane [66]. Oxygen and nutrients are transported across the chorionic membrane into fetal blood and carried to the fetal circulation via the umbilical vein, maternal blood in the intervillous space leaves via the decidual veins and uterine vein [67].

The maternal side of the murine placenta comprises of the maternal decidua, a layer of maternal tissue similar to the human decidua basalis. It also comprises of maternal vessels that traverse it to supply the placenta [63]. A layer of trophoblast giant cells separates the decidua from the junctional zone, which structurally supports the labyrinth throughout its development and comprises spongiotrophoblasts, trophoblast giant and glycogen cells. These cells have endocrine and invasive properties and can also provide an energy source for the developing fetus. This layer is discontinuous due to the presence of trophoblast-lined arterial and venous channels but does not contain any fetal blood vessels. Distal to the junctional zone, the labyrinth forms the interface for materno-fetal nutrient and gas exchange in the murine placenta. Compared to the human placenta, the branches of the main chorionic projections of the mouse placenta are much more interconnected, giving rise to the maze-like or labyrinthine structure. Three layers of trophoblast cells separate the maternal blood spaces from fetal endothelium. Maternal blood spaces are lined by a discontinuous layer of cuboidal, mononuclear cytotrophoblast cells, while the middle and third layers comprise of elongated and multinucleated syncytiotrophoblast cells [68, 69]. Maternal blood enters the maternal blood spaces of the labyrinth through amuscular spiral arteries that

converge together at the trophoblast giant cell layer into a central arterial canal that passes through to the base or fetal side of the placental labyrinth. Similar to the pattern of placental development in humans, remodelling of murine spiral arteries to permit increased blood flow occurs in the first third of pregnancy [70]. Blood then percolates back to the maternal side of the placenta through tortuous, highly interconnected, trophoblast lined sinusoids in the labyrinth [70]. These sinusoids join to form larger channels that traverse the junctional and giant cells layers and lead into large, endothelial cell-lined, maternal venous sinuses in the decidua [70]. Fetal blood passes through the labyrinth in a counter-current direction to the maternal blood. Feto-placental arterioles pass through the apical side of the labyrinth before branching into capillaries that course back towards the base of the placenta. This allows fetal blood to equilibrate with maternal and arterial blood through the trophoblast layers, thereby maximising the umbilical venous nutrient and oxygen delivery to the fetus [70].

1.4 Placental function

1.4.1 *Placental nutrient transport*

A sufficient supply of nutrients from the mother, including glucose, amino acids and fatty acids, is important for optimal fetal growth and development. However, the transfer of substances between the maternal and fetal circulations requires them to pass through the placental barrier [71]. In humans, transplacental transfer involves nutrient transport across the maternal facing microvillous membrane (MVM) of the syncytiotrophoblast, across the cytoplasm and exit to the fetal circulation via the fetal facing basal membrane (BM) [72]. In mice, on the other hand, the mononuclear trophoblast cell layer is highly permeable to solutes with a small molecular weight and hence the second syncytial layer is the initial barrier to materno-fetal exchange [73, 74].

The second and third membranes of the mouse placenta are connected through gap junctions, with the third layer being in close proximity to the fetal endothelium. Studies of isolated apical membrane of the second syncytial layer in mice have shown that it is highly analogous to human MVM [75], while the third placental layer in mice is suggested to be analogous to human BM [76].

The placental transfer of individual substances is dependent on a number of factors including the size of the molecule, its lipophilicity, the rate of blood flow and its transplacental concentration gradient [75]. Small, lipophilic molecules such as respiratory gases diffuse readily across the placenta, and their concentration gradients rapidly equilibrate across the membranes [77]. However larger, hydrophilic and charged molecules cannot readily cross the placenta and hence require membrane transporters, which can transport molecules either down their concentration gradient (facilitated transport) or against their concentration gradient (active transport) [77]. These processes are discussed in greater depth in the following sections, with respect to transport of glucose, amino acids and fatty acids, the main substrates utilised by the fetus.

1.4.1.1 Glucose transport

Transfer of glucose from the maternal to fetal circulations is critical to allow the fetus to meet its energy needs. Glucose concentrations are lower in the fetus than in the mother and thus materno-fetal transfer of glucose across the placenta occurs down a concentration gradient via facilitated diffusion through a family of glucose transporters (GLUTs), primarily GLUT1 (encoded by *Slc2a1*) and GLUT3 (encoded by *Slc2a3*) [78]. GLUT1 is expressed in both the MVM and BM of the human placenta, with approximately 3-fold higher abundance in the MVM compared to the BM [79-81]. GLUT3 is mainly localised to the MVM and its expression decreases across gestation [82, 83].

GLUT1 and GLUT3 are also the primary glucose transporter isoforms expressed in the mouse placenta [84]. Compared to humans, fewer studies in mice have characterised placental localisation of GLUT proteins across gestation. GLUT1 and GLUT3 are co-localised in the placental labyrinth, predominantly in the maternal facing syncytiotrophoblast cell layer [78]. GLUT1 deletion results in embryonic loss at GD12 in mice, while the deletion of GLUT3 results in growth arrest of the embryo around GD8.5 [85]. This suggests that the two isoforms have a dynamic role in supporting fetal and placental growth throughout gestation in both humans and mice, with GLUT1 supporting placental, as well as fetal, growth throughout gestation but GLUT3 predominantly playing a role in the growth of the placento-fetal unit during early gestation [86].

1.4.1.2 Amino acid transport

In contrast to glucose, concentrations of virtually all amino acids are greater in the fetus than in the mother, suggesting that the transfer of amino acids across the placenta is an active process [87]. A range of transporters are utilised, including Na^+ dependent co-transporters which generate an electrochemical gradient via activity of Na^+/K^+ ATPase, which in turn drives these energy demanding processes [88]. One such system is the sodium-coupled neutral amino acid transporter (SNAT) family, involved in the transport of small, neutral amino acids such as alanine, serine and glycine. The family comprises three highly homologous isoforms, SNAT1, SNAT2 and SNAT4, encoded by the genes *Slc38a1*, Slc38a2 and *Slc38a4* [89], and all three isoforms are expressed in both the human and mouse placenta [90-92]. Protein expression of SNAT1 and SNAT2 is abundant in the rat labyrinth [91] with studies in isolated mouse placental vesicles further localising SNAT2 and SNAT4 to syncytiotrophoblast layer II, the maternal-facing plasma membrane [92, 93]. Similarly, all three isoforms are expressed in the placental MVM and BM in humans [90].

The system L transporters, consisting of large neutral amino acid transporter (LAT)1 and LAT2, are also active amino acid transporters, and are sodium-independent [94-96]. In the human placenta, LAT1 is predominantly localised to the MVM, and its expression is higher at term compared to mid-gestation [94, 97]. LAT2 is mainly localised in the BM and facilitates the exchange of non-essential amino acids in the fetal circulation with essential amino acids in the cytoplasm of the syncytium [94]. It has been more difficult to determine the presence and localisation of these amino acid transporters in rodent placentas, and the mode of transfer of amino acids across the two syncytial layers remains to be elucidated [86]. However, one study identified LAT1 and 2 protein expression in the maternal-facing plasma membrane of the second trophoblast layer of the mouse placenta [98].

1.4.1.3 Fatty acid transport

Fatty acids are essential precursors of bioactive molecules such as eicosanoid lipid mediators, and an important source of energy and building blocks for the fetus, with a particularly important role in supporting the developing brain and central nervous system [99, 100]. Although maternal circulating concentrations determine the fatty acid composition delivered to the fetus, the placenta preferentially transfers physiologically important long chain polyunsaturated fatty acids (LC-PUFAs), in particular the omega-3 fatty acids [101, 102]. Fatty acids can diffuse across the placenta down their concentration gradient but their movement is facilitated through a series of transport systems [99].These transporters include fatty acid binding protein of the plasma membrane (FABPpm), fatty acid translocase (FAT/CD36) and fatty acid transport proteins (FATP). FAT/CD36 and FATP transporters are localised in the MVM and the BM of human placental syncytiotrophoblast, suggesting their role in lipid transfer to the fetal circulation [103, 104]. FAT is a multifunctional protein and can bind to free fatty acids, collagen, thrombospondin and oxidized low density lipoproteins [100]. Similar to

the FATP proteins, FABPpm shows higher affinity for LC-PUFAs than other fatty acids, but is only localised to the maternal facing membranes of the human placenta [105].

Several of these fatty acid transport systems have also been identified in the rodent placenta. FABPpm, FAT and FATP are expressed in the rodent placental labyrinth and junctional zones, along with another transporter known as heart-type fatty acid binding protein (hFABP) [106]. Predominant expression of hFABP and FAT in the labyrinth suggests their involvement in the trans-placental movement of fatty acids from the mother to the fetus while FABPpm, FAT and FATP may also have a role in placental fatty acid uptake [106, 107]. The precise localisation of fatty acid systems in the rodent placenta and how lipids pass through the two syncytial layers is, however, still unclear [86].

1.4.2 *Placental hormone production*

The placenta orchestrates the maternal metabolic adaptations of pregnancy by secreting a variety of hormones, including members of the growth hormone (GH) and prolactin (PRL) family, steroid hormones and neuroactive hormones.

1.4.2.1 Growth hormone-prolactin family

One of the major hormone families expressed by the placenta is the growth hormone-prolactin (GH-PRL) family, which comprise structurally-similar genes that are expressed in a species-specific manner [108]. Humans only express a single *PRL* gene, but have an expanded *GH* locus (discussed in later sections), consisting of five genes, including a placentally-expressed GH variant (GH-V) [109]. Conversely, rodents only have a single pituitary-expressed *Gh* gene, but have a large *Prl* locus, consisting of approximately 26 genes that include placental lactogens (PLs), PRL-like hormones, proliferins, and proliferin-related proteins [108, 110]. The rodent placenta expresses

around 22 members of the *Prl* gene family, in the trophoblast giant cells, spongiotrophoblast cells, and invasive trophoblast cells [110-114].

The GH-PRL family promotes maternal metabolic adaptation to pregnancy by inducing β-cell mass expansion by increasing β-cell proliferation and reducing apoptosis in maternal pancreatic islets [115, 116]. These hormones also enhance the expression of glucokinase, hexokinase, and glucose transporter-2 (*Slc2a2*), and activate the serotonin biosynthesis pathway in pancreatic islets to mediate an increase in insulin secretion during pregnancy [1, 115, 117, 118]. This physiological state is essential for adequate glucose transport down a concentration gradient from maternal to fetal circulations [119]. In humans, pituitary GH secretion is diminished by mid-pregnancy when placental production of GH begins to dominate, [120, 121]. An increase in endogenous maternal GH secretion also occurs in rodent pregnancy, but in the absence of the placental growth hormone variant this is derived from the pituitary and is likely driven by a placentally derived factor (discussed in later sections) [122, 123].

1.4.2.2 Steroidogenesis

Early in human pregnancy, the corpus luteum (maintained by the placental hormone human Chorionic Gonadotropin; hCG) synthesises progesterone and oestrogens, until the placenta takes over production of these steroid hormones at about 8 weeks gestation, with increasing production until term [124]. In rodents, the corpus luteum remains the source of these hormones throughout pregnancy [125, 126]. The function of the corpus luteum is regulated by PRL-like or lactogen hormones in mice. Prolactin is secreted by the maternal pituitary until mid-pregnancy in rodents, and is inhibited by the placental secretion of placental lactogens 1 and 2 during mid-late pregnancy (Figure 1.2) [126-131]. Prolactin stimulates expression of oestrogen and luteinising hormone receptors, which increases the sensitivity of the corpus luteum to these hormones [132-134]. Prolactin also promotes differentiation of follicular cells into non-

dividing, progesterone-producing luteal cells, establishing the ongoing progesterone secretion necessary for the inhibition of ovulation, implantation and maintenance of pregnancy (along with oestrogen) [132, 135]. Although a transient increase in oestradiol (produced by follicular and luteal tissue) is observed at the time of embryo implantation, a substantial increase in maternal serum oestradiol concentrations occurs from mid-pregnancy until late pregnancy, with concentrations decreasing rapidly before delivery (Figure 1.2) [136].

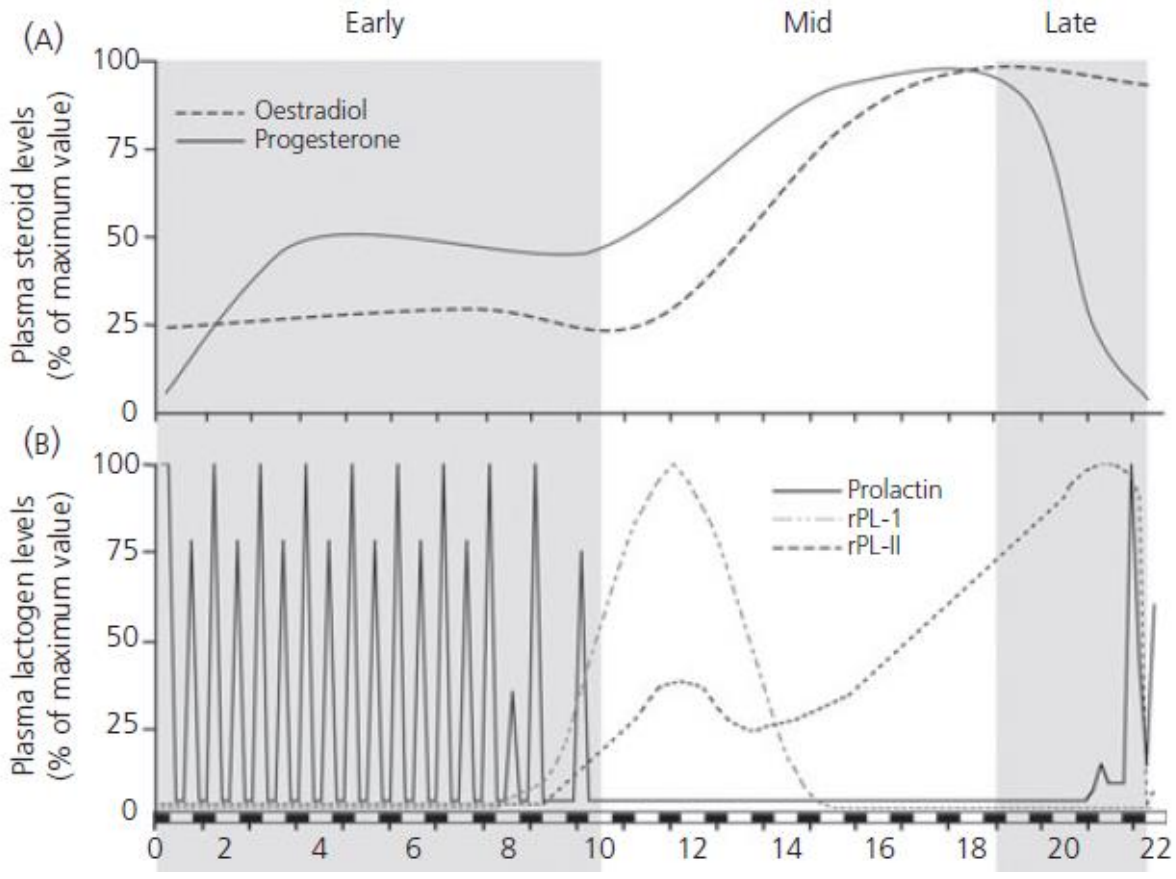


Figure 1.2 *Schematic representation of hormonal profiles during pregnancy in the mouse and rat. During early pregnancy, maternal pituitary derived prolactin (PRL) is secreted in twice-daily surges. During early mid-gestation, the placenta secretes PL-I and then PL-II, which also play roles in inhibiting maternal PRL secretion. This inhibition is lost near the end of gestation resulting in a surge in circulating maternal pituitary PRL. As the overall result of these coordinated events in mice, serum progesterone levels increase with the progression of pregnancy and peak at GD16, rapidly decreasing before parturition [137]. A substantial increase in maternal serum oestradiol (a form of oestrogen) concentrations occurs from mid-pregnancy until late pregnancy, with concentrations decreasing rapidly before delivery [136]. Figure taken from [138].*

Oestrogen (oestradiol in rodents) and progesterone have crucial roles in pregnancy in humans and rodents. Progesterone induces expression of genes that increase uterine receptivity, maintain myometrial quiescence and contributes to maternal immune tolerance through its anti-inflammatory properties. The fall in progesterone prior to parturition allows the ratio of oestradiol to progesterone to increase, which initiates gap junction formation between myometrial smooth muscles cells and permits coordinated contractions during labour [139-142]. Oestrogen also increases uterine receptivity and promotes endometrial growth and differentiation, placental angiogenesis, vasodilation, uteroplacental blood flow and nutrient transport [143-145]. Both hormones also contribute to maternal metabolic adaptations to pregnancy, including facilitating shifts in glucose and insulin homeostasis, lipid handling and appetite regulation [16, 124, 146, 147]. They also promote the development of the mammary gland through proliferation of mammary epithelium by promoting production and secretion of prolactin [148, 149].

1.5 IUGR

1.5.1 *Overview*

Intrauterine growth restriction (IUGR), in which a suboptimal fetal environment prevents the fetus from attaining its genetically determined growth potential, affects 6-12% of babies in developed countries [150]. A lack of international consensus on the diagnostic criteria for IUGR makes the identification of growth restricted fetuses difficult in clinical settings. Recently, the Delphi procedure was used to establish consensus-based definitions for early (>32 weeks) and late (>32 weeks) onset IUGR due to placental insufficiency. Three parameters, abdominal circumference (AC) <3rd percentile, estimated fetal weight (EFW) <3rd percentile or absent end-diastolic flow in

393 the umbilical artery, were accepted as solitary parameters – i.e. any of these
394 characteristics is sufficient to diagnose early onset IUGR, even if all other parameters
395 are normal. Additionally, AC or EFW <10th percentile combined with a pulsatility index
396 >95th percentile in either the umbilical or uterine artery were identified as contributory
397 parameters, which require other abnormal parameter(s) to be present for the diagnosis
398 of IUGR. For late onset IUGR, AC or EFW <3rd percentile were identified as solitary
399 parameters. Three contributory parameters were defined: AC or EFW <10th percentile,
400 AC or EFW crossing centiles by > two quartiles on the growth charts and cerebero-
401 placental ratio <5th centile or uterine artery pulsatility index >95th percentile. Presence
402 of at least two out of the three contributory parameters was agreed as defining late
403 onset IUGR. However, these definitions require information that may not be available
404 in all pregnancies, if repeated ultrasound data is not available. Low birth weight (LBW,
405 <2.5 kg) or small for gestational age (SGA, birth weight <10th percentile for gestational
406 age) as well as ultrasound assessments of fetal growth (abdominal circumference
407 <2.5th percentile) are therefore often used in clinical studies as markers of IUGR in
408 human infants [151]. It must be noted, however, that not all SGA babies are
409 pathologically growth restricted and some may be constitutionally small, while LBW can
410 be the result of prematurity without IUGR [152].

411 LBW and IUGR amplify risks of perinatal mortality more than 5-fold, including a
412 substantial increase in the risk of stillbirth [153]. For those infants who survive, the
413 adverse effects of IUGR persist throughout the life-course, with increased risks of
414 multiple adult diseases including type 2 diabetes and cardiovascular disease [154]. In
415 developed countries, where maternal nutrition is usually not a limiting factor, placental
416 dysfunction is the most common contributor of IUGR and currently cannot be prevented
417 or treated.

1.5.2 *Classification of IUGR*

IUGR can be classified into three types: asymmetric, symmetric and mixed [155-157]. Asymmetric IUGR is associated with a reduced fetal abdominal circumference but normal biparietal diameter, head circumference and femur length. It is the most common type of IUGR and is typically associated with utero-placental insufficiency [158]. This phenotype occurs as a result of redistribution of cardiac output to maintain the growth of essential organs, including the brain and heart, resulting in compromised growth of organs like the kidneys, gut and liver [159, 160]. Symmetric IUGR, where all morphological measures are similarly reduced, was believed to be typically due to genetic disorders or infection intrinsic to the fetus. However, recent work has shown that markers of poor maternal health during early pregnancy, such as low and high body mass index, low socioeconomic status were strong risk factors associated with symmetric SGA [161]. Contrary to asymmetric IUGR, where the onset of growth restriction usually occurs later in gestation, symmetric IUGR is associated with insults that occur during early gestation [157]. This is due to the fact that this stage of fetal development is associated with rapid cellular hyperplasia, as opposed to cellular hypertrophy, and thus insults tend to result in proportionally small fetuses with reduced cell numbers but normal cell size [162]. Fetuses from mixed IUGR, most commonly observed in developing countries, have clinical features of both asymmetric and symmetric IUGR and usually reflect an insult that occurs in early gestation that is further exacerbated later in gestation by placental dysfunction [163].

1.5.3 *IUGR diagnosis*

Measurement of fundal height (in centimetres) between 24-38 weeks of gestation provides an approximation of gestational age and is also used to screen for fetal growth less than or greater than the 10th percentile for gestational age [164]. However, this method has limited accuracy when identifying abnormally grown fetuses, with

maternal obesity further complicating this clinical estimate [165-167]. More appropriate assessment of growth restriction is obtained by biometric measures of fetal biparietal diameter, head circumference, abdominal circumference and femur length by ultrasound [167, 168]. Ultrasonography is also used to obtain an estimate of fetal weight and, if estimated to be below the 10th percentile, further evaluations are considered, including ultrasound assessment of amniotic fluid index and umbilical artery blood flow [169]. Increased umbilical artery resistance may suggest that the pregnancy is complicated by underlying placental insufficiency. Furthermore, absent or reversed end-diastolic flow in the umbilical artery is associated with increased risk of perinatal mortality and will therefore influence clinical decisions about timing of interventions including early delivery [170-174].

1.5.4 *Causes of IUGR*

IUGR can be caused by a number of maternal, placental and fetal factors, including maternal immaturity (adolescent pregnancy) or advanced maternal age, smoking, infections and genetic/chromosomal abnormalities [175-177]. Although the underlying pathophysiological mechanisms in these conditions are different, they often share the same final pathway of suboptimal utero-placental perfusion and fetal supply of oxygen and nutrients [178].

1.5.4.1 Maternal causes

Maternal demographic factors including age, weight, socio-economic status and ethnicity can influence the risk for IUGR in their babies. Women at extremes of reproductive age, especially younger mothers, have a higher risk of experiencing complications like IUGR, while advanced maternal age has been associated with an increased risk of delivering a LBW baby [179, 180]. Due to factors like poor nutritional status, maternal anaemia, lack of access to prenatal care, lower pre-pregnancy weight

and weight gain, women from developing countries have a higher risk of LBW and IUGR compared to women living in developed countries [181-183]. Women living in high altitude areas also experience a chronically hypoxic environment, which probably explains their higher incidence of LBW offspring compared to women living at sea level [184-186]. Substance use and abuse in pregnancy, including maternal smoking, increases the risk of LBW, SGA, preterm birth and perinatal death and is also associated with a greater risk of Sudden Infant Death Syndrome (SIDS) [187, 188]. Similarly, maternal alcohol consumption during pregnancy can cause Fetal Alcohol Spectrum Disorder which is associated not only with reduced fetal growth, but also birth defects and growth and neurodevelopmental issues that persist into adulthood [189]. Maternal medical conditions that alter utero-placental blood flow, including pre-gestational diabetes mellitus, pregnancy-related hypertensive diseases, and cyanotic cardiac disease, as well as autoimmune diseases, can also lead to IUGR [190].

1.5.4.2 Fetal causes

Fetal factors that may cause IUGR include genetic abnormalities, congenital malformations, and fetal infections. Fetal genetics including chromosomal abnormalities account for 5-20% of IUGR cases, and in the majority of these, the growth restriction has an early onset [191, 192]. Fetal infections can result in restricted fetal growth, but this is relatively rare, only accounting for <5% of IUGR cases. Both viral infections, including rubella, cytomegalovirus, herpes and human immunodeficiency virus, and parasitic infections, like toxoplasmosis and malaria, are associated with reduced fetal growth [193, 194]. Malaria is a common cause of LBW and IUGR in developing countries as it reduces uterine and umbilical artery blood flows as well as placental oxygen and nutrient transport capacity [195-197]. Although bacterial infections are less likely to cause IUGR, some reports have linked syphilis, chlamydia, mycoplasma, listeria and tuberculosis with impaired fetal growth [192, 198].

1.5.4.3 Placental causes

Placental dysfunction or placental insufficiency is the most common pathology associated with IUGR, and is broadly defined as alterations in placental oxygen and nutrient transfer capacity which adversely impact fetal growth [199]. Although maternal undernutrition and hypertension can contribute to the development of placental insufficiency, in more than half of the cases placental dysfunction is due to abnormal remodelling of uterine and placental spiral arteries which reduces utero-placental perfusion [200, 201]. Placental insufficiency is often characterised by fetal redistribution of blood flow to vital organs over peripheral organs, in response to hypoxia, and can be detected through altered umbilical, uterine and middle cerebral artery Doppler flows [169, 202]. Certain placental complications, including placental abruption, infarction, circumvallate shape, haemangioma and chorioangioma, although more rare, can also impair the normal function of the placenta and contribute to IUGR or mortality in severe cases [203-207].

1.5.4.4 Placental nutrient transport in IUGR

Facilitated glucose transport across the human placenta through the second half of gestation is primarily mediated by GLUT1 (product of the *Slc2a1* gene). Although fetal hypoglycaemia is associated with IUGR, GLUT1 protein expression, as well as activity in syncytiotrophoblast membranes, is normal in placentas of these fetuses [79, 208]. However, compared to normal placentas, placentas from pregnancies complicated by late-onset IUGR have higher *Slc2a3* mRNA and GLUT3 protein expression on the maternal side of the placental barrier [209]. This may reflect increased placental glucose consumption at the expense of glucose availability for fetal growth [79, 208].

Activity of the System A transporters (SNATs), which mediate the uptake of neutral amino acids (glycine/serine/alanine), is reduced in the MVM of IUGR placentas [208,

210-212]. This decrease in activity is likely due to reduced MVM protein expression of SNAT1 and SNAT2 [212]. Furthermore, the degree of reduction in System A activity is related to the severity of IUGR, as assessed by abnormal pulsatility index in the umbilical artery and fetal heart rate monitoring [213]. In another study, the reduction in placental System A activity is especially notable in IUGR babies who were delivered preterm (28-36 weeks) compared to IUGR babies delivered at term [208]. Additionally, the placental uptake of essential amino acids including leucine, lysine (system L transporters) and taurine (system β transporters) is reduced in MVM and/or BM vesicles isolated from IUGR pregnancies compared to those from uncomplicated pregnancies [214, 215]. Similarly, reduced placental transfer of essential amino acids leucine and phenylalanine in term IUGR pregnancies has been demonstrated *in vivo* using stable isotope techniques [216]. Reductions in activity of placental amino acid transporters are likely associated with decreases in placental amino acid uptake, and contribute to lower plasma concentrations of several amino acids in IUGR fetuses [217].

The characteristically low subcutaneous fat depots and thin appearance of the IUGR fetus are thought to be attributed to reduced fetal fat synthesis and/or placental transfer of free fatty acids [218]. An important first step in the transplacental transfer of free fatty acids to the fetus involves placental lipoprotein lipase. The activity of this enzyme is reduced in the MVM from human IUGR placentas compared to age-matched controls [219, 220] and corresponds to lower fetal/maternal ratios of LC-PUFAs in IUGR pregnancies [221]. Higher protein expression of receptors involved in placental cholesterol uptake (low density lipoprotein-receptor and scavenger receptor class B type-I) [222], as well as placental fatty acid transporters (FATP6 and CD36) [223], provide further evidence of alterations in fetal lipid uptake in IUGR. These appear to be compensatory mechanisms to maintain delivery of cholesterol and vital LC-PUFAs to support processes like placental steroid hormone synthesis and brain development in

the IUGR fetus [223, 224]. However, further studies are required to confirm these associations.

1.5.4.5 Multiple gestation

Multiple gestation is another cause of IUGR, which can reflect maternal (ovulation rate) and fetal (embryo division) factors and leads to placental insufficiency. In these pregnancies, the degree of growth restriction increases in proportion with fetal number, as approximately 55% of twins and 99% of higher-order multiples are LBW compared with 5.2% of singletons [188]. Similarly, the incidence rate of IUGR in singleton pregnancies is around 10% but this increases to 15-41% in twin pregnancies and 50-60% in higher order pregnancies [150, 225-227]. Besides number of fetuses, the risk of IUGR in multiple pregnancies is also influenced by other factors like chorionicity, prevalence of a congenital anomaly or umbilical cord abnormalities, twin-twin transfusion syndrome and maternal nutrition [228]. Poor fetal growth contributes to poor health outcomes in multiple compared to singleton human pregnancies. Twin pregnancies make up only 2-5% of all livebirths but they account for 10-15% of all adverse neonatal outcomes [229, 230]. As a result, twin and triplet neonates have 3 and 9 times longer hospital stay, respectively, compared to singletons [231], and a large proportion of twin (78.2%) and triplet (97.1%) gestations result in Caesarean delivery [231]. One of the major contributors to perinatal mortality in multiples is believed to be the high prevalence of preterm births in this population (~63% in twins and 100% in higher-order pregnancies compared with ~7% in singletons) [188, 232]. As a result, fetuses in multiple pregnancies are also more likely to have respiratory complications immediately after delivery and are more likely to die due to related complications within the first year of life, compared to singletons [231].

1.5.5 *Consequences of IUGR*

1.5.5.1 Short-term consequences of IUGR

IUGR has a number of short-term consequences for the infants. These include a higher risk of preterm delivery, asphyxia and related neonatal complications including respiratory distress syndrome, need for intubation, acidosis, stillbirth and neonatal death [233, 234]. In the early neonatal period, IUGR infants have a higher incidence of low Apgar scores, seizures, polycythemia, hyperbilirubinemia, hypoglycaemia, hypothermia, sepsis, intraventricular haemorrhage, and necrotising enterocolitis [234-236]. As a result of the higher incidence of neonatal complications, growth restricted fetuses often require prolonged support in a neonatal intensive care unit (NICU) and have higher health care costs than babies born at an appropriate weight for gestational age (AGA) [235, 237, 238].

1.5.5.2 Long-term consequences of IUGR

Epidemiological studies have consistently reported associations between LBW and risk of developing a number of complications, including coronary artery disease, type 2 diabetes, central obesity and the metabolic syndrome, later in life [239-243]. IUGR, LBW and SGA babies characteristically exhibit accelerated neonatal growth, known as catch-up growth, during the first two years of life [244, 245]. This period of accelerated growth is associated with preferential deposition of fat (particularly visceral fat), altered insulin secretion, glucose intolerance, hypertension and raised plasma triglyceride concentrations, and is hence a major factor predisposing this population to the development of chronic disease later in life [45, 246-251]. Furthermore, children exposed to a suboptimal intrauterine environment also have reduced head circumference and brain volume due to grey matter loss, as well as impaired white matter development. This is accompanied by microstructural changes that are indicative of reduced myelination and axon injury, often due to inflammation [252-256].

Although reductions in head circumference, a proxy for brain size, are somewhat offset by accelerated post-natal growth, this recovery is often incomplete compared to normally grown infants of the same age [253]. As a result of impaired neurodevelopment, IUGR children often have poorer cognitive and academic outcomes compared to AGA children, with higher incidence of cerebral palsy and behavioural disorders [234, 257-262]. Therefore, the origin of a number of complications associated with IUGR appear to be due to effects of a sub-optimal perinatal environment on the development of fetal organs and systems, including adaptations to survive the intrauterine insult, and these can have life-long impacts [263].

1.5.6 *Treatment of IUGR*

To date, a number of approaches for treating IUGR have been trialled in both pre-clinical and clinical studies. The following section will summarise some of these approaches including those aimed at directly increasing fetal nutrient availability, decreasing fetal oxidative stress and enhancing utero-placental blood flow.

1.5.6.1 Nutrition

As placental insufficiency is associated with reduced supply of nutrients and oxygen to the fetus, a number of animal studies have tested whether direct or indirect nutrient supplementation can improve fetal growth and long-term outcomes. In a hyperthermia-induced sheep model of IUGR, chronic fetal infusion with branched and essential amino acids during late gestation restored fetal insulin secretion and pancreatic islet size [264], but had variable effects on protein accretion rates [265] without any improvements in fetal or placental weights [264, 265]. Direct glucose infusion to growth restricted ovine fetuses, however, was not well-tolerated, as the resulting fetal hyperglycemia resulted in increased fetal oxygen consumption in the absence of an

621 increased fetal oxygen supply, giving rise to fetal hypoxia and acidosis, in the absence
622 of any effects on fetal growth [266].

623 Results of nutrient supplementation are also mixed in human studies. Intra-umbilical
624 supplementation with glucose and amino acids causes an imbalance of amino acids in
625 the fetal plasma and no significant improvements in severely growth restricted fetuses
626 [268]. Later studies combined this method of nutrient supplementation with hyperbaric
627 oxygenation, to increase oxygen diffusion across the placenta. This technique was only
628 tested in two women and results are mainly based on fetal survival, which was only
629 achievable in one of these women [269]. Therefore, attempts to promote fetal growth
630 by increasing nutrient availability appear to result in variable degree of success across
631 species with side effects that may further impair fetal development.

632 1.5.6.2 Antioxidants

633 Maternal antioxidant administration using a range of agents, including allopurinol,
634 vitamin C and melatonin, has been applied in human studies in an attempt to reduce
635 oxidative stress. Of these agents, melatonin is the most well studied and has an
636 established safety profile, strong antioxidant properties and can be easily administered
637 orally. Treatment with melatonin improves vascular endothelial integrity of cultured
638 human umbilical vein cells [270], likely due to the combined effect of melatonin's anti-
639 oxidant and anti-inflammatory properties [271-274]. In rodents and sheep pregnancies
640 complicated by IUGR, maternal melatonin treatment increased fetal oxygenation,
641 uterine blood flow and/or fetal weight [275-278]. Melatonin administration also
642 improved brain development and neuro-behavioural outcomes in growth restricted
643 lambs [218]. However, other studies in sheep found that treatment with melatonin
644 either resulted in no improvement in birthweight [271] or worsened the hypoxia-induced
645 IUGR with further reductions in fetal weight and size [279]. Melatonin is also being
646 assessed as a treatment for IUGR and preeclampsia in human clinical trials.

Preliminary results suggest that melatonin may prolong pregnancy duration and possibly improve fetal growth, but these trials are still ongoing, and therefore clinical efficacy and safety profile remain to be defined [270, 280, 281].

1.5.6.3 Treatments to increase utero-placental blood flow

A technique that improves utero-placental blood flow is the use of vasodilators, with sildenafil citrate being the most widely studied example. Sildenafil inhibits phosphodiesterase type 5, the main enzyme responsible for nitric oxide clearance, and hence prolongs elevation of nitric oxide, leading to vasodilation and relaxation of smooth muscle [282]. The vasodilatory effects of sildenafil on human myometrial vessels from normal and growth restricted placentas have been confirmed *in vitro* as well as *in vivo* [283, 284]. Experimental animal studies have been conducted in a wide range of species, including sheep, rabbits, rats and mice, and sildenafil increased utero-placental blood flow and fetal delivery of nutrients and oxygen, resulting in improved fetal growth in these species, with improved maternal blood pressure also reported in preeclampsia models in rodents [285-288]. However, sildenafil treatment only improves fetal and maternal outcomes in compromised and not normal pregnancies, with its effects being greatest when given orally and continuously [289]. Furthermore, in some animal studies sildenafil treatment caused adverse fetal outcomes [290, 291]. For example, in a sheep IUGR model of single umbilical artery ligation (SUAL), sildenafil was administrated over 24 hr to pregnant ewes between 105-110 days' gestation (term is 150 days) [290]. Outcomes measured over 48 hr after sildenafil administration were indicative of a significant deterioration in fetal well-being, as evidenced by decreased fetal oxygenation, increased fetal CO_2, hypotension, and tachycardia [290]. Maternal administration of sildenafil from mid-late pregnancy in the placental-specific Igf2 knockout mouse model of IUGR also did not improve fetal growth and was associated with increased blood pressure in adult mouse offspring [292].

In light of the positive effects obtained in *in vitro* and in some experimental animal studies, sildenafil citrate was tested as a potential treatment for IUGR in human pregnancies in a multicentre, randomised, placebo-controlled, double-blind trial, the Sildenafil Therapy in Dismal prognosis Early-onset intrauterine growth Restriction (STRIDER) study. This trial only included severely growth restricted babies who had a very low chance of survival without any intervention and may not have been representative of more typical cases of IUGR. Fetal outcomes did not improve in the cohort based in Australia and New Zealand, but no evidence of harm was observed [293]. The STRIDER trial has since been suspended, however, due to the deaths of a number of babies, associated with pulmonary dysfunction [294-296].

Nutritional approaches to increase the abundance of nitric oxide and increase utero-placental blood flow have also been investigated in animal and human studies. Providing protein-restricted pregnant rats drinking water supplemented with L-citrulline, a precursor to the amino acid L-arginine and in turn nitric oxide synthesis increased fetal weight near term by 8%, without changing placental weight [267]. This was attributed to a combination of increased muscle synthesis and the vasodilatory effects of increased nitric oxide in the dam [267]. Similarly, intravenous and/or oral L-arginine supplementation for 7 days to women with pregnancies complicated with IUGR improved fetal weight by up to 6.4% [297]. In another study, intravenous L-arginine to hospitalised pregnant women increased fetal weight by 3.9%, and reduced utero-placental vascular resistance, although the timing and duration of treatment are unclear [298]. In women with gestational hypertension with or without proteinuria, oral L-arginine supplementation reduced diastolic blood pressure and prolonged pregnancy duration [299]. However, no improvements were found in birthweight in the latter study or when oral L-arginine supplements were given to women with severe IUGR [299, 300].

Another interesting approach to increasing utero-placental blood flow and thereby fetal growth is through overexpression of VEGF in uterine arteries, using an adenovirus vector [301]. Preclinical work has shown promising results with improved uterine artery blood flow [301] and fetal weight gain in growth restricted sheep [302, 303], due to improved placental vascularisation. This approach was also tested in guinea pigs, and resulted in a more modest (3%) improvement in weight of growth restricted fetuses compared to non-treated controls [304]. Different methods were used to induce growth restriction between these studies, as the sheep study over nourished adolescent ewes, whereas guinea pig dams were undernourished to restrict fetal growth. Further studies need to be performed to determine whether the effectiveness of this approach is dependent on the experimental model and/or the degree of IUGR. Therefore, there remains a lack of safe and effective strategies for treating IUGR, and animal models are needed to test other possible interventions.

1.5.7 *Animal models of IUGR*

Several different animal models have been developed in order to better understand the complex mechanisms and molecular events that contribute to an adverse intrauterine environment and the alterations in placental physiology leading to growth restricted offspring. These models allow researchers to study the effects of various types and durations of ‘insults’ on maternal and fetal outcomes and hence better understand the multifactorial nature of IUGR. However, due to species differences in placental anatomy, gestation length, maturation stage at birth, and rate of fetal development, the same type of insult can result in different fetal and maternal outcomes depending on the experimental model.

As discussed earlier, the primary cause of IUGR in developed countries is placental dysfunction, which results in a sub-optimal supply of oxygen and nutrients to the developing fetus. This, in turn, can be influenced by a number of factors, including

alterations in maternal or fetal blood flow and/or inability of the placenta to sufficiently alter its morphology (vascularity, barrier thickness/surface area etc.) and function (nutrient transporters) to meet fetal growth demands [200, 305, 306]. Several different approaches to experimentally induce placental insufficiency, and thereby IUGR, have been applied in both small and large animal models, and the most widely used of these models will be described briefly below.

1.5.7.1 Uterine artery ligation

Uterine artery ligation is a commonly used method to experimentally induce placental insufficiency in animals with bicornuate uteri. This process reduces utero-placental blood flow and hence reduces oxygen and nutrition delivery to the fetus, a feature commonly present in clinical IUGR secondary to placental insufficiency [307, 308]. In guinea pigs, rabbits and sheep, SUAL produces asymmetrically growth restricted fetuses [309-312]. Using this model, the average growth restriction relative to controls is 24% in rats [310, 313, 314] and sheep [309, 315, 316], 30% in rabbits [312, 317] and 44% in guinea pigs [318, 319]. SUAL also reduces materno-fetal transfer of glucose, an observation first made in sheep studies and induces fetal hypoxaemia, both of which contribute to reduced fetal growth [320]. Similarly, SUAL in late gestation of rats and guinea pigs decreases placental capacity to transfer glucose and amino acids in conjunction with reduced fetal growth [321, 322].

1.5.7.2 Caruncletomy

Another model of IUGR in sheep is caruncletomy, which involves the surgical removal of the majority of the discrete endometrial implantation sites (caruncles) prior to mating [323, 324]. Limited number of placentomes are formed from the beginning of pregnancy, which constrains placental growth and function, and restricts placental capability to deliver oxygen and nutrients to the fetus [325-327]. Fetuses of

751 carunclectomised ewes are hypoxemic and hypoglycaemic, with higher cortisol
752 concentrations and lower concentrations of insulin, thyroid hormone and prolactin
753 compared to controls [325, 328, 329]. Furthermore, fetal circulating concentrations of
754 the key promoters of fetal growth, insulin-like growth factor (IGF)1 and IGF2, are
755 decreased during late gestation in these fetuses. Ultimately, the restricted nutrient
756 supply and altered hormone concentrations induced by caruncelectomy reduces fetal
757 survival by 14%. Asymmetric growth restriction is observed in the surviving fetuses,
758 with an average of 25% reduction in birth weight relative to control fetuses [330, 331].

759 In addition to the effects outlined above, the increased exposure to glucocorticoids
760 during late gestation in carunclectomised fetuses likely attenuates the activation and
761 regulation of the hypothalamic pituitary adrenal (HPA) axis and impairs fetal glucose-
762 mediated insulin secretion [325, 327, 332]. Enhanced insulin action on glucose and
763 amino acids has been linked to the catch up growth in one-month old IUGR lambs,
764 while increased insulin sensitivity of circulating free fatty acids (FFAs) contribute
765 towards enhanced visceral adiposity, similar to observations in some cases of human
766 IUGR [248, 331, 333]. The period of accelerated growth which occurs after birth is,
767 however, often followed by the gradual development of insulin resistance in these
768 individuals and may contribute to the development of metabolic syndrome and its
769 associated complications (including impaired β-cell function) [334-336], particularly in
770 males [337, 338]. Indeed, by one year of age, the elevated insulin sensitivity observed
771 in IUGR male sheep at one month, is lost and insulin action is actually impaired in
772 these young adult sheep [338].

773 1.5.7.3 Maternal undernutrition

774 Although most cases of IUGR in developed countries are due to impaired placental
775 function rather than maternal undernutrition, the effects of both of these insults on
776 placental signalling and function are similar, suggesting common final pathways of

777 placental responses that are independent of the cause of IUGR. Similar to SUAL,
778 maternal undernutrition in rats, guinea pigs, rabbits and sheep produces growth
779 restricted fetuses near term with evidence of brain sparing [339-342]. Additionally,
780 maternal undernutrition during pregnancy has been associated with several postnatal
781 complications in the offspring, including hypertension, glucose intolerance, increased
782 fat mass and reduced organ weights [342-344]. A large number of studies have
783 investigated the timing and effect of mild (~20-30% less than *ad libitum*), moderate
784 (~50%) and severe (~70%) maternal caloric restriction in a number of animal models
785 [345]. Depending on the timing, duration and severity of the nutrient restriction,
786 decreases in fetal weight compared to controls range from 13-49% in rats [50, 342,
787 346], 11-17% in sheep [317, 347], 10-35% in guinea pigs [339, 348] and 7-10% in
788 rabbits [341, 349].

789 Many of the effects of nutrient restriction are likely mediated through the placenta. In
790 guinea pigs, maternal undernutrition not only reduces fetal and placental weights, but
791 also induces structural modifications in the placenta. Roberts and colleagues found the
792 mean placental barrier thickness for diffusion was higher, and total placental area
793 available for exchange lower, in dams subjected to caloric restriction during pregnancy
794 compared to *ad libitum* fed controls, likely further restricting the delivery of nutrients to
795 the fetus [348]. Reductions in placental functional capacity have also been
796 demonstrated in rats after maternal caloric restriction through reduced trans-placental
797 transfer of radiolabelled isotopes measuring neutral amino acid and glucose transport
798 in late gestation [350]. Consistent with these measures of impaired function, placental
799 GLUT3, SNAT1 and SNAT2 are lower, although SNAT4 protein abundance is higher in
800 pregnant rats during late gestation in response to 50% maternal caloric restriction [351,
801 352]. Although maternal undernutrition in mice has been associated with reduced fetal,
802 as well as placental, weights in some studies, only limited data are available on its
803 effect on murine placental function [353, 354]. In contrast to the studies in rats, 20%

maternal caloric restriction in mice from GD3 onwards did not change placental capacity for glucose transport at GD16 or GD19, although the transport capacity for neutral amino acids was increased at GD19 [354]. Given the discrepancies in the literature, further studies are needed to clarify the effects of different timing and degree of restriction in placental nutrient transport, and to determine whether these are species-specific.

1.5.7.4 Maternal protein restriction

Restriction of maternal protein intake during pregnancy is also associated with modifications in placental function that vary depending on gestational age and species. Jansson and colleagues fed pregnant rats protein restricted (4% protein) or control diets (18% protein) starting from GD2 [355]. They reported lower placental System A transporter activity at GD19 and 21 and lower SNAT2 protein at GD21 compared to dams fed an isocaloric, protein-sufficient diet [355]. However, fetal and placental weights were not reduced in the low protein group until GD21, leading the researchers to conclude that placental transporter changes were a cause, rather than a consequence of IUGR [355]. The group also demonstrated decreased protein expression of SNAT1 and System L transporter activity measured by radioisotopes during late gestation in rat dams fed a low-protein diet compared to control dams [356]. This decrease was linked to inhibition of placental insulin, IGF1, mammalian target of rapamycin (mTOR), and signal transducer and activator of transcription (STAT)3 signalling, positive regulators of placental amino acid transporters [356]. Therefore, these results suggest that maternal protein restriction disturbs placental signalling pathways, which in turn reduces placental capacity for amino acid uptake through downregulation of multiple transporter systems, ultimately reducing fetal weight [355, 356].

Placental adaptations in response to alterations to maternal dietary protein content (casein content 23%, 18% or 9%) have also been reported in mice. In contrast to rat studies, maternal protein restriction (18% or 9% casein in comparison with 23% in the control diet) throughout gestation in mice, resulted in heavier placentas compared to controls [357]. However, in the 18% casein diet, the volume of the placental labyrinth zone, responsible for nutrient exchange was smaller, while that of the endocrine junctional zone was larger compared to controls. In contrast, labyrinth volume was maintained at control values in the 9% casein group, at the expense of junctional zone growth, suggesting that different strategies were employed to match nutrient availability with fetal growth demands between the low protein groups. Furthermore, compared to controls, both protein restricted groups showed increased placental capacity for glucose transport at GD16 but this increase was only sustained until GD19 in the 9% group. Placental capacity for amino acid transport increased at GD16 but decreased at GD19 in the 18% group relative to controls, with no changes observed in the 9% casein group. Given that fetal weight was similar in all groups at GD16 and was only reduced (by 9%) in the 9% casein group at GD19, these data suggest that amino acid availability appears to be more important for fetal growth in mice during late gestation compared to glucose availability [357]. If the protein content of the maternal diet was reduced to 6% (compared with 20% protein diet in controls), from mid-pregnancy in mice, fetuses had lighter placentas compared to fetuses from control dams with evidence of brain sparing near term [358], similar to effects of maternal protein restriction in rats. Although placental function was not measured in this study, maternal protein restriction was associated with a smaller junctional zone relative to controls, possibly to allow the preferential growth of the substrate exchange, labyrinth area [358]. Taken together, these studies demonstrate that changes to maternal diet induce alterations in placental function and capacity to meet fetal growth demands.

1.5.7.5 Hyperthermia-induced placental restriction

Environmental factors such as elevated temperatures can also cause IUGR. Hyperthermia was developed as an IUGR model after observations that sheep in hotter climates have a higher incidence of LBW delivery compared those in more temperate climates [359]. In this model, commencing around day 40 of gestation, pregnant ewes are housed in an environment where the ambient temperature is raised to 40 °C for 12 h, and then decreased to 35 °C for 12 h of each 24 h period with moderate humidity [360]. These conditions raise maternal core body temperature from ~39 °C to 40 °C and results in redistribution of blood flow towards the peripheral vascular system, thereby reducing umbilical and uterine blood flows [361-364]. As a result, fetuses have smaller placentas with reduced glucose and oxygen transport capacity, leading to fetal hypoxaemia and hypoglycaemia, and asymmetrical growth restriction [365-369]. Fetal outcomes in the model are heavily dependent on the timing of insult. Maternal exposure to heat-stress during early pregnancy (~GD0-40) leads to embryonic loss and, consequently, the majority of studies using this model of IUGR expose ewes to high temperatures after GD45 for variable durations [359, 360]. The highest degree of fetal restriction is achieved if maternal exposure to elevated temperature occurs from ~GD40 until late gestation or term (fetal weight ~50% of controls). However, less severe growth restriction is achieved if maternal heat exposure only occurs during mid or late gestation (~30% reduction compared to controls) [369].

1.5.7.6 Genetic models of IUGR

Genetic manipulation of specific genes in murine models have allowed researchers to better understand underlying mechanisms associated with compromised fetal growth in IUGR. A well-studied example in mice is the placental-specific insulin-like growth factor 2 (Igf2) knockout model. In this model, deletion of the labyrinth specific IGF2P0 promoter leads to Igf2 deficiency specifically in the placental nutrient exchange area

[93, 370].The weight of P0 mutant placentas is significantly reduced compared to wild-type placentas from GD13.5 onwards and this precedes the onset of IUGR [93, 370]. As a possible adaptation to reduced placental weight, System A amino acid transport is initially upregulated in IGF2P0 knockout placentas at GD16, allowing fetal growth to be maintained through increased efficiency of secondary active transport compared to wild-type placentas [93, 370]. However, by late pregnancy, the adaptations of the IGF2P0 knockout placentas are insufficient to meet the nutrient demands of the rapidly growing fetus, resulting in growth restriction [370]. Additional stereological analysis revealed further abnormalities in the IGF2P0 knockout placentas, including a 50% reduction in surface area of the trophoblast layers and increase in barrier thickness by 28% compared to wild-type placenta, contributing to reduced diffusional permeability [371]. Together, this causes a 20-25% reduction in weight of IGF2P0 knockout fetuses compared to their wild-type littermates [370, 372]. The reduction in fetal weight becomes even more severe (~50%) if the *Igf2* gene is completely knocked out. Unlike the IGF2P0 knockout mouse, where placental growth restriction precedes fetal growth restriction, placental and fetal growth restriction occur concurrently in the *Igf2* null mice. The placentas from mice with complete *Igf2* ablation support less fetus per gram of placenta with significant reductions in placental amino acid transport compared to their wild-type littermates by term [373]. Thus, both fetal as well as placental *Igf2* appear to be important for facilitating optimal fetal growth through their effects on placental development and function [373].

In another genetic murine model of IUGR, the endothelial nitric oxide synthase (eNOS) gene is globally knocked out [374]. As the encoding enzyme coverts arginine to nitric oxide, and ultimately promotes vasodilation, the deficiency of eNOS results in dysfunctional responses of uterine arteries to vasodilators as well as vasoconstrictors *in vitro* [374, 375]. Furthermore, spiral artery remodelling and impaired feto-placental vascular development associated with reduced VEGF expression are evident in eNOS^{-}

$^{/-}$ mice [376, 377]. Placental System A amino acid transporter activity is also reduced, ultimately contributing to approximately 10% reduction in fetal weight at term compared to wildtype fetuses [374]. These genetic models of IUGR hence highlight the roles of specific genes as important modulators of optimal placental function and fetal growth and allow researchers to better understand and investigate the multifactorial nature of IUGR.

1.5.7.7 Large litter size

In rodents, as in other litter-bearing species such as pigs and rabbits, placental and fetal weight decreases with increasing litter size [378-385]. In twin-bearing sheep, reduction from a twin to singleton pregnancy after implantation results in lambs with birth weight intermediate between that of singleton and twin pregnancies [386, 387]. This suggests that constraint of fetal growth due to increasing litter size likely reflects both reduced placental growth and function and competition for maternal nutrients. Furthermore, similar to humans, the growth trajectory of twin fetal sheep diverges from that of singletons early in gestation [388-391]. Therefore, events that occur around the time of conception or during early pregnancy are likely to play an important part in causing the reduced fetal and placental growth observed in multiple pregnancies [392]. Consequently, improving fetal growth in multiple pregnancies is likely to require different interventions than in singleton IUGR pregnancies, where the primary cause is likely to be poor placental function. However, testing interventions in natural pregnancies in litter-bearing animals is complicated, because the spontaneous variation in litter size is also dependent on maternal factors that determine ovulation rate, which increases with maternal weight, parity and age across multiple species [393-398]. Therefore, there is a need to develop animal models that reflect the intrauterine environment of multiple pregnancies but are not confounded by these maternal factors. This will allow researchers to test potential intervention strategies to prevent or treat IUGR in multiple pregnancies, such as promotion of maternal metabolic

adaptations and placental function through elevated maternal growth hormone (GH) and or IGFs.

1.6 Growth hormone/insulin-like growth factor axis

1.6.1 *Overview of the axis*

GH is a peptide hormone that promotes cell division, regeneration and growth. It is secreted from the somatotrophs of the anterior pituitary in a pulsatile manner due to the alternating stimulatory and inhibitory influence of hypothalamic hormones GH-releasing hormone (GHRH) and somatostatin (SRIF), respectively (Figure 1.3) [399, 400]. The gastric peptide hormone ghrelin, once acylated, also stimulates GH secretion by acting at the level of the hypothalamus and pituitary through the GH secretagogue receptor (GHSR)1-a [401]. GH secretion is further influenced by a broad range of factors, including concentrations of sex steroids (puberty, sex), age, sleep, exercise, stress, insulin, glucose, amino acids and fatty acid concentrations, visceral fat mass and pregnancy (discussed in detail later in this thesis) [402, 403].

GH mediates several important physiological processes, including somatic growth and development, carbohydrate and lipid metabolism, by binding to the growth hormone receptor (GHR). Binding of GH to the GHR induces intracellular signalling through a phosphorylation cascade involving the Janus Kinase/Signal Transducing Activators of Transcription (JAK/STAT) pathway [404]. JAK-STAT activation in turn activates a number of different intracellular signalling pathways which regulate cellular processes including transcription, proliferation and metabolism [405]. GH can also act indirectly by inducing the synthesis of the potent growth and differentiation factor, IGF1, which mediates many of GH's metabolic and growth-promoting effects through endocrine, paracrine and autocrine actions [406].

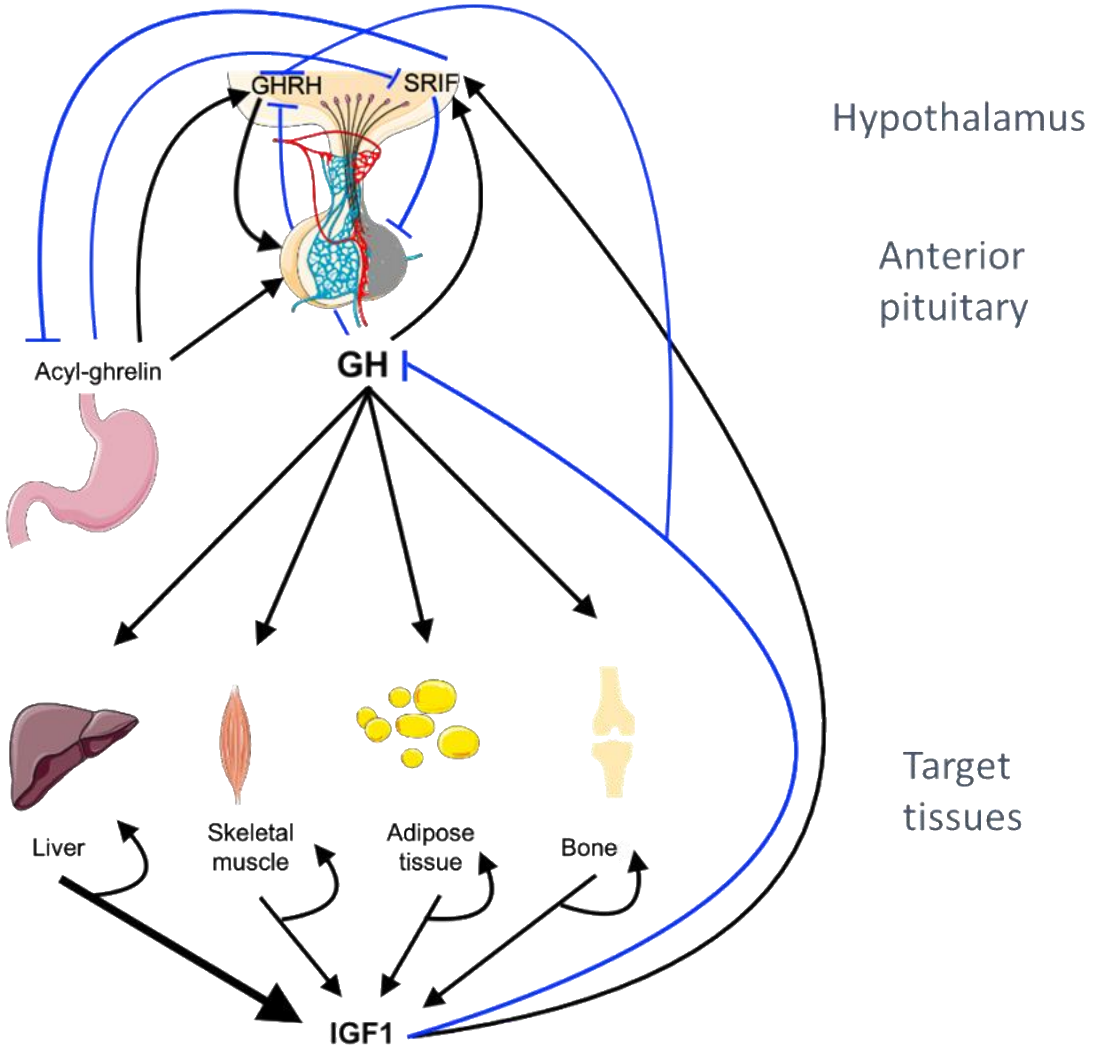


Figure 1.3 *The non-pregnant GH-IGF1 axis: Pulsatile GH secretion is the result of alternating input from GH releasing hormone (GHRH; stimulatory) and somatostatin (SRIF; inhibitory). The acylated gastric peptide ghrelin can also stimulate GH secretion indirectly, by positively inducing GHRH neuronal activity and suppressing SRIF activity and directly, by stimulating the somatotrophs of the anterior pituitary. Once in the circulation, GH has direct actions in several organs and tissues, including the liver, which is also the major site of GH-mediated production of IGF1. IGF1 can exert its actions on tissues in an autocrine or paracrine manner. A number of feedback mechanisms exist to modulate GH secretion. GH suppresses its own secretion by acting on somatotrophs and at the hypothalamus to inhibit GHRH activity and promote SRIF release, which in turn inhibits ghrelin secretion. IGF1 also suppresses GH secretion by inhibiting GHRH and stimulating SRIF secretion.*

1.6.2 *GH releasing hormone (GHRH)*

GHRH is a peptide hormone that positively stimulates pulsatile growth hormone synthesis and secretion by binding to the GHRH receptor [407]. GHRH is produced by neurosecretory cells that originate from the arcuate nucleus of the hypothalamus [408]. The axons of these GHRH neurons project to the median eminence and terminate on the capillaries of the portal system of the pituitary and are hence optimally positioned to regulate GH release [409]. GHRH released from the hypothalamus binds to GHRH receptor on somatotroph membranes. This binding initiates G protein mediated pathways that induce membrane depolarisation and release of GH from secretory granules [410-412].

1.6.3 *Somatostatin (SRIF)*

SRIF is a cyclic tetradecapeptide that has a broad range of actions, including inhibition of GHRH-mediated GH release [413-416]. Within the hypothalamus, the peptide is predominantly synthesised in the anterior periventricular nucleus [417, 418]. These neurons have axonal projections to the median eminence with nerve terminals at the hypophyseal portal vessels [417, 418]. Elevated circulating GH activates these neurons and promotes release of SRIF into the portal vasculature, where it acts on the somatotrophs by binding to membrane-bound high-affinity somatostatin receptors (SSTR) [419].These transmembrane G-protein coupled receptors have 5 subtypes (SSTR1-5) and mediate SRIF's inhibitory action on GH release by inhibiting adenylyl cyclase via G_i [420]. SRIF binding also results in membrane hyperpolarisation [421, 422], which inhibits GHRH-induced exocytosis of GH vesicles but not their biosynthesis [423, 424]. IGF1 mediated stimulation of SRIF and inhibition of GHRH can also contribute towards the suppression of GH release [425].

1.6.4 *Ghrelin*

In addition to the two hypothalamic hormones, GHRH (stimulatory) and SRIF (inhibitory), GH is regulated by the acylated form of the peptide hormone ghrelin which acts at the hypothalamus and pituitary to stimulate GH secretion (Figure 1.3, 1.5). Following its transcription, mature ghrelin mRNA is enzymatically processed into preproghrelin and then proghrelin. Proteolytic cleavage of proghrelin by the enzyme prohormone convertase (PC1/3) gives rise to mature ghrelin, a 28-amino acid peptide [426]. Cleavage of proghrelin also produces a 66-amino acid long carboxyterminal peptide, known as C-ghrelin [427-429], which is further cleaved to generate the 23-amino acid-long peptide, obestatin [430]. This is also biologically active, but since pathways are not the focus of this thesis, these will not be discussed further here. Although acyl-ghrelin has multiple stimulatory actions, including adrenocorticotropic hormone (ACTH) and prolactin release, appetite regulation, gastric acid secretion, gastric motility and cell proliferation, the following sections will focus on its role in GH secretion [431, 432].

1.6.4.1 Tissue distribution of ghrelin

Ghrelin is expressed mainly in the gastric mucosa and gastrointestinal (GI) tract [401, 433], where its abundance gradually decreases from the duodenum to the colon [434-436]. This peptide hormone is produced and secreted predominantly by the X/A-like entero-endocrine cells in the oxyntic glands of the gastric fundus [434, 436-438]. Ghrelin is also found in the pancreas, where it co-localises with a range of cell types including α, β, ε, and acinar cells [439-443], suggesting its potential role in regulating endocrine pancreatic activity [444]. Low-level expression of ghrelin has also been identified in the central nervous system [434], as well as the pituitary and hypothalamic neurons of the arcuate nucleus where ghrelin acts to regulate food intake [401, 445-447]. Other tissues including kidney, adrenal and thyroid glands, breast, ovary,

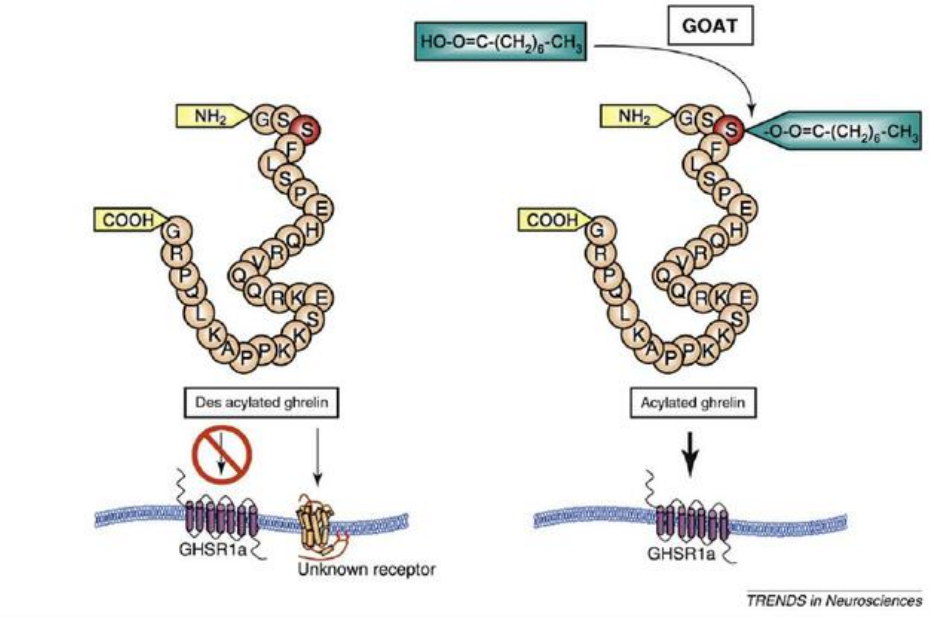
placenta, testis, prostate, liver, gallbladder, lung, skeletal muscle, myocardium, skin and bone also express ghrelin [448, 449].

1.6.4.2 Ghrelin secretion

Under normal physiological conditions, ghrelin secretion exhibits a circadian rhythm with a pre-prandial rise and a post-prandial fall [450, 451]. Due to this secretion pattern, ghrelin was initially believed to act as a meal initiation signal [451]. However, further investigations established that ghrelin secretion actually rises in anticipation of a meal rather than to elicit feeding [452].

1.6.4.3 Ghrelin acylation

In order to mediate a number of its functions, ghrelin undergoes a post-translational modification in the endoplasmic reticulum, catalysed by the enzyme membrane bound O-acyl transferase-4 (MBOAT4, also known as ghrelin O-acyl transferase), which is predominantly co-localised with ghrelin in the GI tract [453]. During acylation, a medium-chain fatty acid (MCFA), typically n-octanoic acid (C8:0), is covalently bonded to the hydroxyl group of serine, the third amino acid in the ghrelin peptide (Figure 1.4) [454, 455]. The addition of an octanoyl (8-carbon) group, or octanoylation, is necessary for ghrelin to bind to the widely expressed GHSR1-a, as this activates downstream pathways, including stimulation of GH secretion in humans and rodents [401, 456, 457].



1043

1044 ***Figure 1.4*** *Octanoylation of ghrelin: In order to bind to growth hormone secratagogue*
1045 *receptor 1a (GHSR-1a), ghrelin is n-octanoylated through the addition of an 8-carbon*
1046 *fatty acid to its third (serine) residue. This process is catalysed by the enzyme*
1047 *Membrane-Bound O-acyltransferase 4 (MBOAT4), also referred to as ghrelin O-acyl*
1048 *transferase (GOAT). The unacylated or des-acyl form of ghrelin lacks the fatty acid*
1049 *group and cannot bind or activate GHSR1-a. Figure taken from [458].*

1050

1051 Acyl-ghrelin has a half-life of approximately 9-13 minutes while that of des-acyl ghrelin
1052 is approximately 30 minutes [459, 460]. Furthermore, acyl-ghrelin is rapidly de-acylated
1053 in circulation [461, 462]. In human sera, butyrylcholinesterase is believed to mediate
1054 ghrelin deoctanoylation/deacylation, while in rodents, carboxylesterase may be
1055 responsible for this process [461]. However, many other proteins in human serum,
1056 including platelet activator factor, paraoxonase, carboxypeptidase, alpha 2-
1057 macroglobulin, acyl-protein thioesterase 1 also show ghrelin esterase activity and may
1058 also potentially deacylate circulating ghrelin [461, 463-465]. Together, rapid clearance
1059 and de-acylation of acyl-ghrelin mean that the ratio of des-acyl ghrelin to acyl-ghrelin in
1060 the circulation is usually high [461, 462].

1.6.4.4 Ghrelin O-acyl transferase; Membrane-Bound O-acyltransferase-4 (MBOAT4)

The enzyme MBOAT4 is one of 16 enzymes that form part of the acyltransferase family, but MBOAT4 is the only member of the family able to acylate ghrelin [466]. This membrane bound enzyme has eleven transmembrane helices and one entrant loop. The C-terminus of the enzyme is located in the cytosol and the N-terminus is in the lumen of the endoplasmic reticulum, where the catalytic activity likely occurs [453, 467]. This enzyme has a broad substrate specificity for fatty acids, and can acylate ghrelin with fatty acids between acetic acid (C2:0) to tetradecanoic acid (C16:0) [453]. The composition of dietary fatty acids is therefore an important determinant of the carbon chain length of the acyl group attached to nascent ghrelin molecules. In mice, the lengths of carbon chains attached to ghrelin correspond to those of the ingested MCFAs or medium-chain triglycerides (MCTs) [468]. However, in the stomach, the majority of ghrelin is acylated with n-octanoic acid (C8:0) [469]. This suggests that octanoic acid has greater enzyme affinity and/or concentration near X/A-like entero-endocrine cells than other MCFAs and MCTs present in the typical human diet [455].

1.6.4.5 Ghrelin-mediated GH secretion

Acyl-ghrelin can promote GH secretion directly by stimulating somatotrophs of the anterior pituitary, and indirectly by acting at the hypothalamus to inhibit SRIF and stimulate GHRH-neuronal activity (Figure 1.5) [431]. Binding of acyl-ghrelin to GHSR1-a activates $G\alpha_{q/11}$ signalling, resulting in activation of phospholipase C (PLC), which in turn hydrolyses phosphatidylinositol 4,5 bisphosphate (PIP_2) located in the cell membrane, contributing to the generation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) [431, 432, 470-472]. IP_3 then binds to its receptor (IP3-R) in the endoplasmic reticulum which releases calcium into the cell, increasing intracellular calcium ion concentrations. IP_3 also enables GH secretion by facilitating mobilisation of GH secretory granules to fuse with the plasma membrane [473]. Elevated intracellular

Ca+ mobilises DAG-activated protein kinase C to the plasma membrane, where it inhibits potassium ion channels, thereby causing membrane depolarisation. Opening of L-type voltage dependent calcium channels is then triggered, which further increases intracellular calcium ion concentration and induces GH secretion [431, 474].

Ghrelin-stimulated GH release is also mediated via ghrelin's interactions with the hypothalamic feeding circuits, particularly through neuropeptide-Y (NPY) / Agouti-related protein (AgRP) expressing neurons in the arcuate nucleus, a region with high density of GHSR1-a (Figure 1.5) [475, 476]. Connection of these neurons with those that express somatostatin in the periventricular nucleus provides a potential pathway of action in the regulation of acyl-ghrelin induced GH secretion [477]. The vagus nerve also appears to play an important role in mediating ghrelin's GH releasing effects, because disruptions of vagal connections in animal models impair GH release in response to central and peripheral ghrelin injections [478]. The endocannabinoid system is likely involved in relaying ghrelin's effects on GH secretion via the vagus nerve, since blockade of peripheral cannabinoid receptor subtype 1 with rimonabant decreases ghrelin-induced, but not GHRH-induced GH secretion but the inhibitory effect of rimonabant on GH secretion is lost in vagotomised animals (Figure 1.5) [477, 479].

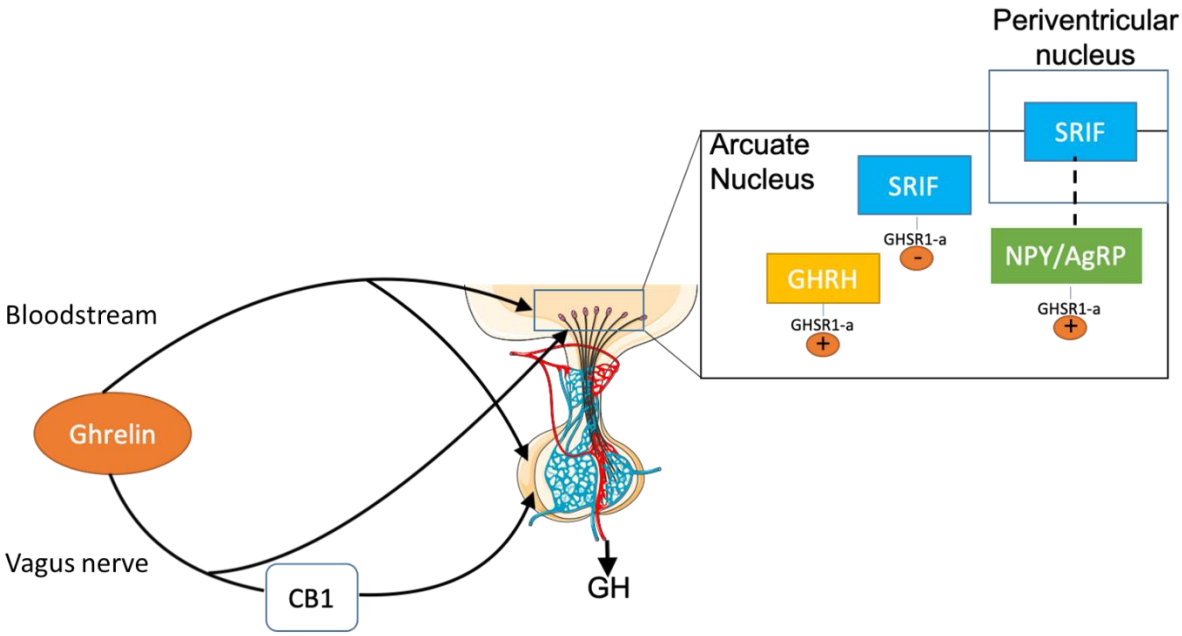


Figure 1.5 *Established and potential pathways of acyl-ghrelin-stimulated GH secretion: Acyl-ghrelin can travel via the bloodstream to stimulate GH indirectly through stimulating GHRH neuronal activity (arcuate nucleus) or directly, by acting on somatotrophs in the anterior pituitary. Additionally, the vagus nerve is necessary for the full GH-releasing activity of ghrelin. Some data suggest that the peripheral cannabinoid receptor subtype 1 (CB1) may be important for relaying ghrelin's action on GH secretion but this receptor does not alter GHRH induced GH secretion, suggesting that it is not involved in ghrelin's activity in the hypothalamus. The NPY/AgRP neurons in the arcuate nucleus, typically involved in mediating ghrelin's orexigenic effects have connections to somatostatin (SRIF) neurons in the periventricular nucleus, and hence may also be involved in regulation of ghrelin-mediated GH secretion through unknown mechanisms. Figure based on information from [431, 475-479].*

1.6.4.6 Des-acyl ghrelin

Due to its inability to bind to GHSR-1a, unacylated or des-acyl ghrelin was originally thought to be an inactive degradation product. However recent work suggests that des-acyl ghrelin is also a hormone, which can either act synergistically with, antagonise the actions of, or work independently from, acyl-ghrelin [480-488]. Furthermore, des-acyl ghrelin can suppress acyl-ghrelin secretion in both humans and animals and therefore

has the potential to inhibit GHSR-1a activation [489]. It has been speculated that des-acyl ghrelin mediates its functions by binding to an unknown receptor and that it may also interact with acyl-ghrelin at this receptor but further studies are required to investigate this directly [486, 490].

1.6.5 *Growth hormone (GH)*

Regulation by GHRH, SRIF and acyl-ghrelin results in episodic, high amplitude bursts of GH secretion, separated by troughs of low-level or basal secretion throughout each 24 h period. Each episode of GH secretion is initiated by a burst of GHRH released into the hypophyseal portal system, preceded by a withdrawal of SRIF's inhibitory input on GHRH neurons and somatotrophs (Figure 1.3) [491-495]. GHRH-mediated GH synthesis during high SRIF tone is believed to allow GH accumulation in somatotrophs which potentiates release of GH pulses when SRIF input is withdrawn and GHRH levels are high [407, 496]. Furthermore, acyl-ghrelin can act directly on the somatotrophs or stimulate GHRH neuronal activity in the hypothalamus to enhance GH pulse amplitude [497-500].

The pattern and abundance of GH secretion is crucial for its biological activity. In hypophysectomised rats, the lack endogenous GH secretion, greater bone growth and weight gain is observed when the same dose of GH given in pulses compared to continuous administration [501, 502]. In children, growth rates and circulating plasma IGF1 concentrations correlate positively with peak, but not basal GH concentrations [503-505].

Both the pattern and magnitude of GH release differ between males and females in a species-dependent manner [513]. In humans, males secrete GH in high amplitude bursts at regular intervals, separated by periods of very low or undetectable GH concentrations, whereas females exhibit more frequent, irregular and lower GH

secretory pulses and have higher baseline GH concentrations [495, 510, 514-516]. In contrast to humans, mean GH concentrations and GH pulse amplitude in blood are higher in male compared to female rats [506, 517-521]. However, female rats have higher interpulse plasma GH concentrations and exhibit a higher irregularity in pulsatile GH secretion compared to male rats [517-519, 522, 523]. Although mean and basal plasma GH concentrations as well as pulse amplitude are also greater in rams (intact males) than ewes (intact females), GH pulse frequency does not differ between the sexes in sheep [524].

The sexual dimorphism in circulating GH likely reflects both acute responses to sex steroids and persistent effects of the steroid hormone environment during early development on the hypothalamo-pituitary axis, which result in sex-specific differences in the number of GHRH neurons and somatotrophs and their sensitivity to post pubertal steroids (reviewed by [525]). Acute changes in circulating steroids likely underlie changes in GH patterns throughout the menstrual cycle in women, where pulsatile GH release doubles during the late follicular stage of the menstrual cycle compared to early follicular phase [509]. Sex steroids regulate circulating GH through multiple pathways. For example, oestrogen administration in women increases circulating GH peak amplitude via suppressing GH negative feedback on hypothalamic GHRH secretion, enhancing SRIF withdrawal-induced GH release, reducing pituitary responsiveness to SRIF and increasing potency to exogenous GHRH [526-529].

1.6.5.1 GH signalling

Once released into the circulation, GH binds to GH binding protein (GHBP), which is produced through the proteolytic cleavage of the extracellular domain of the GHR, and stabilises the hormone and prolongs its bioavailability [530, 531]. GH must be released from GHBP in order to bind to membrane-bound full-length GHR. Binding of GH to a pre-formed GHR homodimer causes rotation of an internal section of the receptor and

repositions the catalytic domain of the associated JAK2 tyrosine kinase [532]. This allows JAK2 to phosphorylate the cytoplasmic regions of the GHR and creates a binding site for Src homology 2 (SHC2) domain-containing proteins. These proteins include the signal transducers and activators of transcription (STAT)1, 3, 5a and 5b proteins, which are phosphorylated by JAK2 and are critical signalling components involved in mediating GH action [404]. The phosphorylated STAT proteins dimerise and are subsequently translocated to the nucleus where they regulate transcription of GH target proteins [533]. Stat5b is the major regulator of GH actions and upregulates transcription of *IGF1*. IGF1 mediates many growth-promoting actions of GH by binding to the IGF1-receptor (IGFR) which stimulates growth in tissues including bone and muscle [534-536].

1.6.5.2 GH target tissues

GH acts on multiple tissues to promote longitudinal growth and regulate metabolism of carbohydrates, lipids and proteins. The liver is a major site of GH action, including promotion of IGF1 secretion [537], and hepatic glucose output through glycogenolysis and gluconeogenesis [538-540]. GH also promotes lipolysis in adipose tissues, in part by upregulating lipase activity and downregulating the expression of glucose transporters in adipocytes [541-545]. While this can reduce adiposity, particularly visceral adiposity, it increases circulating free fatty acid concentrations, which enhances uptake of free fatty acids by skeletal muscle and liver and can contribute towards impaired insulin signalling and ultimately, development of insulin resistance [546-550]. GH also acts directly via GHR in skeletal muscle to promote net protein anabolism by stimulating protein synthesis [551], as well as indirectly via IGF1, which decreases protein degradation [552, 553]. Both GH and IGF1 promote bone formation through the stimulation of osteoblast proliferation and activity [554-557] and bone resorption by inducing osteoclast differentiation and activity [558]. Together, these

actions increase the rate of bone remodelling and ultimately promote bone accumulation [554].

1.6.6 *Insulin-like growth factors (IGFs)*

IGF1 and IGF2 are peptide growth factors that stimulate growth and development of vertebrates through metabolic, mitogenic, anti-apoptotic and differentiating effects on a wide range of cell types [559]. The endocrine, paracrine and autocrine actions of the IGFs are typically exerted through interactions with two different receptors, IGF type 1 (IGF1R) and type 2 (IGF2R) receptors. Additionally, IGFs can also bind to the insulin receptor (IR) and IGF1R/IR hybrid receptors [560-563]. IGF1R has high binding affinity for IGF1, lower affinity for IGF2 and little to no affinity for insulin. Binding of IGF1 to IGF1R activates the phosphatidylinositol 3-kinase (PI3K)- protein kinase B (PKB/AKT) and mitogen activated protein kinase (MAPK) signalling pathways [563-565], whereas binding of IGF2 to IGF2R can either activate G-protein-coupled signalling pathways or result in IGF2 degradation [566].

The bioavailability and actions of the IGFs in circulation is regulated by the family of six insulin-like growth factor-binding proteins (IGFBPs). The majority of serum IGFs are bound to IGFBP-3 in a heterotrimeric complex with acid-labile subunit (ALS), which can also form with IGFBP-5, but not other members of the IGFBP family. These ternary complexes are mainly restricted to the vasculature, and extend the half-life of the IGFs to approximately 15 h, compared to ~10 min for “free” IGF1 not complexed to IGFBP [567, 568]. Most remaining serum IGFBPs are found in binary complexes, which can exit the circulation rapidly but still extend half-life of the IGFs by approximately 20-30 min [567]. Depending on the IGFBP and cell types, IGFBPs either block or promote binding of IGFs to their receptors [569]. IGFBP proteases, on the other hand, can degrade IGFBPs to release IGFs and increase their availability at their receptors [570, 571]. The IGFBPs also have additional roles, including inhibition of insulin-stimulate

glucose uptake in adipocytes (IGFBP-3) [572] and supporting bone tissue growth (IGFBP-5) [573], as reviewed previously [569, 574].

Postnatally, GH is one of the main promotors of hepatic IGF1 synthesis. However, energy status and protein intake are also important regulating factors, and fasting decreases serum IGF1 concentrations [575-578]. During human intrauterine development, the IGFs appear to be less dependent on GH, as congenital GH deficiency is associated with postnatal growth failure, rather than reduced intrauterine growth [579, 580]. IGF1 is more important for fetal growth and loss-of-function IGF1 gene mutations are associated with severe intrauterine growth restriction [581, 582]. In humans, circulating IGF1 levels are low at birth and rise gradually during childhood, peak at puberty, and then gradually decline with age [559, 583]. IGF2 is less well studied but its levels have been reported to increase from birth to puberty and then remain stable [559].

IGF1 mediates the majority of GH's effects on skeletal metabolism, including stimulation of amino acid transport, but can also directly stimulate protein synthesis and is an important inhibitor of protein breakdown [584]. In skeletal muscle, IGF1 acts as a potent stimulator of free fatty acid uptake and oxidation and, together with IGF2, plays a crucial role in skeletal muscle differentiation [585, 586]. Although IGF1 can stimulate pre-adipocyte differentiation, at physiological concentrations its actions on lipid synthesis, lipolysis and glucose transport in mature adipose tissue appear to be limited [587, 588]. IGF1 may also be involved in regulation of carbohydrate metabolism through inhibition of GH secretion and through its insulin-sensitising effects [589]. IGF1 suppresses GH secretion and also enhances insulin suppression of hepatic gluconeogenesis [590, 591]. IGF1 also promotes chondrogenesis and increases bone formation by regulating function of the differentiated osteoblasts [592].

There is substantial evidence that both IGFs play a crucial role in placental and fetal development. Several *in vitro* studies have also shown that these growth factors promote cell differentiation and growth [593-597]. More specific to fetal growth, and discussed in more detail in section 1.7.3 data from *in vivo* studies suggests that maternal IGFs promote fetal growth in part by increasing materno-fetal transfer of nutrients throughout gestation [598-600].

1.7 The GH/IGF axis in pregnancy

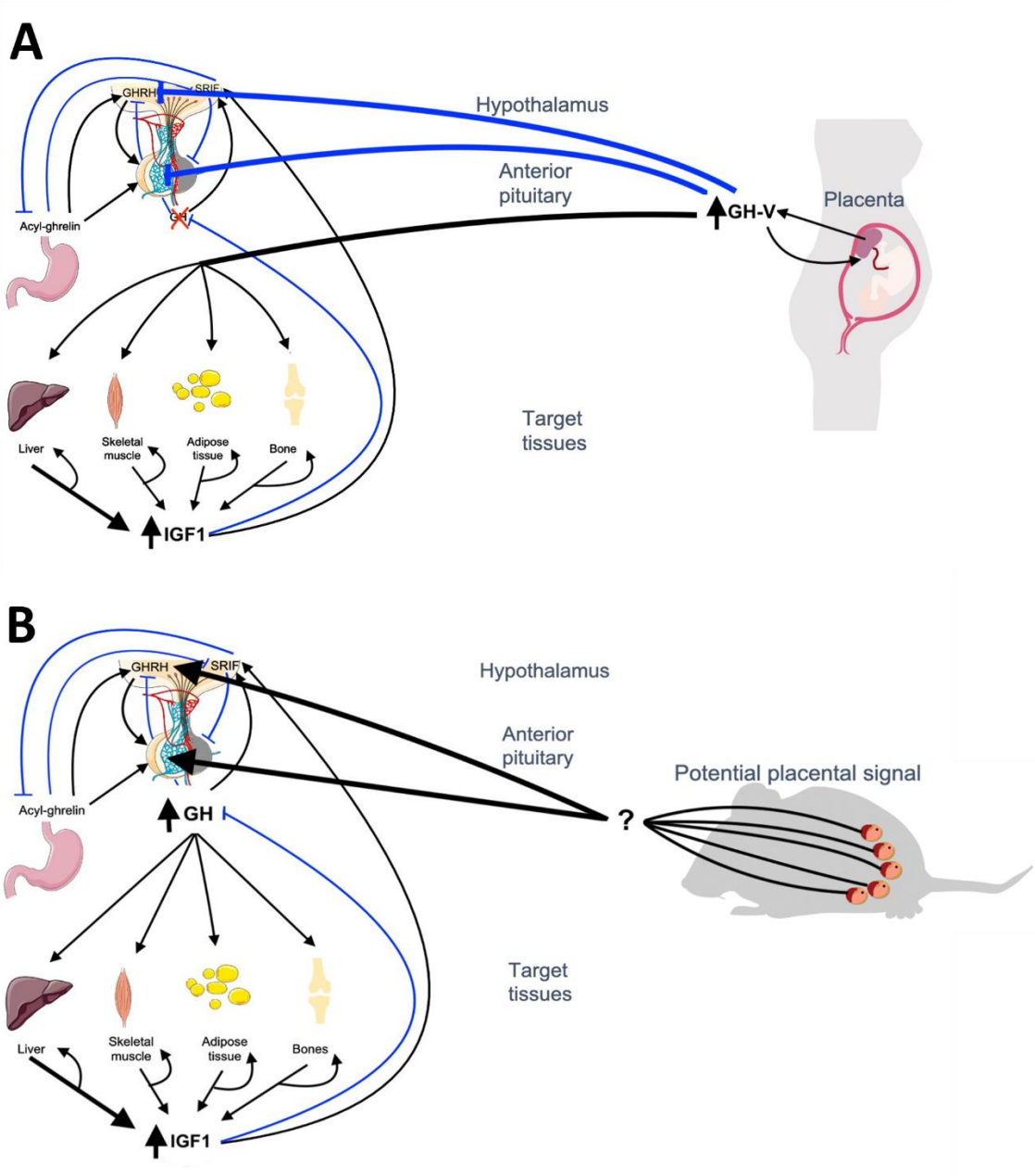


Figure 1.6 *The GH axis during pregnancy (A) Humans: Late in the first trimester, continuous placental secretion of growth hormone variant (GH-V), increases basal maternal GH concentrations. By early in the second trimester, placental GH-V production suppresses pituitary GH secretion, and results in a pattern of elevated, non-pulsatile maternal circulating GH which persists for the remainder of pregnancy. (B) Rodents: Circulating maternal GH concentrations also increase significantly during pregnancy in rodents. Unlike humans, rodents do not express GH-V and maternal circulating GH remains pulsatile and pituitary-derived throughout pregnancy. The pregnancy-associated increase in maternal GH is likely induced by an unidentified placental signal, which acts on the maternal pituitary and/or hypothalamus to enhance GH secretion. Figure based on information from [120, 121, 123]*

1.7.1 *Changes in GH and IGFs during normal pregnancy*

The GH/IGF axis undergoes substantial changes during pregnancy in humans (Figure 1.6A) and most mammalian species studied to date, including rodents (Figure 1.6B). These changes facilitate maternal adaptation to pregnancy in addition to promoting fetal and placental growth and function. During early human pregnancy, pulses of pituitary-derived GH are superimposed on elevated basal GH as a result of continuous release of GH-V, which can be detected by late in the first trimester (Figure 1.7A) [120, 121]. In early pregnancy, at 11 weeks of gestation, circulating GH remains pulsatile and is largely pituitary-derived, whilst pituitary GH secretion becomes suppressed by negative feedback due to placental GH production before mid-pregnancy at around 15-17 weeks of gestation (Figure 1.7A) [120]. Unlike the pulsatile pattern of pituitary-derived GH secretion, placental GH secretion is continuous, resulting in elevated and non-pulsatile maternal circulating GH in the second half of human pregnancy (Figure 1.7A) [120], Circulating GH patterns during pregnancy have also been characterised in rodents (Figures 1.7B and 1.7C). In contrast to humans, circulating GH profiles remain pulsatile throughout pregnancy in rats (Figure 1.7B) [122, 601], as well as mice (Figure 1.7C) [123]. In these species, the increase in average circulating GH concentrations reflects elevated basal GH secretion [122, 123], while the number and magnitude of pulsatile GH secretion remain unchanged throughout murine pregnancy, they decrease in regularity [123, 602]. Despite the importance of GH during pregnancy in many mammalian species, the mechanism driving increased maternal GH in non-human species is not yet completely understood.

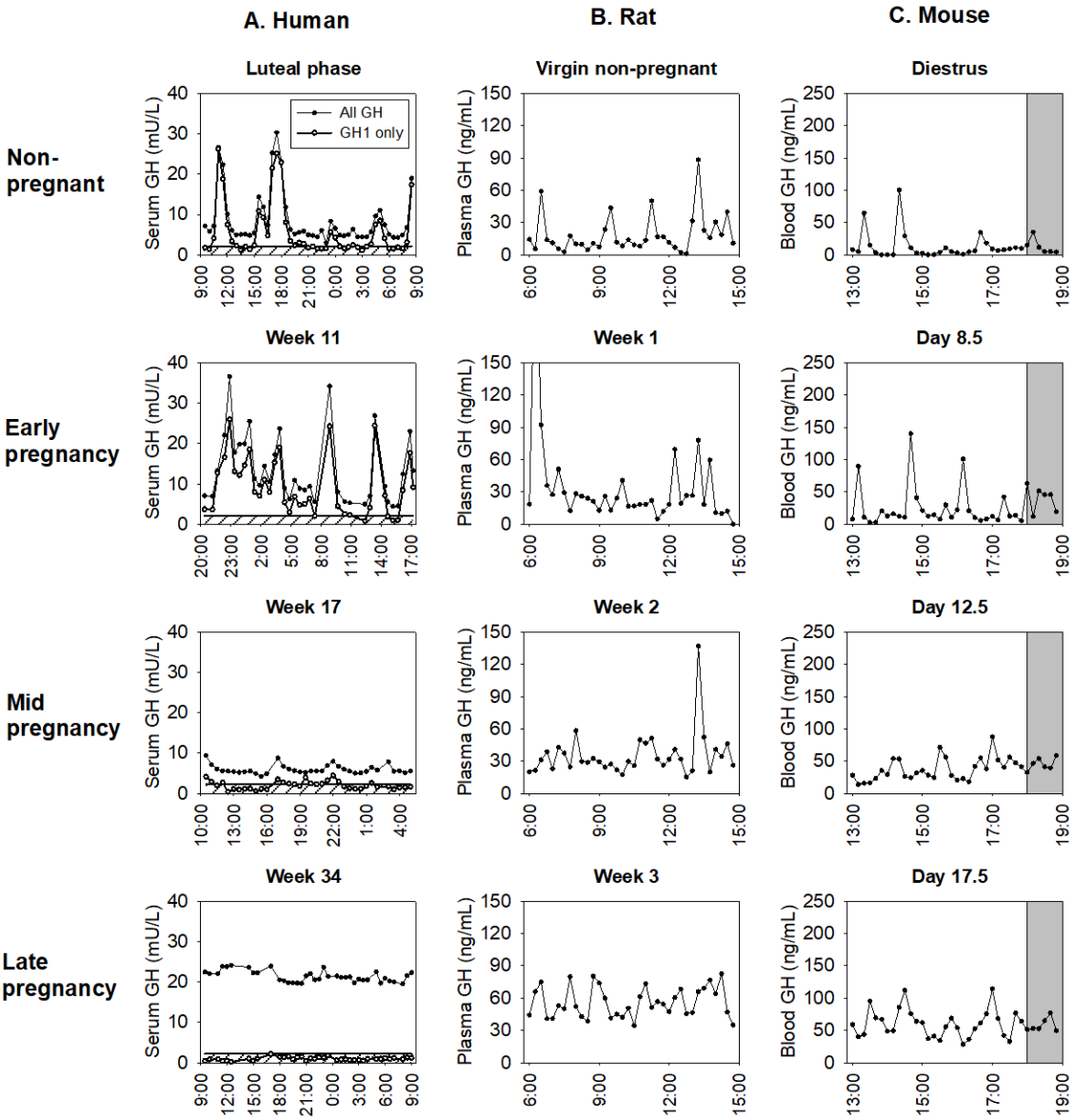


Figure 1.7 *GH secretory profiles in non-pregnant and early, mid and late pregnant humans (A), rats (B) and mice (C). In human pregnancy (A), pulses of pituitary-derived GH are super-imposed on elevated basal GH derived from placental expression of a GH variant (GH-V) by late in the first trimester. Unlike the secretory pattern of pituitary derived GH, GH-V secretion is non-pulsatile and continuous and becomes the main form of circulating GH by mid-pregnancy. In rats (B) and mice (C), total and basal GH concentrations are also elevated by early-mid pregnancy but unlike humans, GH is only produced by the pituitary and GH profiles remain pulsatile throughout the course of pregnancy. Hatched area in A indicates detection limit of the assay used to detect circulating pituitary GH in humans. Shaded areas in C (mouse) indicate sampling during dark period. Profiles have been generated using data from [120, 122, 123].*

Changes in circulating maternal IGF concentrations during pregnancy are highly variable across species [603]. In humans, reported maternal IGF1 concentrations

during the first two trimesters are inconsistent, with some studies reporting a modest or gradual rise in concentrations but others finding no change or decreased concentrations compared to pre-pregnancy levels [604-612]. During the third trimester, however, the majority of studies have reported substantially (45-200%) higher maternal circulating IGF1 concentrations compared with levels in non-pregnant women [604]. In rodents, IGF1 concentrations gradually decrease throughout pregnancy, falling below non-pregnant levels by late pregnancy [613, 614]. In rabbits and guinea pigs, on the other hand, maternal IGF1 gradually rises during the first half of pregnancy, followed by a rapid decline after mid-pregnancy [615, 616], while circulating IGF1 concentrations do not change during pregnancy in sheep and cattle [617-619]. In humans, proteolysis of IGFBPs increases around ~6-8 weeks of gestation [620-624]. Similarly, in rats, most IGFBPs in serum are significantly reduced in the latter half of rat pregnancy, and this likely contributes to faster clearance of maternal IGF1 [614].

In contrast, maternal IGF2 concentrations increase throughout pregnancy in most species, and are higher than circulating concentrations of IGF1 [605, 615, 616, 618, 625]. The placenta is a major source of IGF2 in animals with a haemochorial placenta, where IGF2 is produced by the syncytiotrophoblast layer in direct contact with maternal blood [626-629]. Consistent with the majority of IGF2 being placentally-derived, IGF2 is high in pregnancy but almost undetectable in plasma in non-pregnant rodents postnatally [630, 631]. Although placental IGF1 expression is minimal, other hormones produced by the placenta (like GH-V in humans or placental lactogen) may still influence maternal IGF1 concentrations and actions [629].

1.7.1.1 Potential drivers of increased maternal growth hormone during pregnancy

The GH gene cluster consists of five structurally similar genes, including the pituitary GH variant *GH-N*/ *GH-1*, placental growth hormone *(GH-V),* chorionic somatomammotropin hormone 1 *(CSH1/* hCS-A*),* chorionic somatomammotropin

hormone 2 (*CSH2/* hCS-B*),* and a pseudogene known as chorionic somatomammotropin-like hormone. A related protein, human prolactin (*PRL*), is also included in the GH gene family [632, 633]. As discussed in earlier sections, placental production of GH-V is responsible for the change in maternal GH secretory pattern during human pregnancy (Figure 1.6 and 1.7A). Most other mammalian species, however, do not produce placental GH [634]. The increases in circulating GH concentration and basal GH secretion in pregnant mice [123] coincide with the formation of the chorioallantoic placenta and initiation of placental blood flow [635]. This suggests, therefore, that the pregnancy-associated increase in GH secretion observed in species like rats (Figure 1.7B) and mice (Figure 1.7C) is driven by another placentally-derived factor [123]. A number of candidate placental products might contribute to the increase in GH section. Although lacking the GH-V, endocrine, trophoblast giant cells of the mouse placenta express 22 placental-specific genes related to prolactin (*Prl*) [110, 636, 637]. A number of these prolactin related peptides, including Prl3d1 and Prl3b1, can bind to the Prl receptor and contribute to the increasing maternal glucose concentrations in murine pregnancy [638, 639]. Other potential candidates expressed by the murine placenta include two factors important for endogenous GH secretion, GHRH and ghrelin [640, 641]. Although no studies to date have assessed whether MBOAT4 is expressed in the rodent placenta, GHSR-1a gene and protein expression has been identified in the placenta of rats [642]. It is therefore possible that the increase in basal GH secretion in rodent pregnancy, in the absence of GH-V, may be mediated via placental production and activation of ghrelin, which in turn promotes pituitary GH production throughout pregnancy [123]. However, the relationship between changes in maternal ghrelin and GH production during pregnancy are not clearly understood.

1.7.2 *Responses to maternal GH/IGF treatment in normal pregnancy*

Maternal treatment with GH during pregnancy increases fetal growth in a number of species, provided maternal nutrient intake is not limited. The response is also dependent on the pattern of administration. Continuous GH administration does not alter maternal IGF1 concentrations, fetal or placental weights in rats [643], whereas intermittent (once or twice daily) GH administration significantly increases fetal growth [644-646]. The mechanisms underlying differential effects of intermittent and continuous GH administration on pregnancy outcomes in rats are unclear, but are consistent with pattern-dependent responses to GH in non-pregnant animals and humans (see section 1.6.5 above). In pigs, maternal GH administration via daily injections from early to mid-pregnancy in increased fetal growth and birth weight if the treatment was continued for the majority of gestation [380, 647]. Birth weight was not increased when pigs were treated with GH only in early-mid pregnancy [648], even though fetuses were heavier immediately following treatment [649]. In sheep, sustained release GH administration during the periconceptional or breeding period improved birth weight by approximately 10% [650, 651]. Although some have reported a similar increase in fetal weight with intermittent GH administration to ewes during late gestation, others found an improvement in placental diffusion capacity without any changes in progeny weight [652, 653].

In other studies, exogenous maternal IGF1 treatment alters resource allocation between maternal tissues and the developing conceptus but results vary according to species and gestational age. In rats, maternal IGF1 administration in the second half of pregnancy does not alter fetal or placental weight but increases maternal weight [643, 654, 655]. In guinea pigs, early-mid pregnancy treatment with IGF1 or IGF2 increases fetal and placental weight [656] and improves placental function around mid-late gestation [626]. However, only treatment with IGF1, and not IGF2, decreases maternal

fat mass in this species, supporting the suggestion that IGF1 acts to divert nutrients from the mother to the conceptus, thereby promoting fetal growth at the expense of the mother [626].

1.7.3 *Placental responses to GH and IGF1 function*

Growth-promoting effects of GH may be mediated by the placenta, since maternal GH treatment also increases placental growth and promotes placental function in a number of species [646, 649, 652, 657, 658]. In pigs, maternal GH administration during early-mid pregnancy increases placental weight and surface area [657, 659] with increased protein expression of glucose and amino acid transporters GLUT1 (SLC2A1) and SNAT2 (SLC38A2) in the fetal-facing placental membrane [658] which would be expected to enhance fetal nutrient supply. In sheep, short-term (10 day) maternal GH administration during mid-late pregnancy increases placental capacity for simple and facilitated diffusion (responsible for glucose transport), compared to saline-treated controls [652]. The increase in simple diffusion in this study was greater compared to the effect on facilitated diffusion, suggesting that the improved placental transport capacity was likely the result of increased placental surface area or reduced placental barrier thickness, rather than increased activity of placental nutrient transporters [652].

Maternal treatment with IGF1 and IGF2 during pregnancy also promote fetal growth by enhancing placental growth, fetal nutrient availability and uptake [119, 603, 660, 661]. In guinea pigs, maternal IGF1 infusion during early pregnancy increases placental uptake and transfer of glucose and amino acids at mid pregnancy [661]. This is likely to be mediated in part by increased placental gene expression of *Slc38a2*, suggesting early pregnancy maternal IGF1 concentrations play a role in enhancing mid-gestation fetal nutrient supply and growth [661]. In another guinea pig study, maternal IGF2 treatment increased the volume and surface area of the labyrinth zone in the near term placenta, which is likely to enhance placental capacity for materno-fetal nutrient and

gas exchange during this crucial growth stage [662]. The importance of IGF2 is further highlighted in mouse studies where deletion of the placental-specific P0 *Igf2* promoter, which blocks placental labyrinth-specific *Igf2* production and causes IUGR due to a reduced placental nutrient transfer capacity as discussed in more detail in section 1.5.7.6 [370, 371, 373]. This leads to a 50% reduction in surface area, 28% increase in barrier thickness and a decrease in the overall placental capacity to transport system A transporter substrates, which constributes to 20-25% reduction in fetal weight compared to wild type controls.

The role of IGFs in enhancing placental function is further supported by *in vitro* findings that both IGF1 and 2 promote proliferation, invasion/migration and exhibit anti-apoptotic properties in trophoblast cell lines from humans, pigs and mice, and are therefore likely to promote implantation and overall placental growth [593-597]. The IGFs also enhance the endocrine capacity of trophoblasts in culture, such that the secretion of progesterone, hCG, and placental lactogen by human trophoblasts increases after IGF1 treatment [663, 664]. In mice, IGF2 stimulates the differentiation of ectoplacental cone cells into endocrine trophoblast giant cells, and in ovine trophectoderm cells, IGF2 regulates signalling pathways, suggesting its role in conceptus growth and differentiation [593, 594]. Therefore, the results of both *in vitro* and experimental animal *in vivo* studies support the suggestion that stimulation of the GH/IGF axis can promote fetal and placental growth, as well as enhance hormonal secretion and nutrient transport capacity of the placenta [665].

1.7.4 *Responses to GH and IGF1 treatment in IUGR pregnancy*

Given its effects on growth in normal pregnancies, a number of studies have investigated whether administration of maternal GH or IGF1 can rescue fetal growth in growth-restricted pregnancies. Pigs provide a natural model of IUGR pregnancies, as fetal size and birth weight are constrained by adolescent first pregnancy, limited uterine

capacity, large litter size and limited maternal nutrition [666-669]. Gatford and colleagues previously reported that average piglet weight at birth correlates negatively with litter size in adolescent first pregnancy [380]. However, the impact of maternal constraint and a large litter size on the average progeny birth weight was less pronounced if the mother was treated with GH throughout early to late pregnancy [380]. Similarly, in pregnant sheep with IUGR induced via placental embolisation, twice-daily maternal GH administration increased fetal growth in terms of fetal weight and length [670].

These fetal growth-promoting effects could be mediated by GH acting directly on tissues via its own receptors or indirectly, via increased secretion of IGF1. In rodents increased maternal plasma IGF1 concentrations, either endogenously or exogenously, reduce the impact of maternal constraint induced by litter size on fetal growth [379]. Thus, in both normal dams and dams selected from a low plasma IGF1 line, IGF1 treatment from days 1-19 of pregnancy abolishes the usual negative relationship between fetal weight and litter size in late gestation [379].

1.7.5 *Limitations to clinical treatment with GH or IGF1*

Despite the clear evidence that GH and IGF1 promote fetal growth and placental function, the potential for clinical application of GH or IGF1 administration in humans is limited. This is largely due to the fact that these proteins are not orally active and therefore need to be delivered by injection or infusion. Interventions delivered orally are more acceptable to patients compared to injections, even in pregnancy [671]. Therefore, in this current project, we aim to identify orally active interventions that act directly or indirectly to increase maternal circulating GH, and hence IGF1, to improve fetal growth in IUGR pregnancies. One potential approach to do this is through increasing circulating levels of the acyl-ghrelin, either by direct administration or through a specific dietary intervention.

1.7.6 *Ghrelin and its potential role in pregnancy*

Although limited studies have investigated changes in the ghrelin axis during pregnancy, the available evidence suggests that it has a potential role in facilitating maternal adaptation to pregnancy and promoting fetal growth. Treatment with acyl-ghrelin promotes GH secretion in multiple species [401, 457, 672, 673], while ghrelin administration during pregnancy promotes growth of progeny in rodents [674-676]. Intramuscular administration of acyl-ghrelin to pregnant sheep for the last 10 days of gestation resulted in higher serum GH concentrations compared to non-treated controls [672]. Furthermore, administering acyl-ghrelin 3 times a day, from GD14 until delivery to pregnant rats increased fetal growth by ~10%, while suppressing maternal acyl-ghrelin levels via passive immunisation had the opposite effect [674]. Interestingly, maternal administration of total ghrelin during early pregnancy (GD3 to GD7) alone, reduces birth weight in rats [677], while administration of acyl-ghrelin during the first-half of pregnancy (GD1 to GD11) reduces litter size without altering birthweight [675]. These results suggest that the timing of exogenous elevation in maternal circulating ghrelin may be important for ghrelin's growth-promoting effects. Although not assessed in pregnant animals, it is known that acyl-ghrelin, but not des-acyl ghrelin, can act centrally to stimulate food intake in rodents [476, 678]. Acyl-ghrelin also plays an important role in metabolic adaptations to maintain circulating glucose in response to calorie restriction, particularly in pregnancy [679]. Treatment with acyl-ghrelin might therefore potentially increase fetal growth through direct action on the placenta, by increasing maternal GH and/or by stimulating maternal food intake.

Changes in maternal circulating ghrelin concentrations during gestation occur in a number of mammalian species, albeit not all in the same direction. Total ghrelin concentrations have been reported as lower in pregnant than non-pregnant women and as lower or unchanged in pregnant than non-pregnant rats [642, 680-683]. Variable

evidence exists regarding changes in acyl-ghrelin, with elevated or lower concentrations during pregnancy reported in different studies in women [681, 684], and increased concentrations during late pregnancy in a single study in rats [685]. Although responses to exogenous ghrelin during pregnancy have been previously investigated, the role of endogenous ghrelin during pregnancy remains unclear. Pregnant mice lacking the *Mboat4* gene, which consequently cannot convert ghrelin to acyl-ghrelin [686], still undergo a similar increase in GH during late pregnancy as wild-type mice [686], suggesting that acyl-ghrelin is not required to increase GH during pregnancy. However, compared to wild-type mice, there was a trend for lower median GH concentrations in the knockout mice. Furthermore, in this study GH was only measured in a single maternal blood sample collected at the time of post-mortem which does not provide a precise representation of GH's secretory pattern overtime. Therefore, it is unclear whether a lack of acyl-ghrelin alters the circulating patterns of GH during pregnancy [686].

1.7.7 *Acyl-ghrelin levels can be increased by feeding dietary C8 fatty acids or triglycerides*

The finding that increased acylation of ghrelin can be achieved by a nutritional approach [453, 468, 687] offers great translational potential as a method to increase circulating GH. Feeding mice a diet enriched with the C8 medium chain fatty acid, octanoic acid (C8-MCFA), or the triglyceride containing this fatty acid, glyceryl trioctanoate, increases octanoylation of ghrelin in the stomach and the abundance of circulating acyl-ghrelin [453, 468, 687]. Circulating acyl-ghrelin concentrations as well as plasma GH concentrations in blood pooled from samples collected at 15-minute intervals across a 4-h period, also increased in pigs that were fed a diet enriched with mixed medium chain fatty acids [688]. To date, there has only been one study that has assessed effects of feeding octanoic acid in humans. Enteral feeding of a single meal,

containing 2.8 g of octanoic acid, over a period of 3 h, increased plasma acyl-ghrelin concentrations by more than 30% [689]. Together, these studies provide evidence that dietary C8-MCFA supplementation increases circulating acyl-ghrelin levels in pigs, rodents and humans and that this increases circulating GH, at least in pigs.

In a pilot study conducted in the Gatford lab in 2015-2016 (n=3 mice), dietary supplementation with C8-MCFA increased circulating GH abundance in non-pregnant female mice. Standard lab chow supplemented with 5% octanoic acid increased basal GH secretion >20-fold compared to mice fed a non-supplemented standard chow diet. The pattern of GH secretion remained pulsatile in both groups. This approach to increase maternal circulating GH (which does not require injections or infusions) could be adapted during pregnancy to stimulate placental growth and function and hence, provide a potential pathway for clinical translation. However, no study to date has determined whether, and to what extent dietary C8-MCFA supplementation regulates activation of ghrelin and pulsatile GH profiles in either normal or IUGR pregnancy.

1.8 Hypotheses and aims of thesis

Given the capacity of GH to promote placental and fetal growth, and evidence that dietary C8-MCFA supplementation increases acyl-ghrelin and GH concentrations, two hypotheses were developed to be tested in this project:

1. Feeding C8-MCFA will increase circulating acyl-ghrelin and GH levels in the non-pregnant and pregnant female mouse; and
2. Elevated acyl-ghrelin throughout IUGR murine pregnancy will increase placental growth and/or improve placental function and hence reduce fetal growth constraint associated with IUGR pregnancies.

These hypotheses were tested in the following aims, described in the following three chapters:

1. Determine effects of feeding C8-MCFA on circulating total and acyl-ghrelin and GH profiles in non-pregnant and pregnant mice (chapter 2);
2. Develop methodology to measure placental function in mice using fluorescent tracers (chapter 3); and
3. In order to test potential interventions, develop a mouse model of IUGR (chapter 4);
4. Determine effects of feeding C8-MCFA on circulating total and acyl-ghrelin, fetal and placental growth and function in IUGR mouse pregnancy using the model developed in aim 3.

However, because C8-MCFA supplementation did not increase acyl-ghrelin in non-pregnant mice or in normal pregnant mice, the experiment corresponding to the final aim (aim 4) was not conducted in this thesis.

Chapter 2 Pregnancy, but not dietary octanoic acid supplementation, stimulates the ghrelin-pituitary growth hormone axis in mice

2.1 Overview

The work described in this chapter corresponds to the first aim of this thesis. In this study, we characterised and compared the GH-ghrelin axis in non-pregnant and pregnant mice, and determined the effect of dietary octanoic acid supplementation on this axis in both pregnant and non-pregnant animals.

This chapter has been published in the *Journal of Endocrinology* [602] and has been reproduced exactly as published, with the exception of formatting, which has been modified to be consistent with the rest of this thesis.

Statement of Authorship

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By signing the Statement of Authorship, each author certifies that:

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ii. permission is granted for the candidate in include the publication in the thesis; and

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2.2 Abstract

Circulating growth hormone (GH) concentrations increase during pregnancy in mice and remain pituitary-derived. Whether abundance or activation of the GH secretagogue ghrelin increase during pregnancy, or in response to dietary octanoic acid supplementation, is unclear. We therefore measured circulating GH profiles in late pregnant C57BL/6J mice and in aged-matched non-pregnant females, fed standard laboratory chow supplemented with 5% octanoic or palmitic (control) acid (n=4-13/group). Serum total and acyl-ghrelin concentrations, stomach and placenta ghrelin mRNA and protein expression, *Pcsk1* (encoding prohormone convertase 1/3) and *Mboat4* (membrane bound O-acyl transferase 4) mRNA were determined at Zeitgeber (ZT) 13 and ZT23. Total and basal GH secretion were higher in late pregnant than non-pregnant mice ($P<0.001$), regardless of diet. At ZT13, serum concentrations of total ($P=0.004$), but not acyl-ghrelin, and the density of ghrelin-positive cells in the gastric antrum ($P=0.019$) were higher, and gastric *Mboat4* and *Pcsk1* mRNA expression were lower in pregnant than non-pregnant mice at ZT23. In the placenta, ghrelin protein was localised mostly to labyrinthine trophoblast cells. Serum acyl-, but not total, ghrelin was lower at mid-pregnancy than in non-pregnant mice, but not different at early or late pregnancy. In conclusion, dietary supplementation with 5% octanoic acid did not increase activation of ghrelin in female mice. Our results further suggest that increases in maternal GH secretion throughout murine pregnancy are not due to circulating acyl-ghrelin acting at the pituitary. Nevertheless, time-dependent increased circulating total ghrelin could potentially increase ghrelin action in tissues that express the acylating enzyme and receptor.

2.3 Introduction

Successful pregnancy requires substantial maternal physiological adaptations, including increases in food intake, to maintain maternal health and allow optimal growth of the feto-placental unit to term. Growth hormone (GH) is a key endocrine regulator of growth and metabolism in a range of mammalian species and its secretion changes markedly during pregnancy. In humans, placental synthesis of variant GH in the syncytiotrophoblast increases circulating GH concentrations and progressively suppresses pulsatile pituitary secretion from mid-pregnancy onwards [120]. Although the placentas of most other mammalian species, including rodents, do not produce GH [634], circulating GH does increase during pregnancy in the majority of species investigated to date. In contrast to humans, circulating GH profiles remain pulsatile throughout pregnancy in mice and rats [122, 123, 601]. In these species, the increase in average circulating GH concentration and secretion reflect elevated basal GH secretion, while the number and amplitude of GH pulses remains unchanged [122, 123, 601]. In addition to driving maternal metabolic adaptation to pregnancy, including insulin resistance [119, 690-693], GH likely promotes fetal growth through other mechanisms. Provided maternal nutrition is adequate, intermittent maternal treatment with exogenous GH increases fetal growth in non-human species including rats, pigs and sheep, [380, 646, 649, 652, 657, 658, 670], likely through enhanced placental growth and/or function [646, 649, 652, 657, 658]. Despite the importance of GH during pregnancy in many mammals, the mechanism driving increased maternal GH in non-human species remains less well-known.

The increases in circulating GH concentration and basal GH secretion in pregnant mice [123] coincide with the formation of the chorioallantoic placenta and initiation of maternal blood flow through the placenta [635]. This suggests that placentally-secreted factors are likely to be responsible, either indirectly or directly, for driving the increased

pituitary GH secretion during rodent pregnancy. Ghrelin, a gastric peptide hormone that acts in conjunction with hypothalamic growth hormone-releasing hormone (GHRH) and somatostatin to generate pulsatile GH secretion from the anterior pituitary, is one possible candidate. In mouse stomach, the endoproteolytic processing of the ghrelin molecule from its precursor (proghrelin) into mature ghrelin is facilitated by the enzyme prohormone convertase 1/3 (PC1/3, encoded by proprotein convertase, *Pcks1*) [426]. Ghrelin is secreted in its des-acyl form and requires activation by acylation of the peptide's serine 3 residue with an 8-carbon octanoyl group [401]. This modification is mediated by the enzyme membrane bound O-acyl transferase (Mboat4, also known as GOAT), and allows the activated acyl-ghrelin to bind to the GH-secretagogue receptor [GHSR, 401, 453], stimulating GH secretion. Intravenous administration of exogenous acyl-ghrelin induces GH pulses in humans and rodents [401, 673] whilst mice deficient in ghrelin or Mboat4 have lower amplitude GH pulses than littermate controls [456, 694]. Acyl-ghrelin, but not des-acyl ghrelin, also acts centrally to stimulate food intake [476, 678], and acyl-ghrelin plays an important role in metabolic adaptations to maintain circulating glucose in response to calorie restriction, particularly in pregnancy [679]. Although ghrelin and Mboat4 are predominantly expressed in the stomach [401], ghrelin, Mboat4 and GHSR expression are also detected in the human [449, 695] and rodent [642, 696] placenta. Furthermore, maternal administration of exogenous acyl-ghrelin promotes fetal growth in rats [674]. This raises the possibility that ghrelin could be synthesised by, and act directly on, the placenta, as well as through central actions, to stimulate maternal GH secretion, food intake and fetal growth.

The potential role of ghrelin in pregnancy is supported by evidence that there are changes in maternal circulating ghrelin during pregnancy in a number of mammalian species. However, the reported changes in circulating total and acyl-ghrelin during pregnancy vary between studies. Total ghrelin concentration has been reported as lower compared to non-pregnant levels in humans and as lower or unchanged in

pregnant rats [642, 680-683]. Conflicting evidence is likewise available regarding changes in acyl-ghrelin, with either elevated or lower concentrations during pregnancy in women [681, 684], and elevated during late pregnancy in a single study in rats [685]. Although responses to exogenous ghrelin demonstrate its functional activity during pregnancy, the role of endogenous ghrelin during pregnancy remains unclear. Pregnant mice lacking the *Mboat4* gene, which consequently cannot convert ghrelin to acyl-ghrelin [686], still undergo a similar increase in GH during late pregnancy [686], suggesting that acyl-ghrelin is not required to increase GH during pregnancy. However, in this study GH was only measured in maternal blood collected at post-mortem and, therefore, it is unknown whether the lack of acyl-ghrelin altered the circulating patterns of GH during pregnancy [686]. These pulsatile patterns of circulating GH during pregnancy are important since intermittent, but not continuous, GH administration, improves placental function and increases fetal growth in a number of animal species [643-646].

The finding that increased acylation of ghrelin can be achieved by a nutritional approach [453, 468, 687] offers great translational potential as an approach to increase circulating GH. Oral supplementation with the C8 medium chain fatty acid, octanoic acid, or with the triglyceride containing this fatty acid, glyceryl trioctanoate, induced octanoylation of ghrelin in the stomach and increased abundance of circulating acyl-ghrelin in mice [453, 468, 687]. Similarly, enteral feeding with octanoic-acid enriched formula increased circulating acyl-ghrelin in humans [689]. Further, dietary supplementation with a medium chain triglyceride mixture containing 65-75% octanoic acid increased circulating levels of acyl-ghrelin in pigs [688].

Therefore, the aims of the present study were: (i) to characterise and compare the GH-ghrelin axis in non-pregnant and pregnant mice, and (ii) to determine the effect of dietary octanoic acid supplementation on this axis in both pregnant and non-pregnant animals. We hypothesised that the GH-ghrelin axis would be upregulated during

pregnancy, and that dietary octanoic acid supplementation would increase circulating acyl-ghrelin and GH, and pulsatile GH secretion, in both non-pregnant and pregnant mice.

2.4 Methods

2.4.1 *Ethical approval*

Experimental procedures were approved by The University of Adelaide Animal Ethics Committee (studies one and two, M-2016-186), or South Australian Health and Medical Research Institute (SAHMRI) Animal Ethics Committee (study three, SAM395.19) and carried out in accordance with the Australian code of practice for the care and use of animals for scientific purposes [697].

2.4.2 *Animals and experimental design*

Study one: Virgin female C57Bl/6J 10-week-old female mice were obtained from the Animal Resource Centre, Perth and CBAF1 males were obtained from Laboratory Animal Services, The University of Adelaide. All animals were acclimatised to the facility for at least 7 d prior to commencement of cycle tracking; with estrus cycles tracked for at least 8 d prior to mating. All mice were housed at ~23°C with 12-h:12-h light:dark cycle (lights on at 06:00 h or zeitgeber (ZT0), with *ad libitum* access to water and meat-free rat and mouse diet (14.0 MJ/kg, 20% protein, Speciality Feeds, Glen Forrest, Australia), except when fed experimental diets as detailed below. Female mice were weighed daily throughout the experiment. Estrus stage in females was classified daily by observation of cell types collected by gently flushing the vagina with 10 µL of saline [698]. To generate timed pregnancies, a male was placed in the females' cage overnight when one or both females were in diestrus-to-proestrus transition or proestrus. The presence of a vaginal plug on the following morning was taken as

confirmation of successful mating and designated as 0.5 d of pregnancy (GD0.5). Pregnancy was subsequently confirmed by weight gain and at post-mortem.

The experimental diets were based on prior observations that adding 5 mg/mL octanoic acid to drinking water or feeding a diet supplemented with 5% glyceryl trioctanoate, increased stomach acyl-ghrelin content in mice, whilst dietary tripalmitate did not alter ghrelin activation [468]. The experimental diets were therefore generated by adding 5% (wt/wt) of either octanoic acid or palmitic acid to ground meat-free rat and mouse diet (Speciality Feeds, Glen Forrest, Australia), which was then re-formed into pellets and baked for 4 h at 60 °C prior to feeding. Experimental diets were fed *ad libitum* throughout the remainder of the experiment, commencing at GD0.5 in mated groups and from the same day in age-matched controls, including the day of sampling. To confirm the fatty acid content, samples of each diet were ground, extracted into chloroform and analysed for fatty acid composition as described below.

Samples were collected from pregnant mice (n=8 palmitic acid diet, n=7 octanoic acid diet) at 17.5 d after mating (term is ~19.3 d after mating in this strain [32], and from non-pregnant mice (n=9 palmitic acid diet, n=10 octanoic acid diet) when mice were in diestrus and between 15.5 and 19.5 d after commencing experimental diets. Additional non-pregnant mice (n=2 palmitic acid diet, n=3 octanoic acid diet) did not enter diestrus between 15.5 and 19.5 d after commencing experimental diets and were thus not sampled for GH since secretion patterns change throughout the estrus cycle (Chen Chen, pers. comm.). All other samples were collected from these animals. To minimise stress, all females were handled daily for ~5 min per mouse for ≥14 days before sampling. GH samples were collected from each animal over a continuous 6 h period to allow patterns of GH in the circulation and of GH secretion to be determined. From 13:00 h, ZT7 (lights on at 06:00 h, lights off at 18:00 h, ZT12), 36 sequential tail-tip blood samples (each 2 µL) were collected at 10-min intervals from each mouse, processed and stored at −80°C for later analysis as previously described [123, 699].

Shortly after collection of the final sample (19:00 h to 19:30 h), mice were terminally anaesthetised by i.p injection of Avertin (2,2,2-tribromoethanol and tert-amyl alcohol; Sigma-Aldrich, Missouri, United States) and 0.5 to 1.0 mL of venous blood was collected via retro-orbital puncture. Whole blood (30 µL) from each individual mouse was spotted onto customised PUFAcoat™ collection cards and air-dried for later analysis of free fatty acids [700]. The remaining blood was collected into Eppendorf tubes containing 4-(2-aminoethyl) benzenesulphonyl fluoride (AEBSF; at a final concentration of 2 mg AEBSF per mL whole blood) allowed to clot at room temperature for 30 minutes before centrifugation, and plasma acidified to a final concentration of 0.05M HCl to minimise breakdown of acyl-ghrelin [701]. Following blood collection, mice were humanely killed by cervical dislocation. The uterus was removed from pregnant mice, and the stomach was rapidly collected for processing as described below. The stomach was opened along the greater curvature and rinsed in saline prior to dissection, along the lesser curvature, into two pieces. Mucosal scrapings from the corpus and antrum, the glandular regions of the stomach, were collected from one half of the stomach, snap-frozen in liquid nitrogen and stored at -80°C until further analysis. For the immunohistochemistry experiments, the other half of the stomach was pinned out flat in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PFA-PB) and rocked at room temperature for 3 hours before cryoprotection in 30% sucrose-PB solution overnight. The stomachs were then embedded in optimal cutting temperature compound (Tissue-Tek, ProSciTech, Queensland Australia), frozen and stored at -80 °C until sectioning [702]. The numbers of implantations, fetuses and resorption sites were counted and each fetus and placenta was dissected and weighed. Placentas were alternately snap-frozen in liquid nitrogen or fixed for histological analysis as described below. Complete suppression of GH secretion was observed in two pregnant mice, who had normal litter size and foetal weights at post-mortem. Data for these animals were excluded from all analyses, because a loss of GH secretion occurs

acutely in stressed mice [703], and these two animals were therefore not considered to reflect the normal physiology of pregnancy.

Study Two: In study one, plasma samples were collected from animals at the completion of GH sampling, when most animals had not eaten. Upon analysis, we found no difference in the circulating plasma acyl-ghrelin concentrations between groups. Given the rapid turnover of acyl-ghrelin in circulation [701], we considered it possible that any effects of diet on ghrelin production and release may only have been evident close to the time of feeding. Therefore, in order to determine whether there was an increase in basal circulating plasma acyl-ghrelin levels in mice that had undisturbed access to food overnight, we conducted a second study, with the same interventions, but with samples collected late in the active feeding period (dark phase) for mice [704], and without serial sampling for GH, to minimise disruption to feeding. Animal management, breeding and diets were as described for study one. Animals were humanely killed at 07:30 to 08:00 h (lights on at 09:00 h, lights off at 21:00 h), and blood and tissue samples were collected as described above.

Study three: Since circulating ghrelin was not consistently different between non- and late-pregnant mice in the first two studies, we collected plasma from a third cohort of mice to assess changes in ghrelin abundance throughout pregnancy. Mice were sampled shortly after lights on, at a time when they have recently eaten [705]. Female C57Bl/6J 10-12 week-old female mice were obtained from SAHMRI Bioresources. All mice were housed at ~22°C with 12-h:12-h light:dark cycle (lights on at 07:00 h, with *ad libitum* access to water and standard rodent diet (18.6% protein, 6.2% fat, 44.2% carbohydrate, Teklad standard diet, Envigo, Cambridgeshire, United Kingdom). For timed mating, female mice were pair-housed with a C57BL/6J male at 17:00 h, and pregnancy was confirmed by the presence of a vaginal plug at 07:00 h (assigned as day 0.5 of pregnancy) before being returned to individual cages. Plugged females were randomly assigned to either early (6.5 days, n=8), mid (12.5 days, n=8) or late (18.5 days, n=8)

stage pregnancy end points. Age-matched females that were not housed with a male for mating were used as non-pregnant controls (n=8). Mice were anaesthetised at ZT0-ZT1 by isoflurane inhalation (5% in oxygen) and venous blood was collected from the inferior vena cava and processed as described above to obtain plasma for ghrelin assays. Mice were then humanely killed via decapitation.

2.4.3 *Analysis of fatty acids in feed and blood*

The fatty acid composition of blood and feed samples was assessed as previously described [700, 706]. Briefly, whole dried blood spots from the PUFAcoat™ cards and extracted feed samples were trans-esterified with 2 ml of 1% H_2SO4 in methanol at 70 °C for 3 h. After adding distilled water and heptane to the vial, the samples were shaken using a vortex. Samples were then left standing and the top heptane layer containing fatty acid methyl esters was extracted and analysed using gas chromatography [700]. Abundance of each fatty acid is expressed as a percentage of total fatty acids in the sample.

2.4.4 *Hormone analyses*

Serum concentrations of acyl-ghrelin and total ghrelin were determined by commercial enzyme-linked immuno-absorbent assay (ELISA) kits [EZRGRA-90K, Rat/mouse Ghrelin (Active) ELISA and EZRGRT-91K, Rat/mouse Ghrelin (Total) ELISA respectively; R&D Systems, Minneapolis, MN, USA], in accordance with the manufacturer's instructions. A single ELISA plate was used to measure all samples from each study. Within-assay coefficients of variation were each <10% (study one: total ghrelin: 4.1%, acyl-ghrelin: 7.4%; study two: total ghrelin: 5.8%, acyl-ghrelin: 3.8%). Analysis for GH was performed using an in-house mouse GH ELISA as described and validated previously [699]. Within- and between-assay coefficients of variation were 2.0 % and 16.8 % respectively for a mouse plasma QC sample containing 27.7 ng/mL (n =16

assays). All samples from a single mouse (serial samples for analysis of secretion pattern) were analysed on the same ELISA plate. Kinetics and secretory patterns of pulsatile GH secretion were determined by deconvolution analysis following parameters established previously for GH secretion in mouse [699, 703]. The orderliness and regularity of serial GH serum concentrations were calculated by approximate entropy (ApEn) analysis as described previously; a higher absolute ApEn denotes greater irregularity and indicates a loss in stability of feedback of GH regulation [707].

2.4.5 *Ghrelin and Mboat4 gene expression in stomach and placenta*

Total RNA from snap frozen placenta and gastric mucosal scrapings was extracted using PureLink RNA Mini Kit (12183018A; Invitrogen, Carlsbad, CA, USA), and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative real-time PCR reactions (qPCR) were performed using a 7500 Fast Real-time PCR System (Life Technologies, Carlsbad, CA, USA) and Express One-Step SuperScript qRT-PCR Kit (11781-200, Invitrogen). Predesigned Taqman gene expression assays (4331182, Life technologies) that target *Ghrelin* (Mm00445450_m1), *Mboat4* (Mm01200389_m1), *Pcsk1* (encodes for PC 1/3; Mm00479023_m1) were used. *B2m* (Mm00437762_m1), *Hprt* (Mm01545399_m1) and *Ppia* (Mm02342429_g1) with an expression stability value of 0.035 were used as reference genes for the gastric mucosa, while *Polr2a* (Mm00839502_m1) and *Ubc* (Mm01198158_m1) were identified as suitable reference genes in the mouse placenta [708]. qPCR reactions were carried out under the following conditions: reverse transcription, 50 °C for 15 minutes; initial PCR activation, 95 °C for 20 seconds; PCR cycles 95 °C for 3 seconds, 60 °C for 30 seconds repeated for 40 cycles. Each assay was run in triplicate. Control PCRs were carried out substituting RNase-free water for template RNA. Relative RNA levels were calculated using the delta CT method as described previously [709].

2.4.6 *Immunohistochemistry and histology*

Stomach ghrelin localisation: Stomach tissue was sectioned (10 µm), air dried at RT before being rinsed in PBS + 0.2% Triton X-100 (PBS-TX; Sigma-Aldrich) and blocked with 10% donkey serum at room temperature. To detect ghrelin immunoreactivity, sections were then incubated with rabbit-anti-ghrelin primary antibody (1 in 800 dilution in PBS-TX; Abcam Cat# ab129383, RRID:AB_11159267ab129383, Abcam, Cambridge, United Kingdom) for 20 h at 4 °C and donkey-anti-rabbit 488 secondary antibody (1 in 200 in PBS-TX, Cat# A-21206, RRID:AB_2535792 Thermo Fisher Scientific) for 1 h at RT. The slides were mounted using ProLong Antifade (Life technologies). Slides where primary antibody was omitted showed no labelling and served as negative controls. Slide sections were visualised using an epifluorescence microscope (BX-51, Olympus, Tokyo, Japan) equipped with filters for Alexa Fluor® 488, with images acquired using a CoolSnapfx monochrome digital camera (Roper Scientific, Tuscon, AZ, USA). Ghrelin-positive cells were counted manually on images taken at 10 X magnification by two people blind to treatment. Ten stomach tissue sections were randomly chosen from each mouse. In each section, ghrelin-positive cells were counted in an area of 400 µm × 400 µm in both proximal corpus and distal antrum. The number of ghrelin positive cells in corpus or antrum from each mouse was averaged from the 10 selected sections.

Placental morphology: Histological analyses were performed on two placentae from each dam (palmitic acid n=14 placentas, octanoic acid n=12 placentas). One dam from the palmitic acid group was excluded from this analysis due to a small litter size (a single fetus), however, the placenta was collected and frozen for gene expression analysis. Bisected placentae were fixed in 4% paraformaldehyde (PFA), washed in 1 X PBS over 48 h and stored in 70% ethanol prior to being paraffin embedded. Full-faced placental sections cut to 5 µm thickness were stained with Masson's Trichrome following standard protocols [710]. Areas of junctional zone and placental labyrinth for each placental

section were visualised and measured with NDP.view 2 software (Hamamatsu Photonics, Shizuoka, Japan). Total cross-sectional area and the proportion of junctional zone to placental labyrinth were calculated.

Placental ghrelin localisation: Full-faced sections cut to 5 µm thickness were deparaffinised and rehydrated, using the same placentas as above (palmitic acid n=14 placentas, octanoic acid n=12 placentas). Antigen retrieval was performed by microwaving slides in citrate buffer (0.2% citric acid in distilled water) for 10 min at 150 W. In order to quench endogenous peroxidase, sections were incubated in 3% H_2O_2 in 1 X PBS for 10 min. Non-specific binding was blocked by incubation with 10% goat serum in 1 X PBS for 30 min before addition of rabbit anti-ghrelin antibody (1 in 250 dilution, Abcam Cat# ab129383, RRID:AB_11159267ab129383) and incubated overnight at RT. Bound antibody was detected using biotinylated goat anti-rabbit IgG (1 in 200 dilution, Agilent Cat# E0432, RRID:AB_2313609, DAKO, Carpinteria, CA, USA) for 1 h, followed by streptavidin-conjugated horseradish peroxidase (1 in 500 dilution, Cat# P0397, DAKO) for 1 h, both at RT. Immunolabelling was visualised by incubating sections with diaminobenzidine (Cat# K346811-2, DAKO) and by counterstaining with hematoxylin before mounting in DPX (Sigma-Aldrich). Areas of ghrelin staining were visualised with NDP.view 2 software (Hamamatsu Photonics).

2.4.7 *Statistical analyses*

For studies one and two, effects of diet (palmitic acid supplemented cf. octanoic acid supplemented) and pregnancy status (non-pregnant cf. pregnant) on maternal outcomes were analysed by 2-way ANOVA. For study three, effects of pregnancy stage (non-, early-, mid- and late-pregnant) were analysed by 1-way ANOVA. Where outcomes differed between pregnancy stages, post-hoc analyses were performed using the Bonferroni correction. Effects of diet on fetal and placental outcomes was analysed using repeated measures ANOVA, treating each fetus or placenta as a

repeated measure on the dam. GH secretion data were natural log-transformed to achieve equal variances before analysis. Data are presented as mean ± SEM and P<0.05 was considered statistically significant.

2.5 Results

2.5.1 *Pregnancy outcomes were unaffected by maternal diet*

Maternal weight gain during the study, body and liver weights at post-mortem (Table 2.1) and absolute and relative organ weights (liver, kidneys and spleen, data not shown) were higher in pregnant than non-pregnant mice, and unaffected by diet. Pregnancy outcomes including litter size, fetal weight and placental gross structure were also unaffected by maternal diet (Table 2.1).

2.5.2 *Circulating fatty acid profiles were altered by diet and pregnancy*

As expected, the content of C16:0 (palmitic acid) was higher in the palmitic acid-supplemented feed (4.1 g/100 g feed) than in the octanoic acid-supplemented feed (0.5 g/100 g feed). Similarly, the content of C8:0 (octanoic acid) was higher in the octanoic acid-supplemented feed (1.5 g/100 g feed) than in the palmitic acid-supplemented feed (0.07 g/100 g feed).

Study one: In mice sampled at ZT13, total saturated fatty acid levels were higher in mice fed the palmitic acid than the octanoic acid diet (P=0.014) and higher in pregnant than non-pregnant mice (P<0.001, Table 2.2). Conversely, total monounsaturated fatty acid levels were higher in the blood of mice fed the octanoic acid diet (P=0.006) and in pregnant compared to non-pregnant mice (P=0.046, Table 2.2). Octanoic acid was undetectable in blood, regardless of diet or pregnancy status (data not shown). Circulating palmitic acid (C16:0) comprised a higher percentage of total lipids in mice fed

Table 2.1 *Maternal and pregnancy characteristics (study one ZT13).*

	Non pregnant		Pregnant		P value		
	Palmitic acid	Octanoic acid	Palmitic acid	Octanoic acid	Diet	Pregnancy	D*P
Number of dams	11	13	8	6			
Final body weight (g)	21.3 ± 0.1	21.9 ± 0.3	34.5 ± 1.3	34.0 ± 1.5	0.953	<0.001	0.542
Weight gain (g/d)	0.05 ± 0.01	0.05 ± 0.02	0.83 ± 0.07	0.78 ± 0.10	0.488	<0.001	0.593
Liver (g)	0.75 ± 0.03	0.76 ± 0.02	1.30 ± 0.08	1.20 ± 0.16	0.550	<0.001	0.431
Liver (% of body weight)	2.5 ± 0.5	3.2 ± 0.3	3.8 ± 0.2	3.6 ± 0.6	0.563	0.073	0.306
Litter size			6.4 ± 0.8	7.3 ± 0.8	0.432		
Average fetal weight (g)			1.2 ± 0.1	1.1 ± 0.1	0.626		
Average placental weight (g)			0.1 ± 0.1	0.1 ± 0.1	0.276		
Average junctional zone area (mm^2)			2.5 ± 0.2	2.5 ± 0.1	0.938		
Average labyrinth zone area (mm^2)			5.2 ± 0.2	4.7 ± 0.2	0.158		

Maternal data were analysed using two-way ANOVA and data for fetal outcomes were analysed using 1-way ANOVA. Data are presented as mean ± SEM. Abbreviations: D*P, diet*pregnancy interaction; FA, fatty acid

Table 2.2 *Circulating maternal fatty acid concentrations.*

	Non pregnant		Pregnant		P value		
	Palmitic acid	**Octanoic acid**	**Palmitic acid**	**Octanoic acid**	**Diet**	**Pregnancy**	**D*P**
Study one							
n animals	11	13	8	6			
Total saturated FA (% of total lipids)	42.0 ± 0.2	40.6 ± 0.4	44.1 ± 0.5	43.3 ± 0.5	0.014	<0.001	0.466
16:0 (% of total lipids)	24.7 ± 0.3	22.1 ± 0.2	26.4 ± 0.3	24.3 ± 0.3	<0.001	<0.001	0.307
Total trans-esterified FA (% of total lipids)	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.674	0.978	0.593
Total monosaturated FA (% of total lipids)	16.2 ± 0.3	17.8 ± 0.2	17.5 ± 0.5	17.9 ± 0.3	0.006	0.046	0.092
Study two							
n animals	13	9	5	6			
Total saturated FA (% of total lipids)	44.3 ± 0.3	43.4 ± 0.5	45.1 ± 0.5	46.5 ± 0.5	0.680	0.000	0.022
16:0 (% of total lipids)	28.2 ± 0.2	25.6 ± 0.4	28.3 ± 0.2	28.4 ± 0.5	0.004	0.001	0.002
Total trans-esterified FA (% of total lipids)	0.3 ± 0.02	0.2 ± 0.02	0.3 ± 0.03	0.3 ± 0.02	0.949	0.191	0.485
Total monosaturated FA (% of total lipids)	14.9 ± 0.6	15.1 ± 0.2	14.4 ± 0.4	15.9 ± 0.4	0.141	0.830	0.279

Fatty acids were measured by GCMS in blood collected from non-pregnant and pregnant mice, fed chow supplemented with either 5% palmitic acid or 5% octanoic acid. Mice in study one were sampled between 1 and 2 h after lights off, and mice in study two were sampled between 1 and 0 hours before lights on, at the end of the natural feeding period. Data were analysed by two-way ANOVA and are presented as mean ± SEM. Abbreviations: D*P, diet*pregnancy interaction; FA, fatty acids.

the palmitic acid diet, compared to those fed an octanoic acid diet (P<0.001) and were also higher in pregnant than non-pregnant mice overall (P<0.001, Table 2.2).

Study two: In mice sampled at ZT23, effects of diet on total saturated fatty acids differed between non-pregnant and pregnant groups (interaction P=0.022, Table 2.2). Thus, in mice fed the palmitic acid-supplemented diet, the concentration of total saturated fatty acids was similar between non-pregnant and pregnant mice. However, within mice fed the octanoic acid-supplemented diet, the concentration of total saturated fatty acids was 7% higher in pregnant than non-pregnant mice. Circulating total saturated fatty acid concentrations did not differ between diets within either non-pregnant or pregnant groups. Total trans-esterified and monounsaturated fatty acid concentrations were similar between groups (P>0.05, Table 2.2).

2.5.3 *Circulating GH was higher in pregnant than non-pregnant mice but was unaffected by diet*

Circulating GH was pulsatile in all mice and mean plasma GH concentrations increased during pregnancy (study one, Figure 2.1, Table 2.3). Total and basal GH secretion rates were significantly higher in pregnant mice (P<0.001, Table 2.3) compared to non-pregnant mice, irrespective of diet. The irregularity of GH pulses (approximate entropy; P=0.033) and mode of secretion (P=0.001) were also higher in pregnant mice compared to non-pregnant mice and unaffected by diet (Table 2.3). Pulse frequency, mass of GH released per burst and pulsatile GH secretion rate were similar between dietary groups and between pregnant and non-pregnant animals (Table 2.3).

Table 2.3 *Circulating maternal growth hormone (GH) and parameters of pulsatile GH secretion following deconvolution and approximate entropy (ApEn) analysis.*

	Non pregnant		Pregnant		P value		
	Palmitic acid	Octanoic acid	Palmitic acid	Octanoic acid	Diet	Pregnancy	D*P
n samples	7	10	8	4			
Mean circulating GH (ng/mL)	3.9 ± 1.0	6.1 ± 1.3	11.4 ± 2.7	18.5 ± 9.2	0.155	0.004	0.433
Total GH secretion rate (ng/mL 6h)	169.2 ± 43.0	247.7 ± 58.3	491.6 ± 118.1	819.1 ± 409.1	0.159	<0.001	0.382
Basal GH secretion rate (ng/mL 6h)	18.0 ± 11.3	68.6 ± 24.8	312.2 ± 85.4	404.6 ± 187.3	0.344	<0.001	0.780
Interval between bursts	19.9 ± 10.0	24.3 ± 8.0	26.0 ± 6.7	28.0 ± 10.3	0.343	0.151	0.724
Number of GH pulses/6h	4.1 ± 0.8	4.2 ± 0.6	5.4 ± 0.7	5.8 ± 0.9	0.773	0.073	0.832
Mode of secretory bursts (min)	15.0 ± 2.0	11.3 ± 0.5	7.6 ± 1.2	8.7 ± 1.4	0.353	0.001	0.096
ApEn	0.8 ± 0.1	0.9 ± 0. 04	1.0 ± 0.1	1.0 ± 0.1	0.573	0.033	0.528
Mass of GH secreted/burst (ng/mL)	34.5 ± 8.5	47.8 ± 9.9	39.3 ± 11.0	62.8 ± 31.0	0.187	0.472	0.709
Pulsatile GH secretion rate (ng/mL 6h)	151.2 ± 44.7	179.1 ± 40.0	179.4 ± 40.5	414.5 ± 262.2	0.130	0.130	0.230

Data were analysed by two-way ANOVA and are presented as mean ± SEM. Abbreviations: D*P, diet*pregnancy interaction

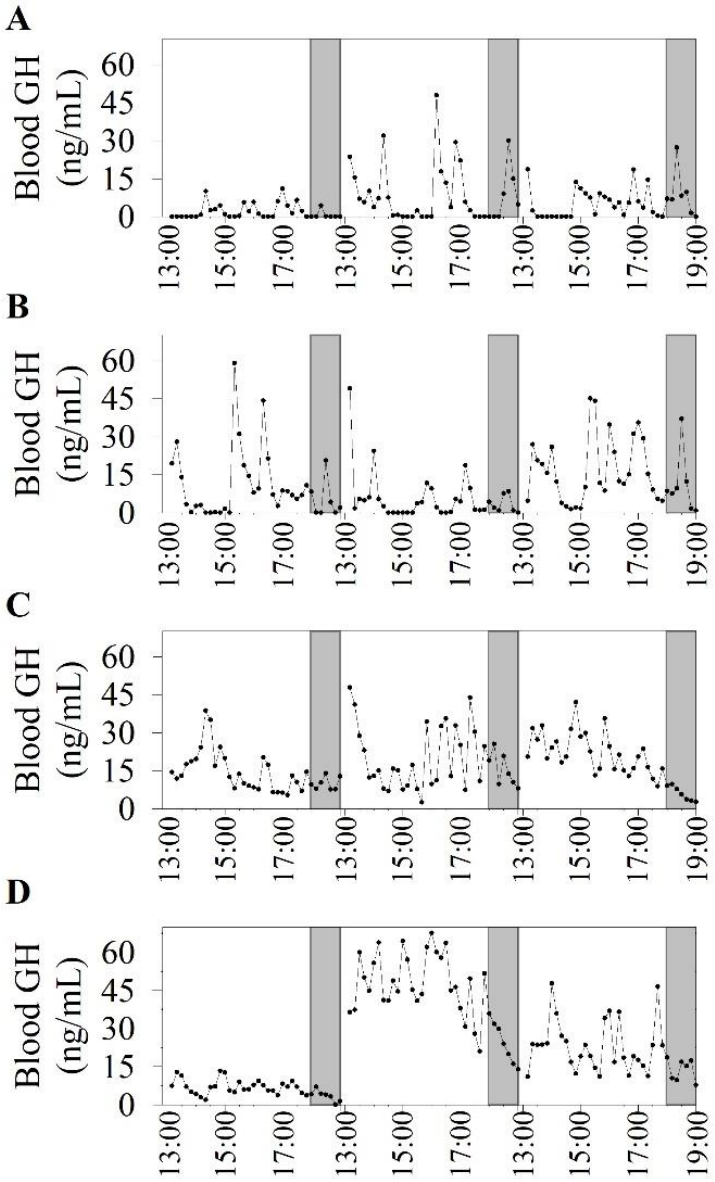


Figure 2.1 *Representative circulating GH profiles in non-pregnant females fed palmitic acid diet; n=7 (A) or octanoic acid diet; n=10 (B), in mice at GD17.5 or age-matched diestrus controls after 15±5 days of eating experimental diets, and in pregnant mice fed palmitic acid diet; n=8 (C) or octanoic acid diet; n=4 (D) at GD17.5. Shading indicates dark period.*

2.5.4 *Total but not acyl-ghrelin is higher in pregnant than non-pregnant mice, depending on time of day*

In mice sampled at ZT13 (study one), serum total ghrelin levels were significantly higher in pregnant than non-pregnant mice (P=0.004, Figure 2.2), and were unaffected by diet, while serum acyl-ghrelin concentrations were similar irrespective of diet or pregnancy status. In mice sampled at ZT23 (study two), total and acyl-ghrelin levels in mice were similar between dietary groups and between pregnant and non-pregnant animals (Figure 2.2).

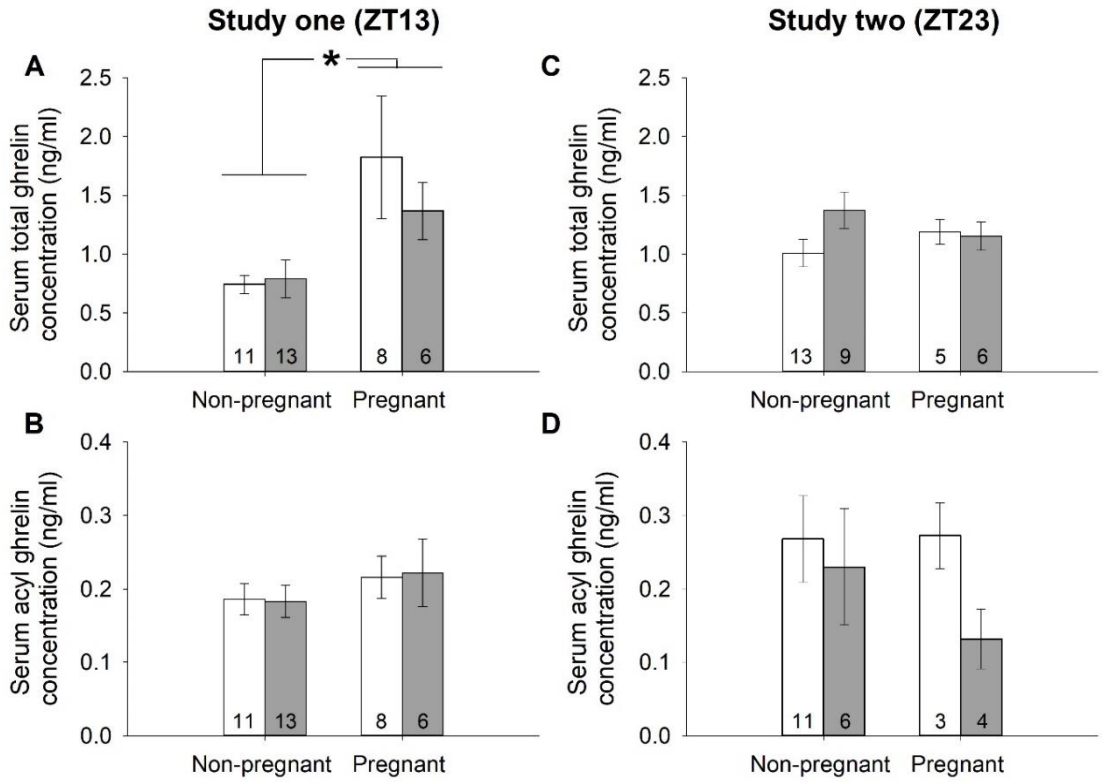


Figure 2.2 *Circulating maternal serum total (A, C) and acyl (B, D) ghrelin in non-pregnant and pregnant mice fed palmitic acid diet (white bars) or octanoic acid diet (grey bars), sampled at ZT13 (study one, A, B) or ZT23 (study two, C, D), in mice at GD17.5 or age matched controls. Data were analysed by two-way ANOVA and are presented as mean ± SEM. Animal numbers are indicated by numerals within bars.*

When assessing changes in ghrelin abundance across pregnancy (study three), serum total ghrelin did not differ between pregnancy stages (P=0.059, Figure 2.3A). In contrast, serum acyl-ghrelin concentrations changed with pregnancy stage (P=0.008, Figure 2.3B). Acyl-ghrelin concentrations were lower in mid-pregnant compared to non-pregnant mice (P=0.007), and did not differ between other pregnancy stages (Figure 2.3B).

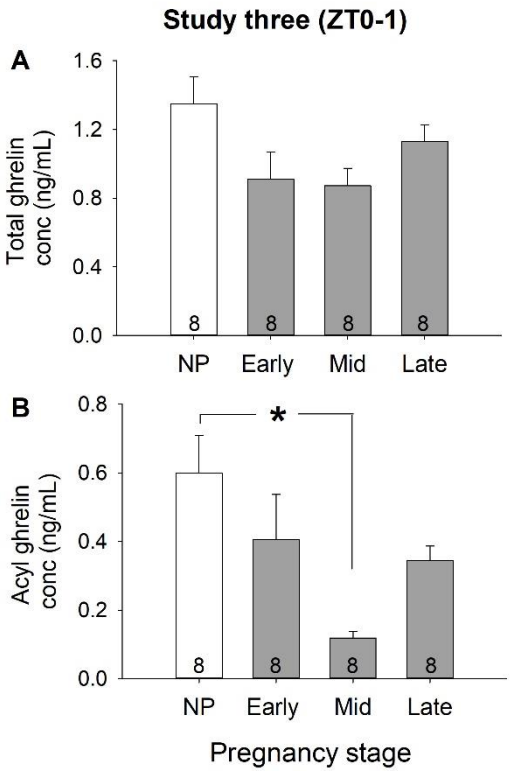


Figure 2.3 *Circulating maternal plasma total (A) and acyl (B) ghrelin throughout pregnancy in non-pregnant (white bars), early, mid and late pregnant (grey bars) mice sampled at ZT0-1 (study three).Data were analysed by 1-way ANOVA and are presented as mean ± SEM. Animal numbers are indicated by numerals within bars.*

2.5.5 *Stomach and placental ghrelin expression may explain higher total but not acyl-ghrelin in pregnancy*

Gastric *ghrelin*, *Mboat4 and Pcsk1* mRNA levels were similar in all groups at ZT13 (study one, Table 2.4). Gastric *ghrelin* mRNA expression was unaffected by pregnancy or diet at ZT23 (study two, Table 2.4). However, the gastric mRNA expression of *Mboat4* and *Pcsk1* was lower in pregnant compared to non-pregnant mice at ZT23 (Table 2.4).

Table 2.4 *Stomach ghrelin, Mboat4 and Pcsk1 mRNA expression.*

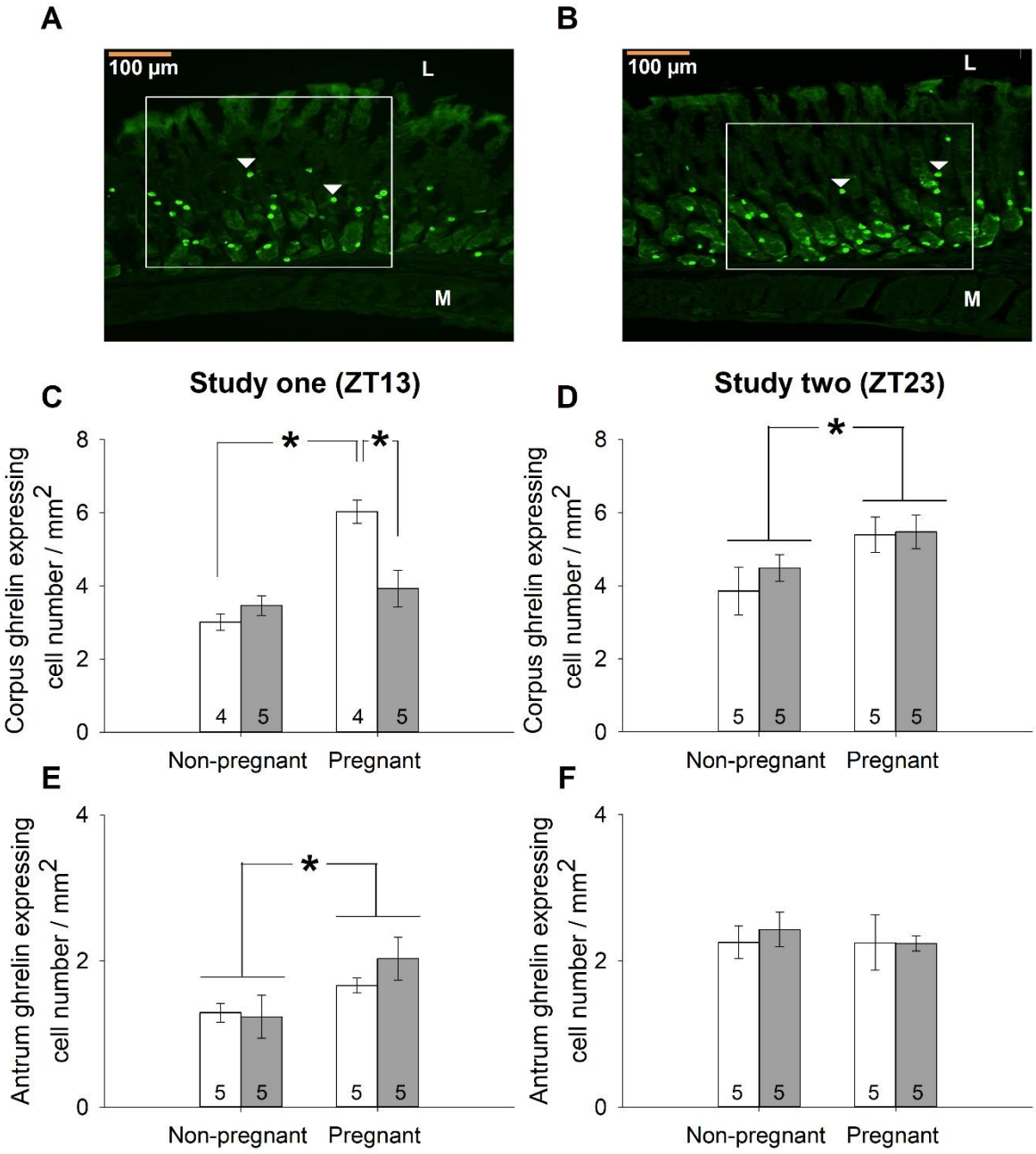
	Non- pregnant		Pregnant		P value		
	Palmitic acid	Octanoic acid	Palmitic acid	Octanoic acid	Diet	Pregnancy	D*P
Study one (ZT13)							
n samples	5	5	5	5			
Ghrelin	5.4 ± 0.3	4.9 ± 0.3	4.9 ± 0.2	4.4 ± 0.2	0.090	0.070	0.976
Mboat4	0.0059 ± 0.0007	0.0061 ± 0.0004	0.0059 ± 0.0002	0.0060 ± 0.0004	0.716	0.927	0.794
Pcsk1	0.015 ± 0.001	0.017 ± 0.001	0.016 ± 0.001	0.015 ± 0.001	0.553	0.829	0.167
Study two (ZT23)							
Ghrelin	3.8 ± 1.0	3.2 ± 0.9	2.7 ± 0.6	2.4 ± 0.6	0.587	0.243	0.825
Mboat4	0.0074 ± 0.0006	0.0067 ± 0.0009	0.0052 ± 0.0004	0.0051 ± 0.0005	0.553	0.007	0.599
Pcsk1	0.021 ± 0.003	0.018 ± 0.002	0.015 ± 0.001	0.016 ± 0.002	0.644	0.049	0.378

Gene expression was quantified using qRT-PCR and is expressed relative to reference genes. Gene expression data were analysed by two-way ANOVA. Data are presented as mean ± SEM. Abbreviations: D*P, diet*pregnancy interaction; *Mboat4*, membrane bound O-acyltransferase, *Pcsk1*, proprotein convertase

At ZT13 (study one), the effects of pregnancy on the density of ghrelin-positive cells in the corpus region of the stomach differed between diets (interaction, P=0.003, Figure 2.4C). In mice fed the palmitic acid diet, the density of ghrelin-positive cells in the stomach corpus was higher in pregnant than non-pregnant mice (P<0.001, Figure 2.4). Pregnancy status did not affect the density of ghrelin-positive cells in the corpus of mice fed the octanoic acid diet.

In pregnant mice, the density of ghrelin-positive cells in the corpus was higher in mice fed the palmitic acid compared to mice fed the octanoic acid diet (P=0.012, Figure 2.4). There was no effect of diet on the density of ghrelin-positive cells in the corpus in non-pregnant mice. In the antrum region, the density of ghrelin-positive cells was higher in pregnant mice than non-pregnant mice (P=0.019), irrespective of diet (Figure 2.4E).

At ZT23 (study two), the density of ghrelin-positive cells in the corpus region was higher in pregnant mice than non-pregnant mice (P=0.023), irrespective of diet (Figure 2.4D). However, the density of ghrelin-positive cells in the antrum region of the stomach at this sampling time was similar in all groups (Figure 2.4F).



2009

2010 ***Figure 2.4*** *Immunohistochemistry for ghrelin positive cells in the corpus of non-*
2011 *pregnant (A) and pregnant (B) mice. Arrows indicate ghrelin-positive cells, L and M*
2012 *indicate lumen and mucosal regions respectively, scale bar = 100 µm. Effects of*
2013 *pregnancy and diet on the density of ghrelin-positive cells in the corpus (C, D) and*
2014 *antrum (E, F) regions of the stomach in mice fed palmitic acid diet (white bars) or*
2015 *octanoic acid diet (grey bars). Samples were collected at ZT13 (study one, C, E) or*
2016 *ZT23 (study two, D, F), in mice at GD17.5 or age-matched controls. Data were*
2017 *analysed by two-way ANOVA and are presented as mean ± SEM. Animal numbers are*
2018 *indicated by numerals within bars.*

2019

Table 2.5 *Placental ghrelin and Mboat4 mRNA expression.*

	Palmitic acid	Octanoic acid	P value
Study one (ZT13)			
n samples	5	5	
Ghrelin	0.0021 ± 0.0001	0.0023 ± 0.0001	0.258
Mboat4	0.0007 ± 0.0002	0.0011 ± 0.0003	0.292
Study two (ZT23)			
n samples	5	6	
Ghrelin	0.0022 ± 0.0001	0.0023 ± 0.0002	0.922
Mboat4	0.0021 ± 0.0005	0.0013 ± 0.0004	0.216

Gene expression was quantified using qRT-PCR and is expressed relative to reference genes. Gene expression data were analysed by 1-way ANOVA. Data are presented as mean ± SEM. Abbreviations: *Mboat4*, membrane bound O-acyltransferase

Placental *ghrelin* and *Mboat4* mRNA levels were measured in pregnant animals and were unaffected by diet in both studies (Table 2.5). In the placenta, cytoplasmic ghrelin immunostaining was mostly localised to labyrinthine trophoblast, and nuclear immunostaining was present in both labyrinth and junctional zones (Figure 2.5). Localisation was unaffected by diet.

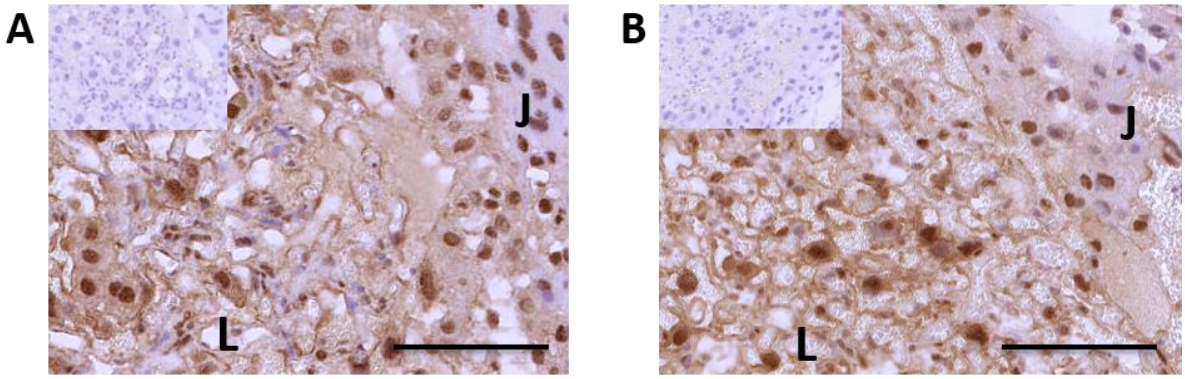


Figure 2.5 *Immunohistochemistry for ghrelin localisation in GD17.5 murine placentas from dams fed palmitic acid (A) and octanoic acid (B) diet. L and J indicate labyrinthine and junctional zones respectively. Scale bar = 100 μm*

2.6 Discussion

In the present study, circulating basal and total GH, as well as total, but not acyl, ghrelin concentrations were significantly higher in pregnant compared to non-pregnant mice. Contrary to our hypothesis, dietary supplementation with octanoic acid did not increase acyl-ghrelin, circulating GH concentration or pulsatile GH secretion in either pregnant or non-pregnant mice.

The increased circulating levels of GH we observed during late pregnancy are consistent with previous studies in mice [123] as well as rats [122, 601]. This further confirms that maternal GH is elevated in multiple species during pregnancy [120, 122, 123, 601] despite the lack of a placentally-expressed GH variant gene in rodents and most other non-primate species [711]. Interestingly, GH was elevated in pregnant mice despite the fact that maternal acyl-ghrelin levels were similar in late pregnant and non-pregnant mice, and actually lower at mid-pregnancy than in non-pregnant females. These observations are consistent with the findings of Trivedi and colleagues, who demonstrated that circulating GH, measured in single samples at post-mortem, was

similarly higher in late pregnant than non-pregnant animals in both *Mboat4*-knockout and wild-type mice [686]. Together, these findings imply that endogenous circulating acyl-ghrelin is not an important regulator of changes in GH during pregnancy.

Total serum ghrelin concentrations were higher in pregnant than in non-pregnant mice, however this was only observed in mice that were sampled at the end of the light phase (study one), and not in those sampled at the end of the dark phase (study two) or early in the light phase (study three). Ghrelin secretion undergoes a pre-prandial increase and a postprandial decline in humans, reflecting its function in stimulating hunger and initiation of meals [450, 451]. Besides the stimulation of food intake, ghrelin decreases energy expenditure in rodents and promotes the storage of fatty acids in adipocytes [712, 713]. The difference in effects of pregnancy on total ghrelin abundance in the light and dark phases might therefore reflect greater diurnal ghrelin variation in pregnancy, possible due to greater nutrient demand and appetite drive [714]. Consistent with food intake patterns in non-pregnant female and male mice [715, 716], the majority of food intake occurs during the dark phase in pregnant mice [3]. Lower acyl-ghrelin concentrations in fed mice at mid-pregnancy (study three) might be a consequence of increasing food intake during pregnancy [3], since postprandial suppression of plasma ghrelin is proportional to the amount of calories ingested [717]. Additional studies are needed to characterise the circadian rhythm in ghrelin and determine whether these change in pregnancy.

The higher circulating total ghrelin concentrations in pregnant than in non-pregnant mice in study one might be explained by greater ghrelin production in the stomach, as well as potentially by placental production. Specific cells in the stomach, particularly X/A-like enteroendocrine cells, are the main site of ghrelin production and acylation in non-pregnant animals [434], and in male mice preproghrelin mRNA expression is substantially higher in the stomach than in other tissues [454]. Although ghrelin mRNA

expression was detected in placenta, its abundance was >1000-fold lower than in stomach, suggesting its contribution to circulating ghrelin is relatively minor, although abundant ghrelin was evident in placentas stained for ghrelin protein. Gene expression of ghrelin in the stomach was not different in pregnant and non-pregnant mice in either study, consistent with similar stomach, hypothalamic and pituitary ghrelin expression in pregnant and non-pregnant mice reported previously [679]. However, the 1.5-fold greater density of ghrelin-positive cells in the stomach antrum of pregnant than non-pregnant mice sampled at ZT13 (study one) suggests a greater capacity to secrete ghrelin, although this was not seen at ZT23 (study two). We saw a similar pattern for the stomach corpus, with greater ghrelin-positive cell density in pregnant than non-pregnant mice at ZT23, although at ZT13 the effect of pregnancy was only significant within the subset fed the palmitic acid diet. The discrepancy between ghrelin gene expression levels and number of ghrelin positive cells in the stomach may be because gene expression was measured from the entire gastric mucosa, whereas ghrelin positive cell count was carried out in specific sections of the stomach. Given the variability within groups and relatively low sample size, we suggest that this is probably a chance finding and not likely to reflect diet-specific outcomes. An increased density of ghrelin-expressing cells is consistent with a recent report in rats, where the density of ghrelin immunopositive cells in the stomach mucosa is increased by ~10% at mid-pregnancy compared to non-pregnant animals, and increases by a further ~30% by the day of birth [683]. We also characterised ghrelin protein expression in the murine placenta. Cytoplasmic expression was primarily localised to the labyrinth (nutrient exchange) region, whilst nuclear expression was evident in both labyrinth and junctional (endocrine crosstalk) regions. This is the first report of ghrelin protein localisation in the rodent placenta, and its presence is consistent with prior reports of ghrelin mRNA expression in placenta of rats and humans [642].

Despite greater circulating total ghrelin in pregnancy, we did not detect greater circulating acyl-ghrelin in pregnant mice, with a lower acyl:total ghrelin ratio in pregnant

compared to non-pregnant mice, and lower circulating acyl-ghrelin at mid-pregnancy. The decreased ratio of acyl to total ghrelin we observed in mice are broadly consistent with those of Tham and co-authors in women, who reported markedly lower maternal acylated ghrelin concentrations during pregnancy than postpartum [681]. These authors suggested that lower acyl-ghrelin during human pregnancy probably reflected decreased acylation, since they found lower rather than higher activity of the key de-acylating enzyme butylcholinesterase in the pregnant group [681]. We observed comparatively lower gastric mRNA expression of *Mboat4* and *Pcsk1* in pregnant mice compared to non-pregnant mice at ZT23. Although the acylation of ghrelin is independent of processing by PC 1/3 [426], lower gastric gene expression of *Mboat4* at ZT23 may explain the lack of subsequent increase in circulating serum acyl-ghrelin concentrations despite higher total ghrelin concentrations in pregnant compared to non-pregnant mice sampled at ZT13. Further experiments are required to determine whether the lower acyl:total ghrelin ratio reflects slower acylation or more rapid deacylation in pregnancy. Despite the lack of elevation of circulating acyl-ghrelin in pregnancy, it is possible that the higher total ghrelin seen before substantial feeding might lead to increased local ghrelin action in tissues expressing both *Mboat4* and the ghrelin-receptor, such as the pancreas, placenta and hypothalamus [454, 679]. Consistent with a potential role for elevated ghrelin during pregnancy, ghrelin receptor mRNA expression increased in hypothalamus and pituitary during rat pregnancy [685]. It is also possible that non-acylated ghrelin has a functional role in pregnancy, since growing evidence suggests that des-acyl ghrelin has GHSR1a independent roles in energy and glucose metabolism [484, 679, 718]. We did not measure des-acyl ghrelin in the present study, but post-test meal concentrations of des-acyl (but not acyl) ghrelin were higher in women with gestational diabetes during late pregnancy and postpartum compared to normal pregnant women [681], suggesting it either regulates or responds to maternal metabolism.

Contrary to our hypothesis, dietary supplementation with octanoic acid did not alter circulating GH or ghrelin concentrations regardless of whether the mice had a full or empty stomach. Although we were able to confirm the presence of octanoic acid in our supplemented diet, we were not able to confirm its presence in the circulation, despite the fact that abundance of other circulating fatty acids were altered in response to the experimental diets. Similarly, Lemarié and co-authors could not detect C8 fatty acids in rat plasma in response to C8-supplementation with 0, 8 or 21% of total fatty acids [719]. This could possibly be due to rapid gastrointestinal hydrolysis and absorption of medium chain-fatty acids, specific transport through the portal vein and rapid beta-oxidation in the liver [720]. The lack of increase in acyl-ghrelin was surprising given that previous studies in mice found increased stomach acyl-ghrelin in male mice using similar dose regimens used in the present study [468]. Furthermore, dietary supplementation with octanoic acid increased circulating acyl-ghrelin concentrations in studies conducted in humans, mice and cattle [687, 689, 721]. However, in other studies, dietary supplementation with octanoic acid increased stomach acyl-ghrelin of mice [468] and stomach octanoic acid content in rats [719] without any changes in circulating acyl-ghrelin secretion. Interestingly, dietary supplementation with medium chain triglycerides (MCT) increased circulating acyl-ghrelin in humans and pigs of both sexes and circulating growth hormone concentrations in pigs [688, 722], suggesting a possible alternate dietary approach to increase circulating acyl-ghrelin abundance. In addition, we did not see any differences in circulating GH patterns in response to diet. Although the current study utilised the established method of characterising patterns of pulsatile GH secretion in mice [699], it was necessary to exclude data from two pregnant animals where pulsatile GH secretion was lost, indicative of an acute response. Pulsatile GH secretion was evident in the majority of mice, indicating that the acclimatisation and sampling protocol were mostly successful in preventing stress responses [703]. Loss of GH secretion in two pregnant mice but no non-pregnant mice on the present study, and from one late pregnant mouse and no non-, early- of mid-pregnant mice in our previous

study [123], suggests that pregnancy may increase susceptibility to handling-induced stress, despite extensive acclimatisation. Together with the need for consistency in the estrous cycle stage of non-pregnant mice, this resulted in a limited sampling size for GH measurements. Therefore, we cannot exclude the possibility of a diet effect on GH based only on the present study. Maternal and fetal weights did not differ with diet, which was not unexpected given that we did not anticipate any restriction of fetal growth in this model of normal pregnancy. Although mice ate both experimental diets readily, we cannot exclude the possibility that food intake may differ between diets and suggest this should be measured in subsequent studies. Future studies may find it beneficial to adapt a dietary approach based on dietary supplementation with medium chain triglycerides containing up to 65-75% of octanoic acid, which has been shown to increase circulating levels of acyl-ghrelin as well as average plasma GH levels in pigs [688].

Overall, increased total and basal GH during murine pregnancy does not appear to be regulated by maternal circulating acyl-ghrelin. Maternal total ghrelin secretion in pregnancy may follow an altered diurnal pattern, possibly due to the pregnancy-associated changes in nutrient demand and energy intake. A higher number of gastric ghrelin expressing cells in pregnant mice may explain the time-dependent higher concentrations of circulating total ghrelin compared to non-pregnant mice. Interestingly, despite elevated total ghrelin, acyl-ghrelin concentrations were not elevated in pregnancy or by dietary supplementation with octanoic acid at the concentrations used in the present study. Our results confirm upregulation of the GH-ghrelin axis in pregnancy, but the mechanisms underlying increases in circulating GH in murine pregnancy remain to be explained.

2.7 Additional information

2.7.1 *Competing interests*

The authors declare that they have no competing interests.

2.7.2 *Authors contributions*

H K, B S M, A J P, C T R and K L G conceived and designed the experiments. H K, B S M, P S S, A J P, H L, M N S, G S C, L H, R L W, J D V, C T R and K L G were involved in the acquisition of data. H K, B S M, A J P, H L, L H, J D V and K L G analysed and interpreted the data. H K and K L G drafted the article. H K, B S M, A J P, H L, L H, R L W, C C, C T R and K L G critically revised the article. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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2.7.4 *Acknowledgments*

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Chapter 3 Validation studies of a fluorescent method to measure placental glucose transport in mice

3.1 Overview

The work in this chapter corresponds to the second aim of this thesis. In this study, we developed and validated a fluorescence-based method to measure placental perfusion in mice using the IRDye 800CW 2-DG, a commercially available glucose analog designed to measure cellular GLUT transporter activity.

This chapter has been published in *Placenta* [723] and has been reproduced exactly as published, with the exception of formatting, which has been modified to be consistent with the rest of this thesis.

Statement of Authorship

Title of Paper	Validation studies of a fluorescent method to measure placental glucose transport in mice.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Kaur, H., Wilson, R.L., Care, A.S., Muhlhausler, B.S., Roberts, C.T., and Gatford, K.L., ***Validation studies of a fluorescent method to measure placental glucose transport in mice***. Placenta, 2019. 76: p. 23-29.

Principal Author

Name of Principal Author (Candidate)	Harleen Kaur		
Contribution to the Paper	Involved in acquisition, analysis and interpretation of data; drafted and critically revised the manuscript; approved of the final version of the manuscript		
Overall percentage (%)			
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	28.06.20

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

I. the candidate's stated contribution to the publication is accurate (as detailed above);

II. permission is granted for the candidate in include the publication in the thesis; and

III. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	11 June 2020

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3.2 Abstract

Proper placental function is essential for optimal fetal growth *in utero.* Placental transfer of nutrients to the fetus can be measured using radiolabelled tracers, but non-radioactive methods have potential advantages. This study aimed to develop a fluorescence-based method to measure placental glucose transport in mice. Time course and localisation of the IRDye 800CW 2-deoxyglucose were recorded (Lumina IVIS Live Imaging System) following tail vein injection into anaesthetised late pregnant mice. Fluorescent signals in placental and fetal tissues were assessed after injecting conscious dams with 10 nmol IRDye 800CW 2-deoxyglucose (3, 30, 60, 120 min) or vehicle. Specificity of dye uptake was determined by comparing uptake of IRDye 800CW conjugated to 2-deoxyglucose or carboxylate, at 2 and 24 h. Finally, we assessed relationships of fetal size and umbilical blood flow velocities with relative dye uptake. In late pregnant mice, uterine fluorescent signal localised rapidly over placentas and remained consistent for >1 h. Signal intensity in whole and homogenised tissues was increased in fetuses and decreased in placentas after 3 minutes and stabilised by 30 min post-injection. Relative fetal dye uptake at 2 and 24 h was greater in littermates with the highest compared to lowest placental efficiency; signals were similar for 2-deoxyglucose- or carboxylate-conjugated dyes. Relative fetal dye uptake correlated positively with fetal weight and placental efficiency and negatively with umbilical artery resistance indices. Fetal uptake of IRDye 800CW correlates with markers of placental blood flow and fetal growth, but does not specifically measure placental glucose transport.

3.3 Introduction

One of the major determinants of intrauterine growth is the placental supply of nutrients to the fetus, which occurs primarily by diffusion and transporter-mediated transfer [724]. The capacity of the placenta to facilitate this nutrient exchange depends on its size, morphology and blood flow as well as the abundance and activity of nutrient transporters [725, 726]. There is now considerable evidence that poor placental function is a major contributor to restricted fetal growth [150]. Although the placenta can adapt morphologically and functionally to fetal signals of nutrient demand [727-729], inadequate placental function can result in intrauterine growth restriction (IUGR) wherein the fetus fails to reach its genetically pre-determined growth potential [213, 730]. Low birthweight (LBW, <2.5 kg) and IUGR fetuses are at a higher risk of fetal mortality and morbidity, both perinatally and later in life [731-735].

One of the most widely used methods of assessing placental efficiency is the measurement of placental nutrient transporter expression and activity. Isolated placental vesicles from human pregnancies complicated by IUGR typically exhibit decreased placental activity of system A amino acid transporters [736]. While not differentially expressed in placentas from human IUGR and normally-grown fetuses [208], placental protein abundance of the facilitated glucose transporter solute carrier family 2 member 1 (Slc2a1, previously known as GLUT1) is lower in experimentally-induced rat IUGR pregnancies at late gestation compared to sham controls [737]. Conversely, in non-manipulated murine pregnancy, small placentas are able to support fetal growth by increasing the expression of nutrient transporters when compared with larger placentas [727]. Near term, at 19 days after mating (e19; GD19.5), although not at e16 (GD15.5), the lightest placentas had 20-40% higher expression of *Slc2a1* and the placental solute carrier family 38, member 2 (Slc38a2/SNAT2), an isoform of the System A amino acid transporter, compared to the heaviest placentas within the litter [727]. Compared with

the heaviest placenta, active transfer of [^{14}C] methyl aminoisobutyric acid (MeAIB) per gram of placenta was ~40-50% greater in the lightest placentas of the litter, at both e16 and e19, when mouse fetuses are growing most rapidly in absolute terms [727]. However, despite greater transported expression of *Slc2a1*, materno-fetal transfer of [^{14}C] glucose per gram placenta did not differ between the lightest and heaviest placentas in the litter [727]. Along with alterations in placental morphology, such changes in placental function are considered to be adaptations that aim to match nutrient delivery to fetal demand. As a result of these alterations, around 30% more fetus is produced per gram of placenta by the lightest than the heaviest placenta in the litter in a normal mouse litter [727]. Consequently, the fetal:placental weight (FW:PW) ratio is used as a marker of placental efficiency [727, 738, 739].

While measuring protein levels can provide an indication of transport capacity, direct assessment of nutrient transfer requires the use of labelled substrates. Alterations in the passive and active placental transport of solutes in experimental models of IUGR has been demonstrated by quantifying the maternofetal transfer of radiolabelled tracers including [^{14}C] inulin, [^{3}H] methyl-D-glucose (MG) and [^{14}C] amino-isobutyric acid (AIB) [93, 661, 729]. An alternative approach to this use of radiolabelled substrates is to use fluorescently labelled compounds. These offer a number of potential advantages over radioisotopes, including fewer constraints on handling and disposal, and the availability of many emission spectra and therefore ability to measure greater numbers of substrates simultaneously [740]. Additionally, live course imaging can be performed by tracking the movement of the label in real-time as well as obtaining better signal localisation and stability [741]. One such commercially available fluorescently labelled substrate is IRDye 800CW 2-DG Optical Probe (LI-COR Biosciences, Lincoln, NE, Lincoln, NE). The fluorescent dye is conjugated to 2-deoxyglucose (2-DG), which is a non-metabolisable glucose analogue that remains within cells after phosphorylation [742]. Uptake of IRDye 800CW 2-DG in human tumour cell lines with high and low metabolic rates and in mouse

adipocytes is dose-dependent and can be blocked by incubation with unlabelled glucose, 2-DG or monoclonal antibodies raised against Slc2a1 [743]. This probe has been previously used to study *in vitro* and *in vivo* glucose uptake in cancer research, including in rodents [743, 744]. To date, there are no non-radioactive methods available to study placental transport. Therefore, the current study aimed to develop and validate a fluorescence-based method to measure placental glucose transport in mice using the IRDye 800CW 2-DG.

3.4 Methods

3.4.1 *Ethics and animal management*

Experiments One and Two: Experimental procedures were approved by the University of Adelaide Animal Ethics Committee (M-2014-168) and carried out in accordance with the Australian code of practice for the care and use of animals for scientific purposes [745]. C57BL/6J virgin female mice aged 10 weeks were obtained from the Animal Resource Centre, Perth and housed at ~23°C with 12 h:12 h light:dark lighting cycle (lights on 06:00 h). Access to water and meat-free rat and mouse diet (14.0 MJ/kg, 20% protein, Speciality Feeds, Glen Forrest, Australia) was *ad libitum* and mice were weighed daily throughout the experiment. Estrous cycle stages were confirmed by daily monitoring of vaginal cell types collected by gently flushing the vagina with 10 µL of saline [698]. To generate timed pregnancies, females in either proestrus or early estrus were placed overnight with a mature adult CBAF1 male. Pregnancy was determined by the presence of a vaginal plug (day 0.5 after mating, GD0.5) and/or weight gain, and confirmed at post-mortem.

Experiment Three: CBAF1 virgin female mice aged 10-12 weeks were obtained from the Animal Resource Centre, Perth and housed at ~23°C on a 12 h:12 h light:dark lighting

cycle (lights on 0700 *h*), with *ad libitum* access to Teklad global soy protein-free extruded rodent diet (irradiated; 13 MJ/kg, 18.4% protein, Envigo, Huntingdon, UK). Mice were weighed daily throughout the experiment. Timed pregnancies were generated and confirmed as described above, with commencement of breeding delayed until ~17-20 weeks of age due to a shift of animal facilities.

Experiment Four: CBAF1 virgin female mice aged 10-12 weeks were obtained from the Animal Resource Centre, Perth and housed and bred under the same conditions as those described above for experiment three.

3.4.2 *Experiment 1 – Live imaged time course*

In order to confirm our ability to detect fluorescent signals over the conceptus regions and the timeframe for the appearance of signals, pregnant mice aged 15-18 weeks at GD17.5 were anaesthetised by intra-peritoneal injection of 20 mg/mL Avertin (2,2,2-tribromoethanol and tert-amyl alcohol; Sigma-Aldrich, Missouri, United States) immediately after being injected with 10 or 20 nmol of fluorescently-labelled 2-DG (LI-COR Biosciences) dye (n=2/dose) diluted in a total volume of 50-100 µL phosphate buffered saline (PBS), intravenously into the tail vein. This was followed by uterine exteriorisation to allow serial live imaging at 3-5 minute intervals under isofluorane anaesthesia for approximately 1 h post-injection using the IVIS imaging system (wavelengths: Ex 760 nm/Ex 790 nm, IVIS Lumina XRMS system, Perkin Elmer). An additional animal was imaged without dye injection to confirm the absence of non-specific signal. Dams were then humanely killed by cervical dislocation. Fluorescent emission signals in a region encompassing the middle conceptus of each horn were measured in each animal throughout each time course. Background signal was measured in an area adjacent to each mouse and was subtracted from the signal measured over the conceptus.

3.4.3 *Experiment 2 – Time course of placental and fetal dye uptake in conscious mice*

In order to determine the time course for placental and fetal uptake of IRDye 800CW 2-DG following maternal tail-vein injection, 16-25 week old dams pregnant at GD17.5 were injected with 10 nmol of IRDye 800CW 2-DG in 50 µL PBS (intravenous, tail vein injection). Injected mice were returned to their home cage for 3, 30, 60 or 120 min (n=2-3/group) before being anaesthetised with Avertin and humanely killed by cervical dislocation. Individual fetuses and placentas were dissected, weighed and imaged using the IVIS imaging system, using settings as described above. Background signal measured in an area adjacent to each tissue was subtracted from tissue fluorescent signals. Immediately after being imaged, all fetuses were snap-frozen in liquid nitrogen, while placentas were alternately snap-frozen or fixed in 4% paraformaldehyde with 2.5% polyvinylpyrrolidone-40 in 70 mM phosphate buffer for 24 h. Samples were subsequently washed in four changes of 1 X PBS over 48 h, incubated in 30% sucrose solution for 2 h before being embedded in Optimum Cutting Temperature compound (OCT, Tissue-Tek, Sakura Finetek Japan Co. Ltd., Tokyo, Japan).

To develop a method to measure fluorescent signals in stored tissues, frozen placentas and fetuses were homogenised in radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris (pH 8.0), 150 mM NaCl, 1% octylphenoxypolyethoxyethanol (IGEPAL), 0.5% sodium deoxycholate, 5 mM Ethylenediaminetetraacetic acid (EDTA) and 0.1% sodium dodecyl sulfate) using the PowerLyzer 24 (Mo Bio Laboratories, Inc. Carlsbad, CA). The placentas were homogenised for 3 cycles of 15 seconds duration each, at 3500 rpm with pause/dwell time of 30 seconds. The fetuses were homogenised using the same settings, except that the cycle number was increased to 10. For each sample, 50 µL of homogenate and RIPA buffer was pipetted in triplicate into an opaque 96-well plate, before being imaged with the Odyssey CLx Infrared Imaging System (LI-COR

Biosciences, Lincoln, NE) using the 800 nm channel at 42 μm resolution and a 4.0 mm focus offset. Background values were measured in wells containing only RIPA buffer. Images were analysed using Image Studio™ Lite (LI-COR Biosciences, Lincoln, NE) image processing software. The triplicate fluorescence emission values (minus background values) from each respective fetus and placenta were averaged and expressed as signal intensity per fetus or placenta. Fetal signals were also expressed relative to placental weight. Relationships between signals obtained using the IVIS imaging system on whole tissues and signals obtained using the Odyssey CLx Infrared Imaging System on homogenised tissues were evaluated by Pearson's correlation.

3.4.4 *Experiment 3 – Specificity of dye uptake*

In order to test the specificity of dye uptake, we compared fetal and placental fluorescent signals after maternal tail vein injection with 0.2 nmol/g body weight of IRDye 800CW 2-DG or IRDye 800CW carboxylate dye (LI-COR Biosciences, Lincoln, NE) in PBS (total volume 50-100 μL) in mice aged 20-21 weeks at GD17.5-GD18.5 of pregnancy. IRDye 800CW carboxylate contains the fluorophore conjugated to carboxylate and is by the manufacturer as a negative control to the IRDye 800CW 2-DG [741, 746]. When injected into tumour-bearing mice, fluorescent signals 18-72 hours post-injection were ~3-fold higher for IRDye 800CW 2-DG than for IRDye 800CW carboxylate, and IRDye 800CW carboxylate is completely cleared from non-tumour-bearing mice within 48 h post-injection [746]. Dams were humanely killed at 2 h post-maternal injection (n=3/group), based on the results of our previous experiments, and at 24 h post-maternal injection (2-DG: n=6; carboxylate: n=4), based on data collection times used in previous *in vivo* studies using this probe [746]. Fetal and placental tissues were dissected, weighed and snap-frozen for subsequent homogenisation and tissue from the fetuses with the highest and lowest placental efficiency (FW:PW ratio) of each litter was imaging using the Odyssey CLx Infrared Imaging System as described above. Data from fetuses within a

litter were treated as repeated measures of the dam. A repeated measures ANOVA was performed to compare fetal dye uptake (fetal fluorescent signal per gram placenta) between dyes and between the littermates with the highest and lowest placental efficiency of each litter (within litter factor), separately for litters collected at 2 and 24 hours.

3.4.5 *Experiment 4 – Relationship between fetal dye uptake and fetal blood flow*

To assess the relationship between fetal dye uptake and indices of fetal blood supply, CBAF1 dams at 14-18 weeks of age were injected (tail vein) with 0.2 nmol/g body weight of IRDye 800CW 2-DG at GD17.5. Approximately 24 h after maternal-dye injection, umbilical artery blood flow velocities were assessed by Doppler ultrasound, using the Vevo 3100 Ultrasound Biomicroscope (FUJIFILM VisualSonics, Toronto, Ontario, Canada) with a 32-55 MHz transducer probe, as described previously [747]. Mice were anaesthetized with isoflurane (5% induction, 1.5% maintenance, in medical air). Doppler ultrasound recordings were taken from the umbilical arteries of at least two, and where possible, four fetuses per dam. Recordings were analysed for peak systolic velocity (PSV), time-averaged velocity (TAV), end diastolic velocity (EDV), and fetal heart rate from 3 consecutive cardiac cycles (Figure 3.1) [748]. Resistance index (RI = [PSV − EDV]/PSV) and pulsatility index (PI = [PSV − EDV]/time averaged velocity [TAV]) were calculated for each fetus. Following Doppler imaging, dams were humanely killed by cervical dislocation, and fetuses and placentas dissected and weighed. Fetal size (abdominal circumference, crown to rump length, and head width) was also measured, prior to snap-freezing of fetal and placental tissues for subsequent homogenisation and imaging using the Odyssey CLx Infrared Imaging System as described above. Relationships of fetal dye uptake (fetal signal per gram placenta) with umbilical blood flow parameters and fetal weight and size were assessed by Pearson's Correlation analysis using data for each individual fetus.

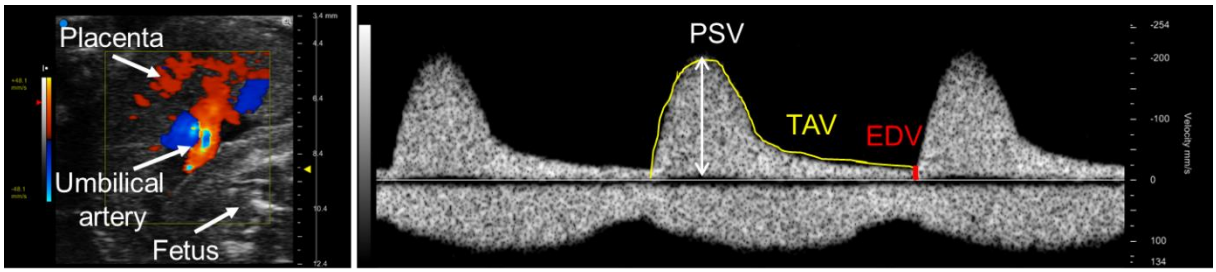

Figure 3.1 *Umbilical blood flow characteristics at GD18.5 in naturally-mated CBAF1 dams were characterised for 3-4 individual fetuses per litter.*

3.5 Results

3.5.1 *Experiment 1 – Live imaged time course*

In the uterine region of anaesthetised GD17.5 pregnant C57Bl6/J mice, fluorescent signal localised rapidly to the placenta after maternal tail vein injection with IRDye 800CW 2-DG (Figure 3.2). Fluorescent signals could be readily detected in the conceptus regions of mice injected with 10 or 20 nmol dye and signal intensity remained stable in individual animals for > 1 h (Supplementary Figure 3.1).

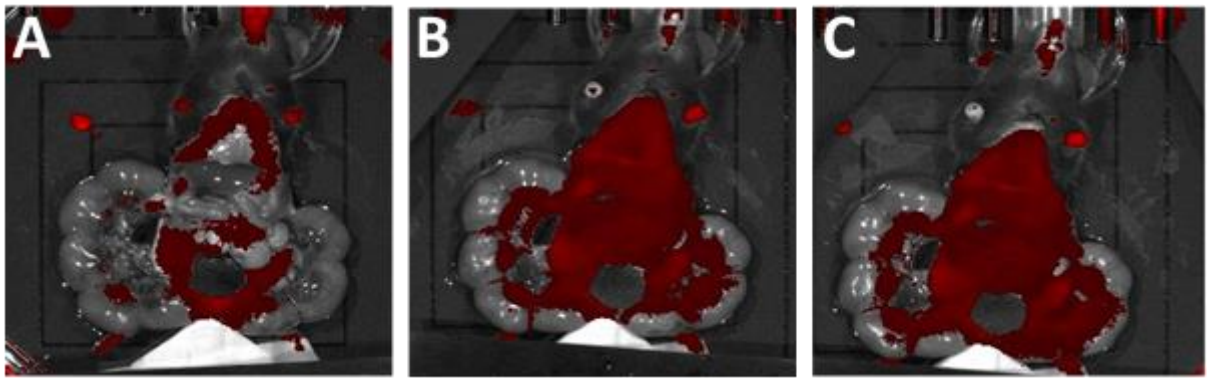

Figure 3.2 *Fluorescent signal detected by the Lumina IVIS Live Imaging System (ex 760 nm/em 790 nm) in anaesthetised C57Bl6/J mice on day GD17.5 of pregnancy at 8 (A), 38 (B) and 68 (C) min after tail vein injection with IR Dye 800CW 2-DG.*

3.5.2 *Experiment 2 – Time course of experimental and fetal dye uptake in conscious mice*

Signal intensity from whole fetuses by IVIS imaging was initially low and placental signals were high at 3 min and then remained stable between 30 and 120 min following maternal tail vein injection (Figures 3.3A and 3.3B). Fluorescence emission measured in homogenised fetuses and placentas followed a similar pattern over time (Figures 3.3C and 3.3D). Fluorescence signals obtained from the same tissues using the two approaches were strongly positively correlated (fetal tissues: P<0.001; R=0.662; placental tissues: P<0.001; R=0.813).

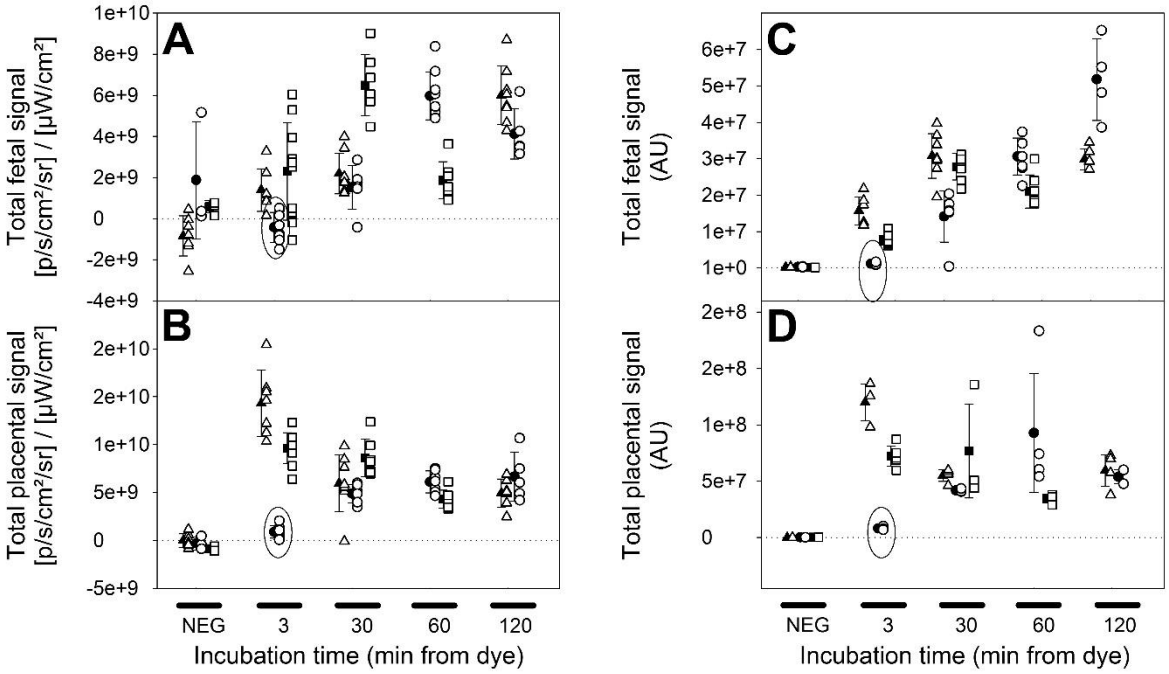

Figure 3.3 *Fluorescent signal was measured by IVIS in whole fetuses (A) and placentas (B) and in homogenised fetuses (C) and placentas (D) by Odyssey CLx (B). At GD17.5 of pregnancy, C57Bl6/J mice were injected with 10 nmol IR Dye 800CW 2-DG via the tail vein and humanely killed at 3 to 120 minutes after injection. Each cluster of symbols represents a separate litter. Filled symbols show litter averages, unfilled symbols show individual fetal and placental data within each litter and the black oval indicates a litter where dye was inadvertently injected into tail sheath (n=2-3 dams and litters for each time point). NEG indicates control animals injected with PBS. Placental fluorescence data from Odyssey measures (D) was only available for frozen placentas from each litter.*

3.5.3 *Experiment 3 – Specificity of dye uptake*

Fluorescent signals were higher in the highest compared to lowest placental efficiency littermates regardless of dye type at 2 h (Figure 3.4A) and 24 h post-maternal injection (Figure 3.4B). Signal intensities were similar in fetuses from dams injected with 2-DG- or carboxylate-conjugated IRDye 800CW at each time point (Figures 3.4A and 3.4B). Differences in signal intensity between lowest and highest placental efficiency littermates did not differ between dye types at either time point (Figures 3.4A and 3.4B).

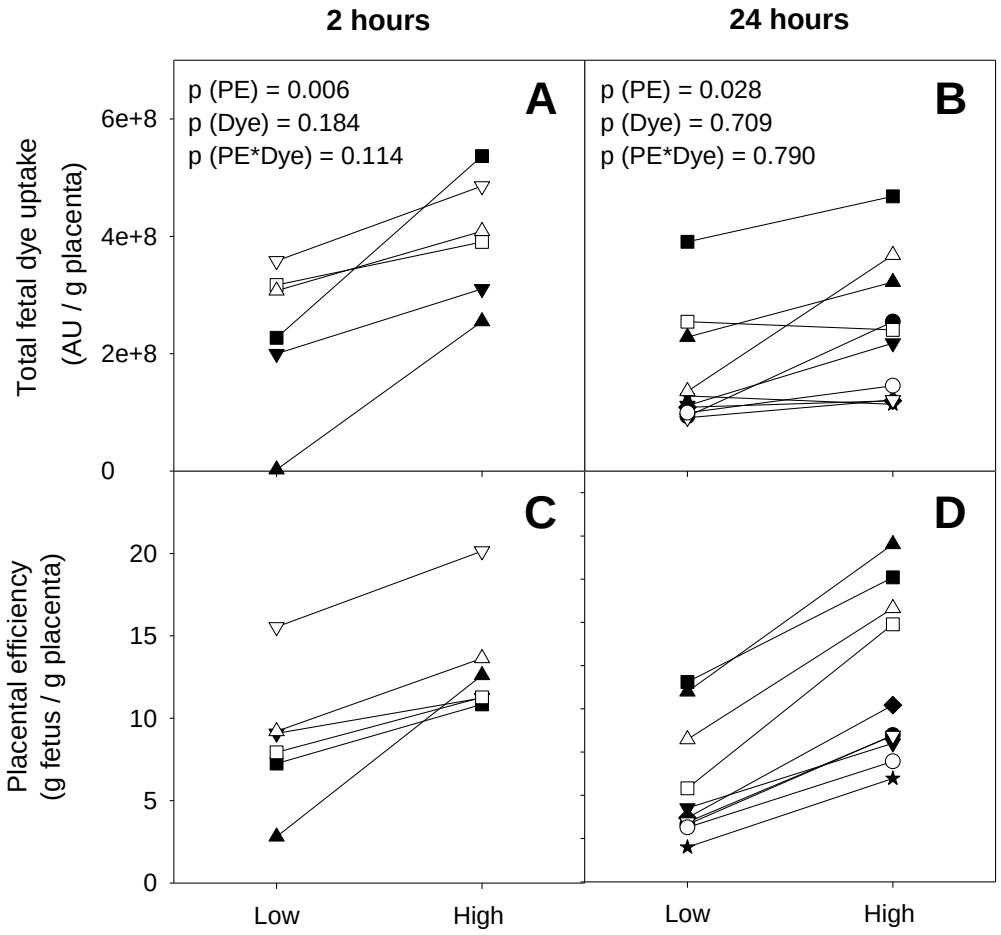

Figure 3.4 *Fluorescent signals (A, B) measured by Odyssey CLx Infrared Imaging System (800 nm channel), in fetuses (from CBAF1 dams at GD17.5-18.5) that had highest and lowest placental efficiency (PE; defined as FW:PW ratio) within the litter. Dams were tail vein-injected with IRDye 800CW 2-DG (black symbols) or IRDye 800CW carboxylate negative control dye (white symbols), and fetuses collected either 2 h (A, C) or 24 h (B, D) post-maternal injection. Panels C and D show corresponding data for placental efficiency – the pairs of littermates on panels A and C and panels B and D share the same symbols. Results are presented as individual data, littermates are linked by solid lines.*

3.5.4 *Experiment 4 – Relationship between fetal dye uptake and fetal blood flow*

Fetal dye signal relative to placental weight correlated positively with fetal weight (Figure 3.5A) and FW:PW ratio (Figure 3.5B), fetal head width (P<0.001, R=0.533) abdominal circumference (R=0.514, P<0.001) and crown to rump length (P<0.001, R=0.582), and negatively with umbilical pulsatility indices (Figure 3.5C) and umbilical resistance (Figure 3.5D) at GD18.5 of gestation. Additionally, relative fetal dye uptake correlated positively with umbilical end-diastolic velocity (P=0.005, R=0.475) and fetal heart rate (P=0.003, R=0.515), but was not correlated with peak systolic velocity (P=0.112, R=0.282).

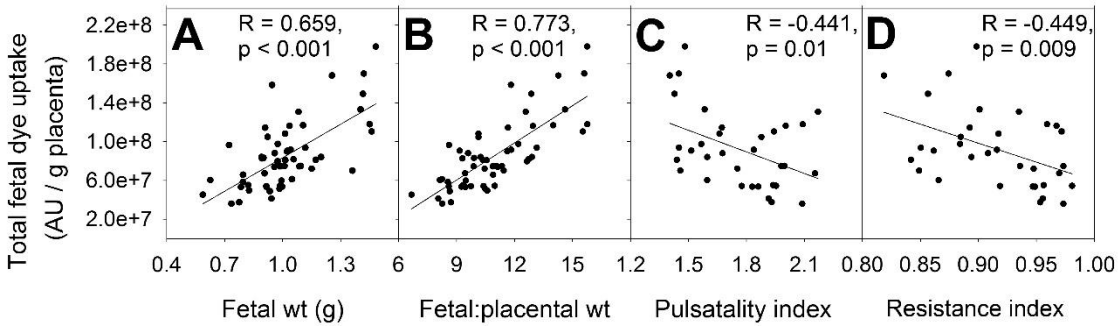

Figure 3.5 *Correlations between fetal dye signal per gram of placental weight and fetal weight (A), fetal:placental weight ratio (B), and umbilical artery pulsatility (C) and resistance (D) indices. Symbols show data from individual fetuses derived from nine independent litters; fetal and placental weight data was available for all 56 viable fetuses and umbilical artery Doppler ultrasound data was obtained in 33 fetuses.*

3.6 Discussion

This is the first study to test the use of a fluorescently-labelled dye for measuring placental nutrient transport. As originally aimed, we developed a method to quantify feto-placental uptake of the near-infrared IRDye 800CW 2-DG dye in term mice. Consistent with reported within-litter differences in uptake of radiolabelled glucose in mice [93, 727], fetal uptake of IRDye 800CW 2-DG relative to placental weight was greater in littermates

with high compared to low placental efficiency, and correlated positively with fetal size. Fetal uptake of dye relative to placental weight also correlated negatively with markers of impaired placental function, including umbilical blood flow resistance indices. Although these data suggested that uptake of IRDye 800CW 2-DG provides a proxy measure of placental function, signals were similar whether the fluorophore was conjugated with carboxylate (negative control) or glucose, implying that uptake is at least in part via other mechanisms than glucose transporters.

Placental efficiency, commonly defined as the ratio of fetal to placenta weight (FW:PW ratio), acts as a proxy measure of placental function up to that stage of gestation [727, 738, 739]. The characteristics of a highly efficient placenta include a large surface area for exchange and appropriate expression and activity of nutrient transporting proteins to ensure optimum nutrient delivery to the fetus [306, 725, 738]. In the present study, we observed differences in fetal uptake of IRDye 800CW 2-DG based on the relative placental efficiency of littermates within near-term mouse litters. Consistent with the results of radioisotope tracer studies [93, 727], the uptake of the 2-DG dye was significantly higher in the fetuses with the highest compared to the lowest placental efficiencies within the litter. Furthermore, we found that fetal accumulation of the dye relative to its placental weight, was positively correlated with fetal biometric parameters at birth, including birthweight, head width, abdominal circumference and crown to rump length. Together, these data imply that the uptake of the fluorescent 2-DG dye provides an indirect measure of nutrient delivery to the fetus. Ultrasonography is often used in clinical practice to monitor placental function by assessing the resistance to blood flow in the feto-placental unit. In normal pregnancies, low impedance in the umbilical artery allows continuous and stable forward flow throughout the cardiac cycle [749]. Maternal, placental or environmental conditions that negatively influence uterine blood flow are often detected by abnormal umbilical artery Doppler velocimetry, and are associated with pregnancy complications including IUGR and preeclampsia [750, 751]. In these

complications, fetal delivery of nutrients that cross the placenta by diffusion is often reduced due to low uterine blood flow [752, 753]. Hence, as part of the current study, we recorded and analysed fetal umbilical arterial waveforms in late gestation (GD18.5) in our final experiment. Even though our investigations focussed on normal mouse pregnancy, and did not include studies of experimental IUGR, we found that lower resistance indices in the feto-placental unit were associated with higher fetal uptake of IRDye 800CW 2-DG per gram of placenta.

Although IRDye 800CW 2-DG uptake was greater in high than low placental efficiency littermates and correlated positively with markers of fetal growth and negatively with umbilical resistance indices, total and differential uptake of the fluorophore were similar regardless of whether it was conjugated to 2-DG or carboxylate. This was unexpected, since in non-pregnant mice, uptake and retention of IRDye 800CW 2-DG 24 h post-injection is ~3–4-fold higher than that of IRDye 800CW carboxylate in kidneys, muscle and liver [743]. Similarly, uptake and retention of these dyes differ in murine cancer. In mice with unilateral brain tumours, fluorescent signals imaged in anaesthetised animals 24 h after injection with IRDye 800CW 2-DG were also ~3-fold greater in the tumour region than in the contralateral brain region, thought to reflect higher glucose metabolic demand within the tumour [741]. Fluorescent signals after injection with IRDye 800CW carboxylate were ~3-fold lower than those in mice injected with the 2-DG-conjugated dye, and were not different in tumour and non-tumour sides of the brain [741]. In our initial live imaging studies, we observed high fluorescence intensity in the bladder area of dams injected with the 2-DG dye (data not shown), suggesting rapid renal clearance. Although the renal threshold for glucose in mice has not been clearly stated in literature, the available data suggest that in rodents, glucose excretion through urine only occurs at concentrations >22.2 mmol/L (400 mg/dL) [754]. The maximum amount of the dye injected to dams in this study was 20 nmol, which in a blood volume of approximately 2 mL in a heavily pregnant 35 g mouse should increase circulating glucose by

approximately 0.01 mmol/L. Therefore the dye administration itself is not expected to have caused the mice to reach the renal clearance threshold. When measuring the tissue distribution of the IRDye 800CW carboxylate in male and female rats, the highest degree of uptake occurred in the kidneys, regardless of sex [755], also suggesting renal clearance of the fluorophore. In the same study, uptake of the IRDye 800CW carboxylate was markedly higher in the ovaries compared to the testes [755]. SLC2A1 transporter-dependent uptake of IRDye 800CW 2-DG has been previously validated *in vitro*, where it can be inhibited by addition of antibodies raised against SLC2A1, as well as by addition of unlabelled glucose or 2-DG [743]. This is interesting, given that IRDye 800CW 2-DG is estimated to be too large to pass through the predicted three-dimensional model of the SLC2A1 channel [756]. Given these data, and the findings of the current study, it is possible that IRDye 800CW 2-DG is taken up by transporters that are not glucose-specific, and that are co-expressed in the kidneys and the female reproductive tract in rodents. However, further cell assays will need to be performed in order to investigate the mechanisms involved in the cellular uptake of the IRDye, including the placenta. Confirmation that placental IRDye 800CW 2-DG transport is glucose transporter-independent requires inhibition studies using unlabelled glucose or 2-DG or antibodies to glucose transporters in placental cells or cell lines, similar to previous studies in tumour cells [743].

In conclusion, the lack of differential uptake between 2-DG- and carboxylate-conjugated dyes indicate that the IRDye 800CW 2-DG is not able to specifically measure placental glucose uptake via glucose transporters in mice. The use of radio-labelled tracers therefore remains the optimal method to measure placental transport of specific nutrients. However, in term mice, IRDye 800CW 2-DG signals can differentiate between littermates of high and low placental efficiencies, and its relative fetal uptake correlates positively with fetal growth parameters and negatively with markers of impaired placental function. This establishes a fluorescence-based method to indirectly

measure overall placental function and nutrient supply to the fetus in murine pregnancy.

3.7 Additional information

3.7.1 *Acknowledgements*

We thank FUJIFILM VisualSonics Inc. for loan of a VEVO 3100 ultrasound. We would also like to acknowledge the assistance of Adelaide Microscopy for data generation and image analysis.

3.7.2 *Funding*

This study was supported by funding from the Channel 7 Children's Research Foundation (project ID 171401). HK is supported by an Australian Government Research Training Program PhD scholarship. BSM is supported by a Career Development Award (APP:1038009) and ASC is supported by an Early Career Fellowship (APP:1092191) from the National Health and Medical Research Council of Australia. CTR is supported by the Lloyd Cox Professorial Research Fellowship from University of Adelaide. Financial support for the purchase of the VEVO 3100 ultrasound was provided by the Ian Potter Foundation (project 20190089).

3.7.3 *Disclosures*

The authors declare no conflicts of interest.

3.7.4 *Supplementary information*

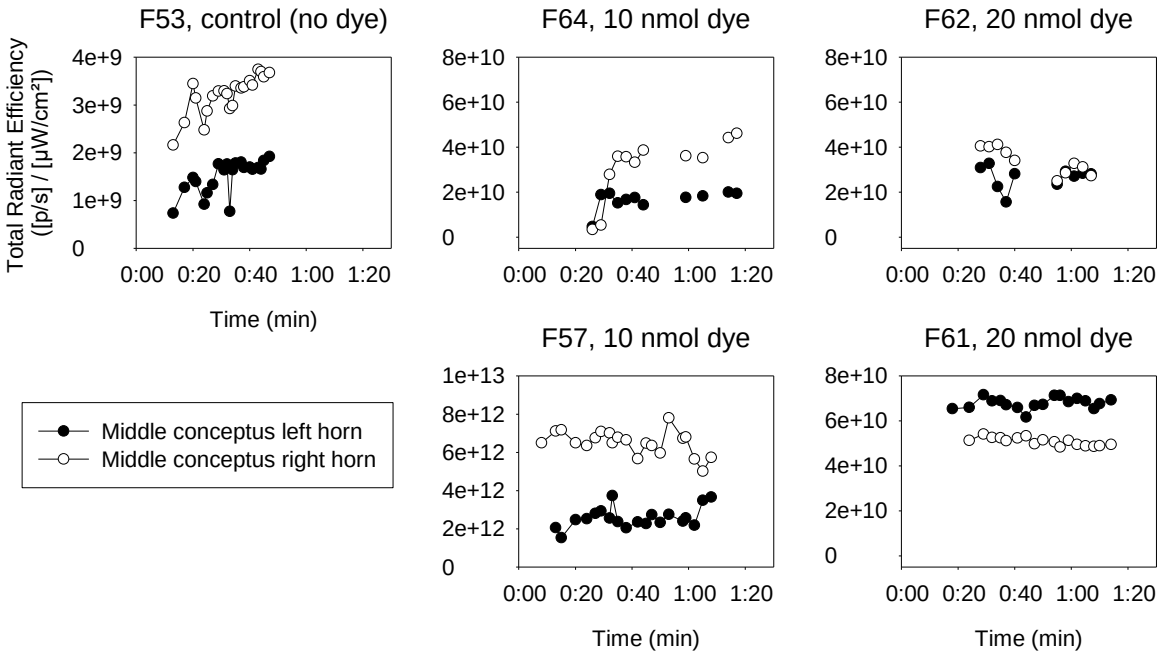

Figure 3.6 (Supplementary) *Fluorescent signal detected by the Lumina IVIS Live Imaging System (ex 760 nm/em 790 nm) in individual anaesthetised C57Bl6/J mice on day GD17.5 of pregnancy after tail vein injection with IR Dye 800CW 2-DG. Y axis shows fluorescence signal measured in the regions over the middle left and middle right conceptus of each uterine horn. X axis shows minutes after dye injection. All data points for each animal were taken under the same imaging settings (missing points for animals F62 and F64 were from images taken at different settings or reflect technical problems). Due to varying imaging settings used for different animals during these pilot studies, signal intensity should not be directly compared between animals.*

Chapter 4 A sexually-dimorphic murine model of IUGR induced by embryo transfer

4.1 Overview

The work described in this chapter corresponds to the final aim of this thesis. In this study, we established and characterised a model of variable fetal constraint due to increasing litter size induced through embryo transfer, in which to test intervention strategies to prevent or treat IUGR in multiple pregnancies. We also assessed whether responses to increasing litter size in our experimental model were sex-specific.

This chapter has been submitted to *Reproduction* (manuscript number: REP-20-0209) and a revised version is currently under review. The chapter included in this thesis is the resubmitted version, with additional edits made in response to suggestions of the thesis examiners.

Statement of Authorship

Title of Paper	A sexually-dimorphic murine model of IUGR induced by embryo transfer
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Authors: Harleen Kaur, Alison S Care, Rebecca L Wilson, Sandra G Piltz, Paul Q Thomas, Beverly S Muhlhausler, Claire T Roberts, Kathryn L Gatford Submitted to *Reproduction* on 15th April 2020

Principal Author

Name of Principal Author (Candidate)	Harleen Kaur		
Contribution to the Paper	Involved in acquisition, analysis and interpretation of data; drafted and critically revised the manuscript; approved of the final version of the manuscript		
Overall percentage (%)			
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	28.06.20

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Alison S Care		
Contribution to the Paper	Conceived and designed the experiments; was involved in acquisition, analysis and interpretation of data; critically revised the manuscript; approved of the final version of the manuscript		
Signature		Date	12/06/20

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Name of Co-Author	Rebecca L Wilson		
Contribution to the Paper	Involved in acquisition, analysis and interpretation of data; critically revised the manuscript; approved of the final version of the manuscript		
Signature		Date	

Please cut and paste additional co-author panels here as required.

Name of Co-Author	Sandra G Piltz		
Contribution to the Paper	Involved in acquisition of data, critically revised the manuscript; approved of the final version of the manuscript		
Signature		Date 16/06/20	

Name of Co-Author	Paul Q Thomas		
Contribution to the Paper	Conceived and designed the experiments; approved of the final version of the manuscript		
Signature		Date	16.6.20

Name of Co-Author	Beverly S Muhlhausler		
Contribution to the Paper	Conceived and designed the experiments; was involved in analysis and interpretation of data; critically revised the manuscript; approved of the final version of the manuscript		
Signature		Date	11/06/2020

Name of Co-Author	Claire T Roberts

2623

Contribution to the Paper	Conceived and designed the experiments; was involved in analysis and interpretation of data; critically revised the manuscript; approved of the final version of the manuscript		
Signature		Date	11.6.2020

Name of Co-Author	Kathryn L Gatford		
Contribution to the Paper	Conceived and designed the experiments; was involved in acquisition, analysis and interpretation of data; drafted and critically revised the manuscript; approved of the final version of the manuscript		
Signature		Date	11 June 2020

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4.2 Abstract

Animal models are needed to develop interventions to prevent or treat intrauterine growth restriction (IUGR). Fetal growth rates and effects of *in utero* exposures differ between sexes, but little is known about sex-specific effects of increasing litter size. We established a murine IUGR model using pregnancies generated by multiple embryo transfers, and evaluated sex-specific responses to increasing litter size. CBAF1 embryos were collected at gestational day 0.5 (GD0.5) and 6, 8, 10 or 12 embryos were transferred into each uterine horn of pseudo-pregnant female CD1 mice (n=32). Fetal and placental outcomes were measured at GD18.5. In the main experiment fetuses were genotyped (*Sry*) for analysis of sex-specific outcomes. The number of implantation sites (P=0.004, P=0.003, respectively) and litter size (P=0.058, P=0.001, respectively) correlated positively with number of embryos transferred, while placental weight correlated negatively with litter size (both P<0.01). The relationship between viable litter size and fetal weight differed between sexes (interaction P=0.002), such that fetal weights of males (P=0.002), but not females (P=0.233), correlated negatively with litter size. Placental weight decreased with increasing litter size (P<0.001) and was lower in females than males (P=0.020). Our results suggest that male fetuses grow as fast as permitted by nutrient supply, whereas the female maintains placental reserve capacity. This strategy reflecting sex-specific gene expression is likely to place the male fetus at greater risk of death in the event of a “second hit”.

4.3 Introduction

Intrauterine growth restriction (IUGR), where a suboptimal fetal environment prevents the fetus from attaining its genetically determined growth potential, affects 6-12% of pregnancies in developed countries [150]. Babies born from IUGR pregnancies often have a low birth weight (LBW, <2.5 kg), although this can also result from prematurity without IUGR [152]. In developed countries, one the most common causes of IUGR is placental insufficiency [150]. This typically affects the fetus in the later stages of gestation (>32 weeks) and results in an asymmetric reduction in fetal biometric parameters like abdominal circumference, while the growth of the fetal brain is relatively preserved [757]. LBW is associated with increased risks of both neonatal complications, including still birth, and persistent impacts on health, including ~20-30% increased risks for cardio-metabolic diseases [153, 154, 188, 263]. There is currently no cure or prevention for IUGR and robust animal models are needed to develop and test interventions.

In humans, the risk of LBW increases in multiple births; 5.2% of singletons, 55% of twins and 99% of higher-order multiples in Australia were LBW in 2017 [188]. In mice, as in other litter-bearing species such as pig, placental and fetal weights decrease as litter size increases [378-381]. Seminal studies demonstrated restricted fetal growth due to maternal constraint in the context of cross-breeding of horses [758] and, embryo transfer experiments in horses [759, 760] and pigs [761] that remove confounding effects of imprinting. More recently, the embryo transfer technique was also used in rabbits to demonstrate that fetal size is larger in smaller litters [762]. These studies clearly show that fetal growth is limited by maternal size, despite evidence of some compensatory adaptations to placental size and structure [759]. The mechanisms for constrained fetal growth due to increasing litter size is likely to differ somewhat from those seen in singleton pregnancies. However, larger litters are subject to both

reduced placental growth and function and competition for maternal nutrients, since reducing litter size from twin to singleton post-implantation only partially restores fetal growth in sheep [386].

Litter size increases as a greater number of embryos are transferred to pseudo-pregnant mice [763], producing a range of litter sizes independent of these maternal confounders, although fetal and placental growth have not been reported in this model Our primary aim was therefore to establish and characterise a model of variable fetal constraint due to increasing litter size induced through embryo transfer. In human pregnancies, median weights of male fetuses are higher than female fetuses at all gestational ages up to term [36, 38]. Their rapid growth strategy is believed to put male infants at a higher risk of preterm birth and neonatal mortality compared to female infants, and fetal and placental responses to an adverse environment also differ between sexes in humans and experimental animal models [54, 55, 764-766]. Therefore, our secondary aim was to assess sex-specific responses to increasing litter size in our experimental model. We hypothesised that within our model, as litter size increased, there would be a decrease in fetal and placental weights. Furthermore, we expected the degree of growth restriction to be more pronounced in male compared to female fetuses near term.

4.4 Materials and methods

4.4.1 *Ethics and experimental design*

Experimental procedures were approved by the University of Adelaide Animal Ethics Committee (M-2016-186) and carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes [697]. Mice used in this study were housed at ~23 °C with 12-h:12-h light:dark cycle (lights on 06:00 h),

with *ad libitum* access to water and meat-free rat and mouse diet (14.0 MJ/kg, 20% protein, Speciality Feeds, Glen Forrest, Australia).

4.4.2 *Animal experiments*

Fetal and placental outcomes were assessed in recipient dams that received between 12 and 24 embryos to induce variable litter sizes (Figure 4.1). Virgin female CBAF1 mice at 21-25 d old were purchased from the University of Adelaide breeding colony. Mice aged approximately 4-5 weeks old were super-ovulated by intraperitoneal (i.p.) injection of pregnant mare's serum gonadotropin (Folligon, 5 IU; Intervet Australia, Victoria, Australia), followed 46-48 h later by an i.p. injection of human chorionic gonadotropin (hCG, Chorulon, 5 IU, Intervet Australia) to induce ovulation. These mice were then housed 1:1 with proven fertile CBAF1 males, and 1-cell embryos were collected by humanely killing the mouse and flushing the tract, 23 h after hCG injection. Embryos were vitrified, thawed and transferred to pseudo-pregnant CD1 females [767] in the South Australian Health and Medical Research Institute (SAHMRI) facility. All transfers were conducted by the same operator.

To induce pseudo-pregnancy, mature (7-10 week old) CD1 female mice purchased from SAHMRI breeding colony (selected due to their availability as well as the wide use of this strain as recipients in embryo transfer protocols) were mated at estrus to vasectomised male CBAF1 mice by housing two females with a male overnight. The following morning, females with a vaginal plug were anaesthetised using gas anaesthesia (isofluorane, 2% O_2 at 2 L per minute) and analgesia (single dose of 0.1 mL Buprenorphine (Temgesic) administered subcutaneously at the rate of 0.05-0.1 mg/Kg). To access each uterine horn, a small cut was made in the skin and muscle wall, and the upper part of the reproductive tract was gently exteriorised. Either 6, 8, 10 or 12 embryos were transferred to the ampulla of each uterine horn using a fine pipette, resulting in transfer of a total of 12, 16, 20 and 24 embryos per dam,

respectively (n = 8 dams per group, n = 32 dams in total). The reproductive tract was then returned to the abdominal cavity and the skin closed with surgical clips. Females were pair-housed after recovery and wound clips were removed 7-10 d post-surgery [767]. Transfer was noted as unsuccessful during the procedure in one mouse receiving 6 embryos per horn, and data from this animal was excluded from analyses of the relationship between number of embryos transferred and litter size.

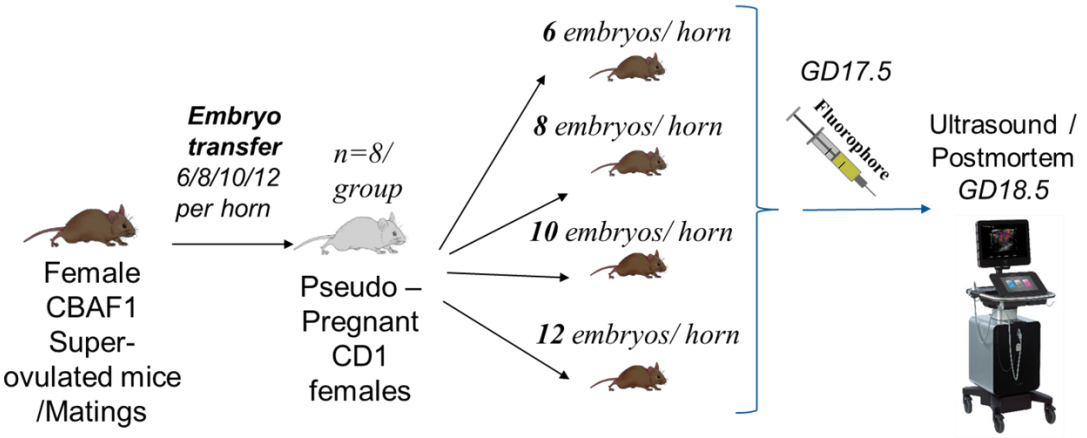


Figure 4.1 *Experimental design*

At GD17.5, 0.2 nmol/g body weight of IRDye 800CW 2-deoxyglucose (IRDye 800CW 2-DG) was injected via the tail vein of each pregnant mouse [723], which were then returned to pair-housing (Figure 4.1). Fetal uptake of IRDye 800CW 2-DG correlates positively with placental efficiency, and fetal growth parameters. However, the fetal fluorescence signal is similar for fluorophore conjugated to glucose or carboxylate (negative control), so should be interpreted as a measure of overall placental function and nutrient supply to the fetus in murine pregnancy, not as a marker of fetal glucose accumulation [723].

Approximately 24 h later, at GD18.5 (Figure 4.1),, the mice were anaesthetised with isoflurane (5% induction, 1.5% maintenance, in medical air). Blood flow velocities of the

uterine artery (n=30 dams*)* and from the umbilical arteries of at least two, and where possible, four fetuses per dam (n=66 male fetuses; n=49 female fetuses) were assessed *in vivo* using an ultrasound biomicroscope (Vevo 3100, VisualSonics®, ON, Canada) as described previously [747, 768]. These measurements were transabdominal, and therefore Doppler ultrasound of umbilical vessels could only be performed for fetuses located adjacent to the maternal ventral surface. Fetal location was marked on the skin of the anaesthetised dam using permanent texta during ultrasound. At post-mortem, conducted immediately after ultrasound of anaesthetised dams, the maternal abdomen wall was opened carefully without moving the uterus, and marks were made on the uterus to indicate the studied fetuses. The fetuses located at these positions were dissected and weighed before other fetuses to ensure accurate identification. Peak systolic velocity (PSV), end diastolic velocity (EDV), time-averaged velocity (TAV) and heart rate averages were obtained from a minimum of three consecutive cardiac cycles. Resistance index (RI = [PSV − EDV]/PSV) and pulsatility index (PI = [PSV − EDV]/TAV) were calculated. Following Doppler imaging, dams were humanely killed by cervical dislocation, and fetuses and placentas were dissected and weighed. Placental and fetal weight (n=157 males; n=141 females) were measured for all pregnancies. Every alternate placenta per dam was fixed as described above. Additional fetal size measures (abdominal circumference, crown to rump length, and head width) were made on all individual fetuses by a single researcher using cotton thread and Vernier callipers in all pregnancies after the initial 10 post-mortems (n=22 dams; n=105 male fetuses; n=100 female fetuses). After completion of fetal size measures, anaesthetised fetuses were killed by decapitation, and fetuses and fetal tails were snap-frozen.

4.4.3 *Determination of fetal sex*

DNA was extracted from fetal tails using QuickExtract DNA Extraction Solution (Epicentre, Madison, Wisconsin, USA). PCR reactions for *Sry* were made up of 20 µL mastermix containing 0.2 µL Taq polymerase (Platinum™ Taq DNA Polymerase, Invitrogen), 10 µL FailSafe™ PCR Enzyme and 2X PreMix Buffer D (Epicentre) and 10 µM *Sry* primers (Forward: 5' AACAACTGGGCTTTGCACATTG 3', reverse: 5' GTTTATCAGGGTTTCTCTCTAGC 3' (final concentration 0.5 nM) and samples were run for 33 cycles (94 °C 1 minute, 60 °C 1 minute, 72 °C 72 seconds) followed by 9 minutes at 72 °C on a GeneAmp® PCR system 9700 (Applied Biosystems, Waltham, Massachusetts, USA). Male fetuses were determined by the presence of *Sry*. Female fetuses were defined by a negative *Sry* reaction, together with the presence of intact DNA, confirmed by a positive reaction for *Myogenin*, assessed using the same protocol and using forward and reverse primers for *Myogenin* (Forward: 5' TTACGTCCATCGTGGACAGC 3', reverse: 5' TGGGCTGGGTGTTAGTCTTA 3') [769]. DNA was re-extracted and PCR repeated for any sample where *Myogenin* was not detected. Outcomes of the PCR were validated by gel electrophoresis on a 2% agarose gel.

4.4.4 *Placental morphology and function*

Frozen fetuses were homogenised and fluorescence-imaged at 800 nm using the Odyssey CLx Infrared Imaging System [723]. An index of placental transfer of IRDye 800CW 2-DG, which correlates positively with placental efficiency and negatively with umbilical cord resistance index, was calculated as total fetal fluorescence at 800 nm divided by placental weight [n=154 males, n=140 females, 723].

Bisected placentas (n=2-6 per dam) were fixed in 4% paraformaldehyde (PFA), washed in 1 X PBS over a 48 h period and stored in 70% ethanol prior to being paraffin

embedded. Full-faced mid-sagittal placental sections cut to 5 µm thickness were stained with Masson's Trichrome following standard protocols [710]. Areas of junctional zone and placental labyrinth for each placental section were visualised and measured with NDP.view 2 software (Hamamatsu Photonics, Shizuoka, Japan). Total cross-sectional area and the proportion of junctional zone to placental labyrinth were calculated for each placenta.

4.4.5 *Statistical analyses*

Relationships between numbers of embryos transferred and numbers of implantation sites and fetuses in each experiment were assessed by Pearson's correlation. Effects of fetal sex (fixed factor) and litter size (covariate) on maternal, fetal and placental outcomes were analysed using the mixed models procedure in SPSS (version 24.0, IBM, New York, US) and including dam as a random variable to account for the common maternal environment. Fetal dye uptake data was not normally distributed and was log transformed for analysis but non-transformed data is presented in Figure 4.3 for ease of interpretation. Previous studies have reported that murine fetuses at either end of the uterine horns are heavier than those located near the middle of the uterine horns [770]. In preliminary analyses we therefore also added fetal position within the uterine horn as a covariate; this was not significant and was removed from final models. Because effects of litter size on fetal weight differed between males and females, fetal morphometric data was also analysed separately within each sex. Relationships between fetal dye uptake and umbilical blood flow parameters and fetal weight and size were assessed by Pearson's Correlation analysis using data for each individual fetus. Figures show points for individual litters or fetuses/placentae, except for Figure 4.4, where data are presented as mean ± SEM, and $P<0.05$ was considered significant for all analysis.

4.5 Results

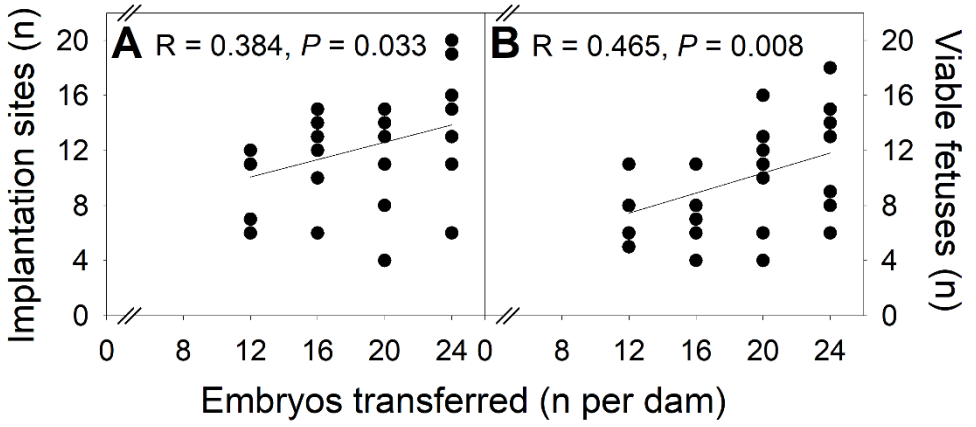


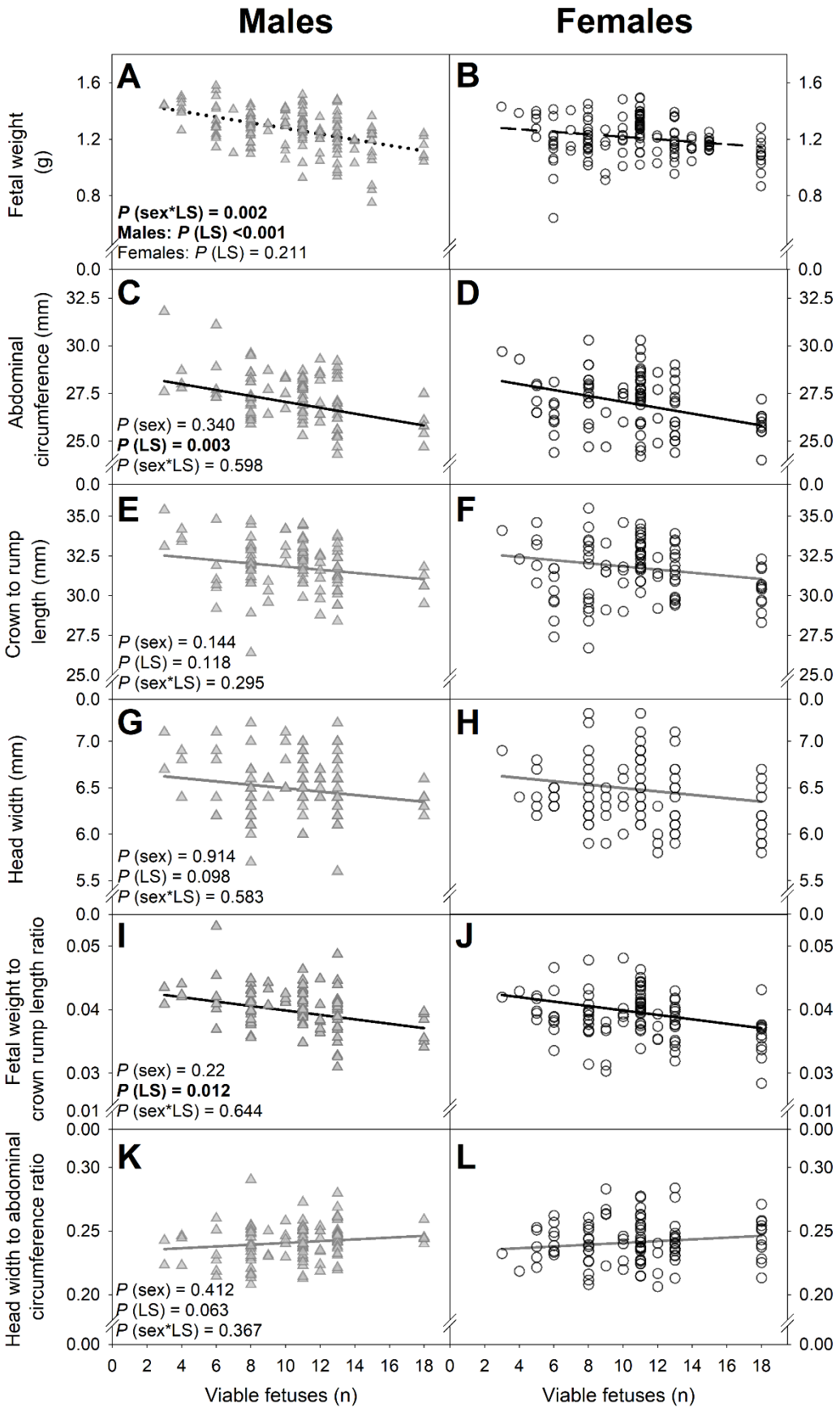
Figure 4.2 *The numbers of viable implantation sites in pilot study (A) correlated positively with the number of embryos transferred in mice. Total number of viable fetuses in the litter (B) also increased with the number of embryos transferred. P and R values are derived from Pearson's correlation analysis; n=31 litters.*

4.5.1 *Increased litter size induces sex-specific fetal IUGR*

Both the number of implantation sites (P=0.033, R=0.38, Figure 4.2A) and litter size (P=0.008, R=0.47, Figure 4.2B) correlated positively with number of embryos transferred.

The relationship between litter size and fetal weight differed between males and females (interaction P=0.002), such that fetal weight was negatively correlated with litter size in male (P<0.001; Figure 4.3A), but not female (P=0.25; Figure 4.3B) fetuses. In contrast, abdominal circumference, fetal head width and crown to rump length did not differ between sexes (all P>0.05). As litter size increased, fetal abdominal circumference decreased overall (P=0.003), within males (P=0.003; Figure 4.3C and within females (P=0.035; Figure 4.3D). Crown to rump length and fetal head width were not related to litter size overall (P>0.1, Figures 4.3E, F and 4.3G, H), respectively), or within each sex. Markers of IUGR were observed in fetuses from larger litters.

Specifically, as litter size increased, fetal weight to crown rump length ratio decreased overall (P=0.012), and in males (P=0.007; Figure 4.3I) but not females (Figure 4.3J). Similarly, with increasing litter size fetal head width to abdominal circumference tended to increase overall (P=0.0634), and increased in males (P=0.026; Figure 4.3K) but not females (Figure 4.3L).

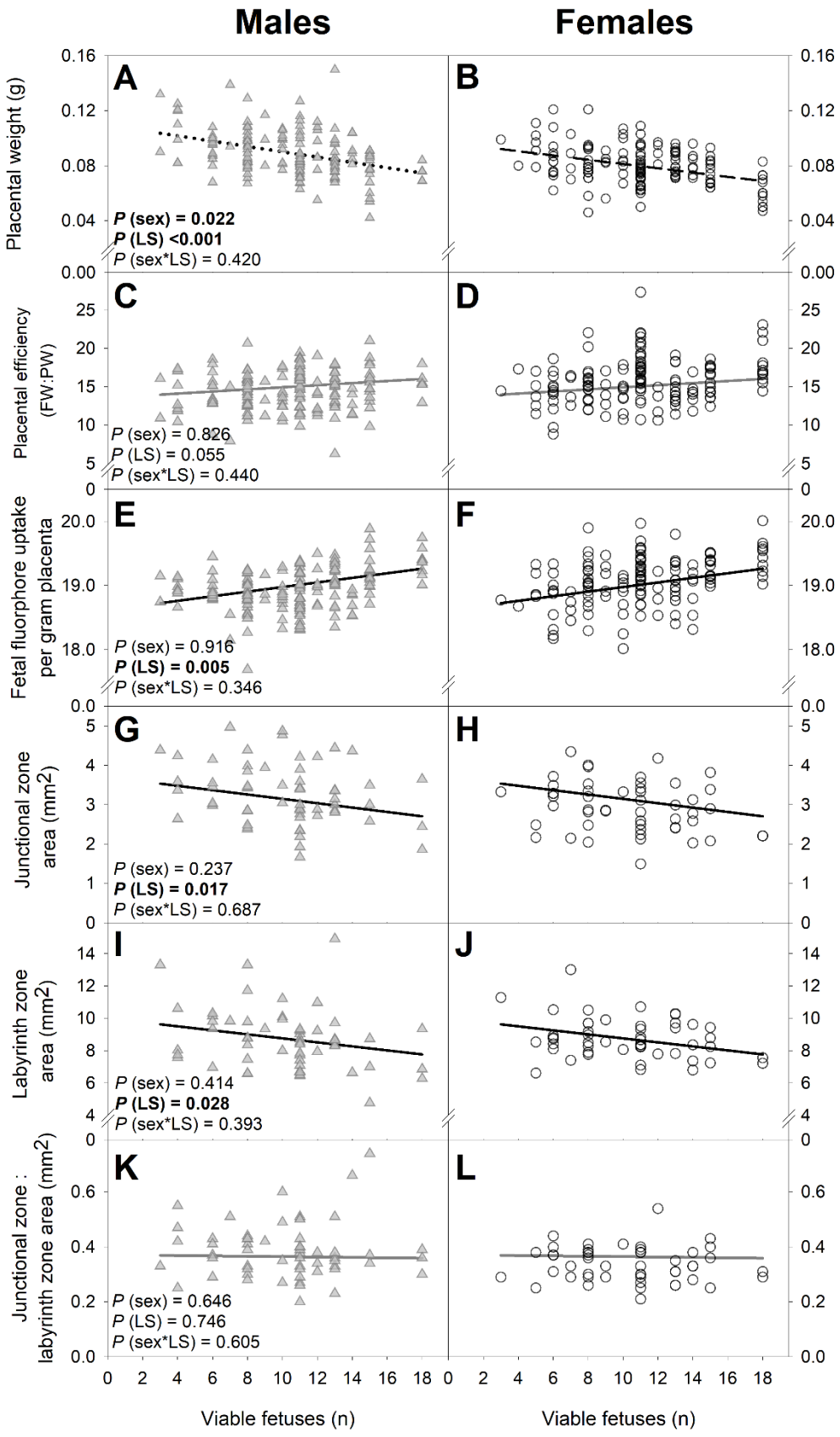

Males
Females
A
B
C
D
E
F
G
H
I
J
K
L
Fetal weight (g)
Abdominal circumference (mm)
Crown to rump length (mm)
Head width (mm)
Fetal weight to crown rump length ratio
Head width to abdominal circumference ratio
P (sex*LS) = 0.002
Males: P (LS) <0.001
Females: P (LS) = 0.211
P (sex) = 0.340
P (LS) = 0.003
P (sex*LS) = 0.598
P (sex) = 0.144
P (LS) = 0.118
P (sex*LS) = 0.295
P (sex) = 0.914
P (LS) = 0.098
P (sex*LS) = 0.583
P (sex) = 0.22
P (LS) = 0.012
P (sex*LS) = 0.644
P (sex) = 0.412
P (LS) = 0.063
P (sex*LS) = 0.367
Viable fetuses (n)
Viable fetuses (n)

2842

Figure 4.3 *Effects of litter size on fetal weight differed between sexes in mice. Weights of males (A), but not females (B), correlated negatively with litter size. Abdominal circumference (C, D) decreased with litter size but crown to rump length (E, F) and head width (G, H) were unchanged, regardless of fetal sex. With increasing litter size, all fetuses were thinner (I, J) with evidence of brain sparing (K, L). P values are derived from mixed model analyses of individual fetal data (weight n=298, other size measures n=205). Symbols show data from individual male (grey triangles; A, C, E, G and I) and female (open circles; B, D, F, H and J) fetuses. Regression lines show correlations for combined sexes (black solid lines for significant correlations, grey solid lines for non-significant correlations); where sex or sex*litter size interactions were significant, correlations are shown separately for male (dotted line) and female (dashed line) fetuses.*

4.5.2 *Increased litter size impairs placental size and function similarly in both sexes*

Placental weight correlated negatively with litter size (P<0.001) and was lower in females than males (P=0.022; (Figure 4.4B and 4.4A, respectively), but the effect of litter size on placental weight did not differ between sexes. Placental efficiency tended to correlate positively with litter size (P=0.055), and the effect of litter size on placental efficiency was similar in males and females (P>0.4, Figure 4.4C, D). Similarly, fetal dye uptake per gram of placenta correlated positively with litter size (P=0.005), and this relationship was similar between sexes (P>0.9, Figure 4.4E, F). Placental labyrinth (P=0.028) and junctional zone (P=0.017) areas correlated negatively with litter size, regardless of fetal sex (interactions: all P>0.4) and did not differ between male and female placentas (all P>0.2, Figures 4.4G, H and 4.4I, J, respectively). The ratio of junctional to labyrinth zone areas was unaffected by litter size or sex (each P>0.7, Figure 4.4K, L). Pulsatility and resistance indices for the umbilical artery did not change with litter size (P>0.70 for both). Interestingly, the pulsatility index in the umbilical artery was lower in female than male fetuses (P=0.043, Figure 4.5B), with a similar trend for resistance index (P=0.054). Uterine artery resistance and pulsatility indices did not correlate with litter size (P>0.05, data not shown).


Males
Females
Placental weight (g)
Placental efficiency (FW:PW)
Fetal fluorphore uptake per gram placenta
Junctional zone area (mm2)
Labyrinth zone area (mm2)
Junctional zone : labyrinth zone area (mm2)
A
B
C
D
E
F
G
H
I
J
K
L
P (sex) = 0.022
P (LS) <0.001
P (sex*LS) = 0.420
P (sex) = 0.826
P (LS) = 0.055
P (sex*LS) = 0.440
P (sex) = 0.916
P (LS) = 0.005
P (sex*LS) = 0.346
P (sex) = 0.237
P (LS) = 0.017
P (sex*LS) = 0.687
P (sex) = 0.414
P (LS) = 0.028
P (sex*LS) = 0.393
P (sex) = 0.646
P (LS) = 0.746
P (sex*LS) = 0.605
Viable fetuses (n)
Viable fetuses (n)

2874

2875

Figure 4.4 *Placental weight (n=298) decreased with increasing litter size and was lower in females (B; open circles) compared to their male littermates (A; triangles), but effects of litter size did not differ between sexes in mice. Placental efficiency (fetal:placental weight; C, D; n=298) and perfusion (E, F; n=295) increased while both junctional zone (G, H; n=108) and labyrinth zone (I, J; n=108) areas decreased with litter size, irrespective of fetal sex. Ratio of junctional zone to labyrinth zone area (K, L; n=108) was not affected by litter size or fetal sex. P values are derived from mixed model analyses of individual placental data, and data for fetal fluorophore uptake per gram placenta was log-transformed for analysis but non-transformed data is presented for ease of interpretation. Symbols show data from individual (A, B, C, D, G, H, I, J, K and L) or within sex litter averages (E and F) for male (grey triangles; A, C, E, G, I and K) and female (open circles; B, D, F, H, J and L) fetuses. Regression lines show correlations for combined sexes (black solid lines for significant correlations, grey solid lines for non-significant correlations); where sex or sex*litter size interactions were significant, correlations are shown separately for male (dotted line) and female (dashed line) fetuses.*

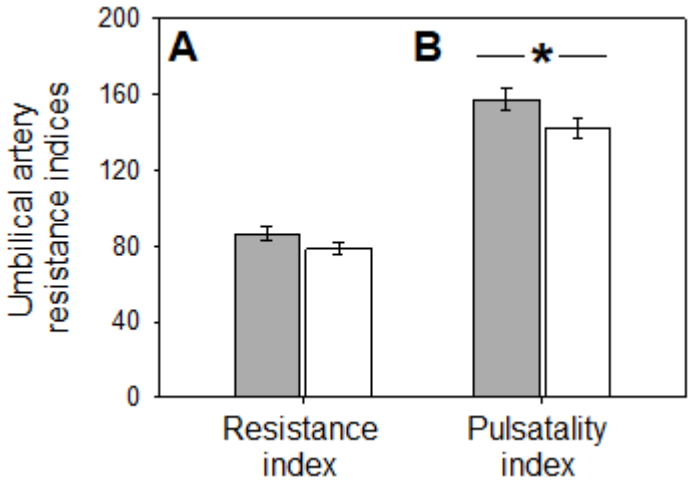


Figure 4.5 *Umbilical artery resistance (A) and pulsatility (B) indices from male (grey bars; n=66) and female (white bars; n=49) mouse fetuses at GD18.5. Data is represented as mean ± SEM. P values are obtained from mixed model analysis of individual umbilical artery measurements.*

4.6 Discussion

We have shown that litter size increases linearly as more embryos are transferred to recipient female mice, at least up to a total of 24 embryos. Increasing litter size in this model induces an IUGR phenotype, with thinner fetuses and evidence of brain sparing

near term. Furthermore, we have shown for the first time that fetal responses to increasing litter size are sex-specific, with constrained body weight, thinness and brain sparing evident in male but not female fetuses as litter size increased. Male placentas were heavier than female placentas, but the effects of increasing litter size on placental weight and function did not differ between sexes. We have therefore developed a model of variable fetal constraint independent of maternal confounders that affect ovulation rate (including breed or size), which can now be utilised to evaluate interventions.

In this present study, we report for the first time that increased litter size achieved by increasing the number of embryos transferred in mice is associated with increasing degree of fetal and placental weight reduction near term, independent of maternal confounding factors. This adds to previous evidence that the litter size of mice could be nearly doubled by transferring increasing number of zygotes into recipient mice, although fetal and placental outcomes were not characterised in this study [763]. Also consistent with our findings, Gluckman and colleagues reported a negative relationship between litter size and fetal growth in mice [379]. In contrast to the present study, authors transferred similar numbers of embryos into each dam, rather than generating a range of litter size by differential transfer, and the range of litter size that they observed may therefore potentially reflect maternal factors that alter implantation rate. Consistent with the results of Johnson and co-authors, who transferred up to 25 embryos per dam [763], we did not see any evidence of increasing fetal deaths as we transferred more embryos into each uterine horn. This implicates variable implantation rate as the cause of variable litter sizes amongst dams that received the same number of embryos within the present study. We predict that fetal deaths might increase if the numbers of embryos were increased further to exceed the natural litter size range of the recipient strain, resulting in more resorptions, but this requires further experimentation.

Our findings of restricted fetal growth with increasing litter size in mice are consistent with observations in non-litter bearing species like humans and sheep, where the occurrence of multiple gestations reduces fetal growth rate relative to that of singletons [388-390]. The fetal growth trajectory of twins diverges from that of singletons around 8 weeks in humans, and a slower fetal growth in twins persists in late gestation in both humans and sheep [388-391]. Similar to our results, fetal length in late gestation decreased with increasing fetal number in rabbit pregnancies generated by embryo transfer, although within a limited range of litter sizes that were all below those of natural pregnancies (8-10 pups), since a maximum of 6 embryos were transferred to each recipient [762]. Transfer of limited embryo numbers may explain the lack of relationship between litter size and placental length in this rabbit study, since low litter sizes would have resulted in limited competition for implantation area [771]. In contrast, placental weight and litter size correlated negatively in embryo-transfer murine pregnancies in both the present study and that of Gluckman and colleagues, where larger ranges in litter size were generated [379]. Reduced birthweight in multiple pregnancies is similarly accompanied by reduced placental weight in humans [772], and sheep [387, 773].

A particularly interesting and novel finding of the present study was that the effect of an increasing litter size on fetal growth was sex-specific. In smaller litters, male fetuses weighed more than their female littermates in late gestation. It is now well established in humans, sheep and rodents that the average weights of male fetuses are heavier than females at an equivalent gestational age [36-39]. In the present study, as litter size increased, the weight of male fetuses decreased while weights of female fetuses remained stable. Other markers of asymmetric growth restriction, i.e. weight:crown-rump length (thinness) and fetal head width:abdominal circumference (brain sparing), were also evident with increasing litter size in male fetuses but not their female littermates. Two hypotheses may explain this sex-specific relationship. Firstly, growth

strategies may differ between sexes, such that females maintain a reserve by not growing to their full genetic capacity, even in smaller litters where competition for nutrients is likely within a normal range. The maximal growth strategy in males may mean their growth is limited by the available nutrient supply when either faced with an external insult or limitations to supply. Although we did not observe sex differences in fetal:placental weight ratio, which is considered a marker of placental efficiency [739], we did not measure placental nutrient transport in our late gestation pregnancies, which would provide a direct, stage-specific measure of placental function. Evaluating these outcomes in male and female placentas under “normal” conditions and in response to an *in utero* challenge might also provide useful insight. This hypothesis of a female placental “reserve” capacity is also consistent with reports, that although IUGR is more prevalent in females [48], males have higher rates of spontaneous preterm birth and perinatal death if there is a perturbation in pregnancy [44, 49-53].Secondly, female fetuses (and placentas) may be better able to adapt to an *in utero* environment where competition for nutrients is high, in order to maintain growth. This is supported by human and animal studies which demonstrate that in response to maternal undernutrition, female placentas undergo a higher number of changes in gene expressions compared to male placentas [781]. These changes in placental gene expression likely act as a buffer to protect the female against in utero perturbations and possibly reduce the risk of adult onset of diseases [782].

In addition to reduced fetal and placental weights, we found that increasing litter size in mice induced asymmetric IUGR in a sex-specific manner. Male fetuses in larger litters were thinner and showed evidence of brain sparing, regardless of sex. Skeletal growth, measured as crown-rump length, was not reduced with increasing litter size in the present study, suggesting that restriction in multi-fetal litters occurs later in gestation, after the spine develops [783, 784]. Similarly, a previous study in humans reported no differences in skeletal mass between asymmetrically growth restricted and AGA

fetuses and neonates [783]. This could be in part due to compensatory mechanisms in IUGR which favour the formation of mineralised bone, such as the downregulation of Dickkopf-1 (DKK-1), a protein involved in the inhibition of osteoblast differentiation [785]. However, the effects of IUGR become apparent during adulthood, as growth restricted individuals are predisposed to reduced peak skeletal size and mineralisation in later life [783]. In humans, asymmetrical IUGR is also common in twins [390, 391, 774] and characteristic of IUGR fetuses [775]. This growth pattern is often associated with placental dysfunction, which impairs fetal oxygen and nutrient supply to the fetus, especially during late gestation when absolute fetal growth is at its fastest [776]. In these fetuses, cardiac output is redirected towards maintaining growth of vital organs like the brain and heart, further impairing the growth of other organs including the liver and kidneys, contributing to the disproportionate fetal growth [160]. Structural and functional changes in the organs that accompany asymmetric growth restriction underlie adverse health outcomes that emerge in adolescent and adult life, including arterial hypertension and chronic renal failure [49, 777, 778]. Asymmetric fetal growth is also observed in a number of animal models of IUGR, including maternal undernutrition in rabbits, mid-late gestation uterine artery ligation in rats, and reduction of placental exchange surface (through endometrial caruncle removal or repeated uteroplacental embolisation) in sheep, which likewise restrict rapid fetal growth in late gestation [310, 331, 341, 780].

An additional major finding of the present study was that sex-specific relationships between litter size and fetal growth were not explained by sex-specific changes in placental size or function. The overall increase in placental efficiency and function as litter size increased in our study, is likely an adaptation aimed at optimising or maintaining fetal growth in the face of limited nutrient supply [43, 725, 727, 786]. Although in the present study placental size, efficiency and dye transfer (a marker of perfusion) increased similarly in males and females, it is possible that transport of

specific nutrients might be differentially affected and hence explain the sex-specific constraint of fetal growth in large litters. Fetal susceptibility to adverse outcomes in a number of pregnancy complications appears to be sex-dependent [53] and is largely orchestrated by the placenta, which is genetically identical to the fetus [42]. A number of different human and animal studies show that placentas may undergo sex-specific adaptations to the same *in utero* stressors [787]. In males, placental adaptations appear to promote continued growth while in females, these adaptations are focussed on promoting placental development and fetal survival [54]. Although umbilical blood flow measures were not related to litter size, we did see some evidence of greater uteroplacental resistance in male compared to female fetuses near term. Although we were unable to assess placental microstructure in the present study, it has been suggested that greater vascular resistance at term in mice may reflect decreased surface area of the maternal blood spaces in the labyrinth [788, 789]. Decreased area for exchange might therefore contribute to IUGR in male fetuses. Variable structural adaptations, including increased fetal villi length, placental surface density and volume fraction have been reported in inter-breed embryo transfer pregnancies in horses, which may partially compensate for decreased placental weight and enable partial maintenance of fetal growth in the face of maternal constraint [759, 790]. However, further studies will be needed to characterise placental adaptations to variable litter size within our IUGR model.

An important strength of our study is analysis by fetal sex, shown here to be an important determinant of fetal growth responses to variable maternal constraint. An additional strength of our model is removal of maternal factors that occur in studies of natural variation in litter size. In naturally-conceived pregnancies, the spontaneous variation in litter size is dependent on maternal factors that determine ovulation rate, which increases with maternal weight, parity and age [393-398]. Our approach of transferring variable numbers of embryos removes factors that affect ovulation rate as

confounders. This does, however, require the additional periconceptional exposures of super-ovulation, embryo culture and vitrification, which may also affect fetal development [791]. Whether these effects differ with litter size or between sexes is, however, unknown.

In summary, we have generated a murine model of variable fetal constraint reflective of multiple birth, with sex-specific fetal outcomes. Explanations for this must lie in the genetic differences between males and females conferred by the sex chromosomes and resultant hormonal differences. This model can be used to understand the mechanisms associated with IUGR induced by multifetal pregnancies, as well as to test possible interventions in future studies. Such analyses could compare the slope of the relationships between fetal and placental outcomes and litter size, as used here to compare sex-specific correlations, or could compare outcomes in larger and small litters specifically. We suggest also, that potential therapies identified in the mouse model should be validated in other models prior to translation in humans, given the potential differences in fetal demand and placental signalling between twins or triplets in humans compared to higher-order multiples in mice. The availability of animal models of variable fetal constraint in which to evaluate interventions has substantial clinical relevance, given the substantial increases in the risks of perinatal death and lifelong adverse effects on health in IUGR infants and the current lack of effective treatments or preventative therapies.

4.7 Additional information

4.7.1 *Competing interests*

The authors declare that they have no competing interests.

4.7.2 *Authors contributions*

HK, ASC, RLW, BSM, CTR and KLG conceived and designed the experiments. HK, ASC, RLW, SGP and KLG were involved in the acquisition of data. HK, ASC, RLW, BSM, CTR and KLG analysed and interpreted the data. HK and KLG drafted the article. All authors critically revised and approved the final version of the manuscript and agree to be accountable for all aspects of the work.

4.7.3 *Funding*

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4.7.4 *Acknowledgments*

We thank University of Adelaide Laboratory Animal Services and SAHMRI Bioresources for providing excellent animal care.

Chapter 5 General discussion

IUGR increases risks of perinatal death >5-fold and has lifelong adverse effects on health. There is currently no treatment to prevent or cure IUGR. Both GH, and its main mediator, IGF1, play crucial roles in facilitating maternal adaptations to pregnancy and their circulating concentrations change significantly during pregnancy in a number of species. In normal, as well as IUGR pregnancies, exogenous maternal GH administration in patterns replicating endogenous secretion has shown promising effects for promoting fetal growth. Therefore, in this project, I tested a novel approach to stimulate the mother's own production of hormones that promote placental function and fetal growth. This involved investigating whether diet-induced increases in acyl-ghrelin elevated circulating maternal GH levels and promoted fetal growth in normal murine pregnancies (chapter 2). Novel tools to test the effectiveness of this potential intervention were also established. We developed and validated a method to measure placental uptake of nutrients via non-radioactive tracers (chapter 3). In the final experiment, a murine model of IUGR was established with the use of embryo transfer techniques. In this model, sex-specific impacts of varying degrees of litter size-induced prenatal constraint on fetal and placental growth were (chapter 4).

5.1 Summary of results

Contrary to our original hypotheses, dietary supplementation with octanoic acid at concentrations used in this study did not elevate circulating acyl-ghrelin concentrations. Furthermore, circulating maternal acyl-ghrelin concentrations did not increase during pregnancy and were actually lower in mid-pregnant compared to non-pregnant mice, suggesting that acyl ghrelin is unlikely to be the main mediator of elevated maternal GH concentrations during murine pregnancy. However, the observed time-dependent

increase in maternal circulating total ghrelin concentrations and gastric ghrelin expression indicate possible involvement of ghrelin in the regulation of maternal energy homeostasis and energy intake during pregnancy.

As per the second aim of this thesis (chapter 3), a method was developed to quantify feto-placental uptake of the near-infrared IRDye 800CW 2-DG in late pregnant mice. Fetal uptake of this fluorescent tracer correlated positively with markers of placental blood flow and fetal growth, suggesting that it provides a proxy measure of placental function. However, the signals obtained were similar whether the fluorophore was conjugated with glucose or carboxylate (negative control), implying that the uptake mechanism is not restricted to glucose transport. The methods established in this chapter have the potential to provide a marker of overall placental perfusion and can be adapted to test the suitability of other fluorescent tracers.

In chapter 4, in order to test potential interventions (aim 3), we established a murine model of IUGR. In this model, embryo transfer-generated pregnancies were used to create a range of litter sizes, and hence prenatal constraint, to replicate the intrauterine environment in multi-fetal pregnancies. Interestingly, the relationship between litter size and fetal weight was sex-specific, such that increasing litter size restricted the weight of male, but not female, fetuses at term. This sex bias in fetal growth could not be explained by the effects of increasing litter size on placental size and perfusion (measured using methods established in chapter 3), suggesting that other sex-specific adaptations are responsible for slowing the growth of female fetuses to increase their resilience to insults later in gestation. Whether this sex-specific fetal growth response would lead to male-biased fetal deaths or growth restriction in males if litter size was increased further, consistent with sex-specific effects of perinatal insults on human fetuses, remains to be established. Additional work is also needed to define sex-specific impacts throughout gestation and up to birth, since in other models, impacts of restricted placental function [370] and maternal undernutrition [354] on fetal growth increase with gestational age.

This chapter therefore describes a model of variable fetal constraint that is largely independent of maternal confounding factors, reflective of growth restriction in multifetal pregnancies, and with sex-specific fetal outcomes, which can be utilised to evaluate potential interventions.

5.2 Strengths, limitations and future directions

Studies reported within this thesis are the first to describe a number of outcomes related to the GH-ghrelin axis in murine pregnancy. These include quantifying circulating abundance of total and acyl-ghrelin at several stages of the light-feeding cycle and at multiple stages of the murine pregnancy. This is also the first report of alterations in gastric ghrelin expression and placental ghrelin expression in murine pregnancy. Ghrelin's role in regulating food intake and energy homeostasis is well-established in non-pregnant humans and animals. Although not previously investigated, the time-dependent alterations in ghrelin expression and secretion observed in this study are suggestive of ghrelin's potential role in the regulation of maternal energy expenditure and food intake during pregnancy. Furthermore, as ghrelin acts at the level of the hypothalamus and pituitary to increase GH secretion [401, 446], measurements of ghrelin receptor expression and activation in these regions would be a valuable addition to future work.

One of the main limitations we faced while assessing the effects of dietary supplementation on changes in growth hormone profiles in non-pregnant and pregnant mice was a small sample size. Suppression of GH secretion was observed in three otherwise normal pregnant mice, likely due to the stress associated with the sampling period [703]. Data from these animals could therefore not be included in our analysis, reducing the sample size/power of the data and hence potentially masking any diet effects on GH secretion. A further limitation is that while we were able to detect octanoic acid in the feed using gas chromatography analysis, we were unable to detect

octanoic acid in the circulation of the mice using this same method. It is possible that this reflects the rapid metabolism of MCFAs [719, 792-794]. The lack of increase in circulating acyl-ghrelin in response to octanoic acid supplementation in our studies was, however, surprising given that published studies have reported increased stomach acyl-ghrelin in *male* mice when fed similar concentrations of octanoic acid [468]. Our work is the first to test this dietary approach in female mice. Given that estrogen administration decreases the number of ghrelin-producing cells, gastric ghrelin gene expression and plasma ghrelin levels in ovariectomised rats, further investigation should be conducted to test whether steroid hormones can explain the lack of changes in circulating acyl ghrelin observed in the female mice in our study in response to dietary supplementation with octanoic acid. Furthermore, variable effects of octanoic acid or octanoic-containing triglyceride supplementation on circulating acyl-ghrelin have been reported in recent literature. In a few studies, dietary octanoic acids increased circulating acyl-ghrelin in humans, mice and cattle [687, 689, 721]. In other studies, although dietary octanoic acid supplementation increased stomach acyl-ghrelin in mice [468, 795] and stomach octanoic acid content in rats [719], an increase in circulating acyl-ghrelin was not observed [468, 719, 795]. There are recent reports show that dietary supplementation with medium chain triglycerides (MCT) increases circulating acyl-ghrelin in humans and pigs of both sexes [688, 722], as well as growth hormone in pigs [688] Thus, supplementation with MCTs is a potential strategy to increase circulating maternal acyl-ghrelin concentrations. In the current study, the daily consumption of food pellets by pair-housed mice allocated to the octanoic acid diet was not noticeably different to those given the palmitic acid diet. However, in future, a more precise quantification of the amount of food consumed by mice may also allow better understanding of the relationship between dietary intake and circulating acyl and total ghrelin concentrations at different time points.

The study described in chapter 3 is the first to attempt to quantify placental and fetal uptake of nutrients using a non-radiolabelled tracer, which can potentially be used to measure placental function in response to an intervention. However, a major limitation of this method was that, unlike radio-labelled tracers, the fluorophore tested in this study did not reflect the specific uptake of glucose by placental and fetal tissues. The lack of specificity of this probe for glucose transporters is likely influenced by the molecular size of the probe (18-23Å), as it was probably too large to be taken up by the GLUT1 transporters (with a predicted channel size of 15 Å × 8 Å) [743, 756]. Therefore, *in vitro* cell assays will need to be performed in future studies in order to investigate the specific mechanisms involved in the cellular uptake of the 2-DG dye, including in the placenta. Confirmation that placental IRDye 800CW 2-DG transport is glucose transporter-independent would also require inhibition studies using unlabelled glucose, 2-DG or antibodies to glucose transporters in placental cells or cell lines, similar to previous studies in tumour cells [743].

Finally, the IUGR model described in chapter 4 is the first to utilise embryo transfer techniques to generate litter sizes larger than those observed in normal mouse litters and to subsequently measure the effect of this prenatal constraint on fetal and placental outcomes. A strength of this approach to generate variable litter size and therefore constraint is the avoidance of maternal confounders that impact ovulation rates and therefore variation in spontaneous litter size. Our protocol does however require exposure of embryos to hormones required for maternal super-ovulation (in donors), as well as to embryo culture. Although these exposures happen in all pregnancies, it is possible that these induce some of the adverse effects observed in human IVF pregnancies, including lower birthweight and differential methylation of genes critical for growth [791]. This approach does not confound our analyses of litter size effects, since all embryos are similarly exposed. However, we suggest that future intervention studies should include a naturally-mated control group as a reference. The

findings of this study also highlighted the importance for animal models of IUGR to analyse data by fetal sex. Our observation of sex-specific fetal growth responses to the constraint due to increasing litter size is consistent with evidence from humans, indicating that males and females can respond differently to the same intrauterine environment [44, 49-53], which in turn may influence the nature of clinical interventions. For example, following antenatal betamethasone treatment, the activity of the glucocorticoid metabolising enzyme 11 beta-hydroxysteroid dehydrogenase-2 (11βHSD2), is increased in female but not male placentas [796], whereas higher placental production of radical oxygen species is observed in preterm males compared to preterm females [797]. The resultant increase in cortisol inactivation in females may protect them from the glucocorticoid-induced effects of reactive oxygen species production [796, 797], which are associated with conditions like bronchopulmonary dysplasia that are more prevalent in premature males than in females [798, 799]. The mechanisms contributing to the sex-bias in fetal growth in our model are not clear but are likely to involve sex-specific morphological and functional placental adaptations. This study is the first to investigate sex-specific placental uptake of a non-metabolisable glucose analogue in growth restricted rodent pregnancies. However, no study to date has investigated whether placental uptake of other nutrients, including amino acids, differ between male and female growth restricted mouse fetuses. Although not possible in the current study, it would also be ideal to also measure changes in placental microstructure, as suboptimal placental structural development may explain our observations of greater uteroplacental resistance in male fetuses at term and, possibly, the greater growth restriction in this group. It would also be interesting to further develop this model and test whether the growth restricted mice undergo postnatal catch-up growth in a sex-specific manner and whether this is accompanied by glucose intolerance and impaired β-cell function, similar to that observed in human IUGR. However, it must be highlighted that models using litter-bearing species may not accurately reflect the environment of human multifetal

pregnancies, as the greater number of placentas in mice may mean more signals to the mother that induce maternal adaptations to pregnancy, although these were not measured in this study.

5.3 Conclusion

Although the dietary approach tested in this project did not provide the expected results, evidence from recent literature suggests that dietary supplementation with triglycerides or even direct acyl-ghrelin administration may be more successful at promoting maternal circulating acyl-ghrelin and GH levels, and possibly fetal growth. Through our IUGR model, we have established the means to test the effectiveness of these, and other interventions or treatments aimed at promoting fetal growth, including maternal melatonin administration and local VEGF overexpression in uterine arteries, especially in the setting of IUGR in multifetal pregnancies. Effects of interventions on fetal and placental responses to constraint can be assessed by comparing the slope of the relationship between litter size and fetal growth in treated and untreated dams. Additionally, we have also developed methods to measure the impact of these interventions on placental function, a crucial determinant of fetal growth. This method can be applied by other researchers who wish to measure the placental uptake of a broad range of nutrients, but are not able to use radioactive tracers, for example due to facility constraints. Together, the work described in this thesis provides the means to test and develop potential interventions aimed at promoting placental function and fetal growth in the setting of prenatal constraint and hence, minimise the risk of perinatal death and lifelong effects on health in IUGR infants.

Chapter 6 References

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Chapter 7 Appendices

Appendix 1 Pregnancy, but not dietary octanoic acid supplementation, stimulates the ghrelin-pituitary growth hormone axis in mice

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Pregnancy, but not dietary octanoic acid supplementation, stimulates the ghrelin-pituitary growth hormone axis in mice

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Abstract

Circulating growth hormone (GH) concentrations increase during pregnancy in mice and remain pituitary-derived. Whether abundance or activation of the GH secretagogue ghrelin increase during pregnancy, or in response to dietary octanoic acid supplementation, is unclear. We therefore measured circulating GH profiles in late pregnant C57BL/6J mice and in aged-matched non-pregnant females fed with standard laboratory chow supplemented with 5% octanoic or palmitic (control) acid (n = 4–13/group). Serum total and acyl-ghrelin concentrations, stomach and placenta ghrelin mRNA and protein expression, *Pcsk1* (encoding prohormone convertase 1/3) and *Mboat4* (membrane bound O-acyl transferase 4) mRNA were determined at zeitgeber (ZT) 13 and ZT23. Total and basal GH secretion were higher in late pregnant than non-pregnant mice (P < 0.001), regardless of diet. At ZT13, serum concentrations of total ghrelin (P = 0.004), but not acyl-ghrelin, and the density of ghrelin-positive cells in the gastric antrum (P = 0.019) were higher, and gastric *Mboat4* and *Pcsk1* mRNA expression were lower in pregnant than non-pregnant mice at ZT23. In the placenta, ghrelin protein was localised mostly to labyrinthine trophoblast cells. Serum acyl-, but not total, ghrelin was lower at mid-pregnancy than in non-pregnant mice, but not different at early or late pregnancy. In conclusion, dietary supplementation with 5% octanoic acid did not increase activation of ghrelin in female mice. Our results further suggest that increases in maternal GH secretion throughout murine pregnancy are not due to circulating acyl-ghrelin acting at the pituitary. Nevertheless, time-dependent increased circulating total ghrelin could potentially increase ghrelin action in tissues that express the acylating enzyme and receptor.

Key Words
- mouse
- pregnancy
- octanoic acid
- ghrelin
- growth hormone

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Appendix 2 Validation studies of a fluorescent method to measure placental glucose transport in mice

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Validation studies of a fluorescent method to measure placental glucose transport in mice

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ABSTRACT

Introduction: Proper placental function is essential for optimal fetal growth *in utero*. Placental transfer of nutrients to the fetus can be measured using radiolabelled tracers, but non-radioactive methods have potential advantages. This study aimed to develop a fluorescence-based method to measure placental glucose transport in mice.

Methods: Time course and localisation of the IRDye 800CW 2-deoxyglucose were recorded (Lumina IVIS Live Imaging System) following tail vein injection into anaesthetised late pregnant mice. Fluorescent signals in placental and fetal tissues were assessed after injecting conscious dams with 10 nmol IRDye 800CW 2-deoxyglucose (3, 30, 60, 120 min) or vehicle. Specificity of dye uptake was determined by comparing uptake of IRDye 800CW conjugated to 2-deoxyglucose or carboxylate, at 2 and 24 h. Finally, we assessed relationships of fetal size and umbilical blood flow velocities with relative dye uptake.

Results: In late pregnant mice, uterine fluorescent signal localised rapidly over placentas and remained consistent for > 1 h. Signal intensity in whole and homogenised tissues increased in fetuses and decreased in placentas after 3 min and stabilised by 30 min post-injection. Relative fetal dye uptake at 2 and 24 h was greater in littermates with the highest compared to lowest placental efficiency; signals were similar for 2-deoxyglucose- or carboxylate-conjugated dyes. Relative fetal dye uptake correlated positively with fetal weight and placental efficiency and negatively with umbilical artery resistance indices.

Conclusions: Fetal uptake of IRDye 800CW correlates with markers of placental blood flow and fetal growth, but does not specifically measure placental glucose transport.

1. Introduction

One of the major determinants of intrauterine growth is the placental supply of nutrients to the fetus, which occurs primarily by diffusion and transporter-mediated transfer [1]. The capacity of the placenta to facilitate this nutrient exchange depends on its size, morphology and blood flow as well as the abundance and activity of nutrient transporters [2,3]. There is now considerable evidence that poor placental function is a major contributor to restricted fetal growth [4]. Although the placenta can adapt morphologically and functionally to fetal signals of nutrient demand [5–7], inadequate placental function can result in intrauterine growth restriction (IUGR) wherein the fetus fails to reach its genetically pre-determined growth potential [8,9]. Low birthweight (LBW, < 2.5 kg) and IUGR fetuses are at a higher risk of fetal mortality and morbidity, both perinatally and later in life [10–14].

One of the most widely used methods of assessing placental efficiency is the measurement of placental nutrient transporter expression and activity. Isolated placental vesicles from human pregnancies complicated by IUGR typically exhibit decreased placental activity of system A amino acid transporters [15]. While not differentially expressed in placentas from human IUGR and normally-grown fetuses [16], placental protein abundance of the facilitated glucose transporter

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