DISSERTATION

THE PHYSIOLOGICAL RAMIFICATIONS OF CHORIONIC SOMATOMAMMOTROPIN RNA INTERFERENCE

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ABSTRACT

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Intrauterine growth restriction (IUGR), a condition by which the fetus fails to reach its growth potential in utero affects 6-8% of pregnancies worldwide. Not only is IUGR a leading cause of infant morbidity and mortality, but it also increases the likelihood of developing metabolic diseases in adulthood. Chorionic somatomammotropin (CSH; aka placental lactogen) is the most abundantly produced placental hormone and is part of the growth hormone/prolactin gene family. Deficiencies in CSH have been causally linked to IUGR, however, the process by which CSH regulates uteroplacental function and fetal growth remains to be elucidated. CSH deficiency in both humans and sheep can result in two distinct phenotypes: 1) pregnancies with IUGR, and 2) pregnancies with normal fetal and placental weights. Both categories of CSH deficient pregnancies experience some shared metabolic perturbations.

Thus, to investigate the biological roles of CSH in regulating fetal and placental physiology, a series of experiments using lentiviral-mediated RNA interference (RNAi) were designed to test the physiological ramifications of CSH RNAi. These experiments included measuring the effects of CSH RNAi on blood flow and nutrient uptakes in pregnancies with both normal weight and IUGR phenotypes (studies 1 and 2), determining how the maternal-fetal glucose gradient would impact placental glucose

transfer (study 3), and assessing transcriptomic changes in CSH RNAi pregnancies (study 4). To generate pregnancies for each study, the trophectoderm of hatched blastocysts (9 days of gestational age; dGA) were infected with lentiviral-constructs expressing either a scrambled control (Control RNAi) or CSH-specific shRNA (CSH RNAi), prior surgical transfer into synchronized recipient ewes. The pregnancies were then surgically fitted with maternal and fetal catheters between 120-126 dGA and underwent subsequent metabolic studies near term (130-136 dGA) to assess blood flow and nutrient transfer. Study 3 pregnancies also underwent a maternal hyperglycemic clamp following the baseline metabolic study. The CSH RNAi pregnancies for study 4 were generated in the same manner as the studies 1-3 but were not surgically instrumented.

Study 1 assessed the physiological consequences of CSH RNAi in a cohort of normal weight CSH RNAi pregnancies. Uterine blood flows were reduced ($P \leq 0.05$) even in the absence of fetal growth restriction in CSH RNAi pregnancies, but umbilical flows were not impacted. Uteroplacental glucose utilization (μmoL/min/kg placenta) was increased ($P \leq 0.05$) in CSH RNAi normal weight pregnancies, whereas umbilical glucose uptake (μmoL/min/kg fetus) was reduced. These results demonstrated that CSH RNAi has significant physiological ramifications, even in the absence of IUGR.

Study 2 determined the ramifications of CSH RNAi in a cohort of IUGR pregnancies. CSH RNAi reduced ($P \leq 0.05$) both fetal and uterine weights as well as umbilical blood flow (mL/min). This ultimately resulted in reduced ($P \leq 0.01$) umbilical IGF1 concentrations, as well as diminished umbilical nutrient uptakes ($P \leq 0.05$) in CSH RNAi IUGR pregnancies. CSH RNAi also reduced ($P \leq 0.05$) uterine nutrient uptakes as

well as uteroplacental glucose utilization. These data suggested that CSH is necessary to facilitate adequate blood flow for the uptake of oxygen, oxidative substrates, and hormones essential to support fetal and uterine growth.

Study 3 assessed the impacts of altering maternal-fetal glucose gradients on placental glucose utilization and transfer. While basal uterine glucose uptakes were suppressed ($P \leq 0.05$) in CSH RNAi pregnancies, increasing the maternal-fetal glucose gradient via maternal hyperglycemic clamp removed the differences in uterine glucose uptakes between treatments. However, umbilical glucose concentrations in CSH RNAi fetuses remained diminished ($P = 0.06$) in spite of maternal hyperglycemia. Furthermore, relative uteroplacental oxygen utilization (mmol/min/kg placenta) increased ($P \leq 0.05$) during the maternal hyperglycemic clamp, suggesting greater placental oxidation of substrates. This may be explained in part by lower maximal glucose transfer during maternal hyperglycemia ($P \leq 0.05$) in CSH RNAi pregnancies. Fetal insulin concentrations were reduced in CSH RNAi pregnancies, both during basal conditions ($P = 0.01$) as well as during maternal hyperglycemia ($P = 0.10$). These data suggested that CSH likely plays a key role in modulating placental metabolism that ultimately promotes maximal placental glucose transfer. Furthermore, these data hint at a role for CSH in maintaining fetal insulin sensitivity to glucose.

Study 4 performed RNA-sequencing on cotyledonary tissue. 442 differentially expressed genes (DEGs) were identified with a $Q \leq 0.10$. 346 DEGs were found ($Q \leq 0.10$) between CON vs. CSH-IUGR and 96 DEGs ($Q \leq 0.10$) between CON vs. CSH-normal weight with 36 common DEGs between analyses. Analysis of CON vs CSH-IUGR pregnancies revealed perturbations ($P \leq 0.05$) in protein processing, regulation of

cAMP, cell proliferation, carbon metabolism, and metabolic pathways. Furthermore, 185 genes in the following six functional categories were also identified (P≤0.01): cell-to-cell signaling, cellular movement, protein synthesis, cell death, lipid metabolism and hematological development and function. These data suggested that CSH RNAi perturbs metabolic and cellular function regardless of fetal weight. Together, these four experiments provide evidence that two of the key biological roles of CSH are 1) promoting blood flow and nutrient uptake by the uteroplacental unit and 2) regulating uteroplacental oxidative metabolism.

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CHAPTER I: LITERATURE REVIEW

PLACENTAL PHYSIOLOGY

The placenta is a remarkable transient organ, responsible for modulating fetal growth as well as maternal-fetal interactions. It has long captivated the mythos of the ancient world and the intellects of philosophers. Ancient folklore across Australasia, the Pacific Islands, and Africa deemed the placenta to possess supernatural properties (Long, 1963), with the ancient Egyptians going as far as to consider the placenta part of Pharoah's soul, honoring it as such in their ceremonial artwork (Seligman and Murray, 1911). Philosophers also contemplated the purpose and beauty of this intriguing organ. Aristotle is largely credited with first describing and naming the various placental membranes such as the chorion and amnion, and Claudius Galeneus (Galen) famously detailed his thoughts about the perfusion of the placenta to supply the needs of the growing fetus (reviewed in Longo and Reynolds, 2010). Even Leonardo da Vinci was captivated by the beauty of the uteroplacental unit, depicting it in his anatomical sketches (reviewed in Longo and Reynolds, 2010). Clearly, there is both historical and scholarly precedent for studying this fascinating organ.

Crudely, the placenta functions as an exchanger, transferring substrates and heat between maternal and fetal circulation. More broadly, the placenta coordinates fetal growth by regulating nutrient transfer and gas exchange, facilitating maternofetal communication, and by camouflaging the fetus from the maternal immune system. Compared to other organs, one remarkable attribute of the placenta is it not only functions as the conduit for the transport nutrients, but also must prioritize its own

oxidative demands. Thus, it is not surprising that when placental dysfunction or insufficiency occurs, the health of the growing fetus and sometimes, the mother, can be compromised. To more completely understand the complexities of the organ responsible for modulating fetal growth, the biological process of placentation should be examined.

Placentation in the Human

Placentation begins early in embryonic development (6-7 days post coitus; dpc), upon invasion of the blastocyst into the uterine endometrium (reviewed in Frank, 2011). The blastocysts consist of an outer layer of trophoblast cells which gives rise to the placenta, and an inner cell mass which develops into the fetus and umbilical cord (reviewed in Frank, 2011). Fetal contributions to the placenta include the allantois, which is derived from the fetal hindgut, as well as mesenchymal cells which are essential for fetal villi branching and development (reviewed in Rhodes et al., 2011; Frank, 2011). Prior to invasion, the trophoblast is constituted by cytotrophoblast cells which undergo subsequent proliferation to form a double layer of cells, the outer layer of which differentiates into syncytiotrophoblast cells (reviewed in Frank, 2011). During the prelacunar period, a robust increase in syncytiotrophoblast volume occurs, which is driven by continuous proliferation of the inner cellular trophoblastic layer and its subsequent fusion with daughter cells (reviewed in Frank, 2011). The lacunar period (8-13 dpc) is marked by the appearance of vacuoles in the syncytiotrophoblast to form lacunae, separated by trabeculae, which eventually develops into the intervillous space (reviewed Rhodes et al., 2011; Frank, 2011). Both the lacunae and trabeculae are

covered by two uninterrupted syncytial layers; the trophoblastic shell (endometrial facing) and the primary chorionic plate (blastocyst cavity facing; reviewed in Frank, 2011).

Fetal villi development

Fetal villi formation begins at the primary chorionic plate, by trophoblastic proliferation inside the trabeculae, which give rise to the anchoring villi by driving longitudinal growth and branching (reviewed in Frank, 2011). Primary villi are formed when the anchoring villi protrude into the lacunae (reviewed in Frank, 2011). At 12-13 dpc, the lacunae first begin to be perfused by maternal blood as the trophoblastic shell begins eroding maternal endometrial vessels (reviewed in Rhodes et al., 2011; Frank, 2011). Invasion of the extraembryonic mesenchyme into the primary villi between 15-20 dpc, transform them into secondary villi (reviewed in Rhodes et al., 2011; Frank, 2011). The extraembryonic mesenchyme also promotes angiogenesis by facilitating the secretion of angiogenic growth factors or by differentiating into angiogenic and hematopoietic precursor cells (reviewed in Frank, 2011). From 18-20 dpc, the first fetal capillaries develop within the mesenchyme which marks the formation of tertiary villi (reviewed in Rhodes et al., 2011). Tertiary fetal villi formation occurs concurrently with the invasion of the vascularized allantois (fetal) into the chorionic plate, fusing with the intravillous capillary bed (reviewed in Frank, 2011). By 5 weeks post conception, a primitive but complete fetoplacental circulation is established (reviewed in Rhodes et al., 2011). By 6 weeks post conception, the basal lamina begins to form around this fetoplacental capillary network which undergoes dynamic modifications to form

endothelial tubes within the larger villi (Ahmed & Perkins, 2000). Tertiary villi are transformed into immature intermediate villi and after 15 weeks post conception, into stem villi (reviewed in Rhodes et al., 2011). When the length of the fetal villi has been exceeded by the intermediate villi vessels, the formation of terminal villi occurs (Ahmed & Perkins, 2000). The placental vessels of terminal villi are in such close proximity to the intervillous space, such that only syncytiotrophoblast cells separate the placental and maternal blood supplies to facilitate nutrient transfer and waste exchange (Ahmed & Perkins, 2000). Furthermore, to increase the maternal blood supply to the intervillous space, extravillous trophoblast invade the maternal spiral arteries and begin remodeling them to make them wider and less vasoactive (Moll et al., 1988; Kaufmann and Castellucci 1997; reviewed in Frank, 2011).

Angiogenesis and vascular development

The exact shape of the fetal villous tree is dictated by a variety of factors that dynamically shift across pregnancy. The first half of gestation, when oxygen tension is extremely low, is characterized by primarily branching angiogenesis but transitions to mostly non-branching angiogenesis from 26 weeks until term (Ahmed & Perkins, 2000). The process of angiogenesis is orchestrated by the sophisticated interplay of many angiogenic and angiostatic factors. Angiogenic promoting factors include fibroblast growth factors (acidic and basic), angiopoietins 1 & 2 (ANGPT1 & ANGPT2), placental growth factor, transforming growth factor α, tumor necrosis factor α, and the vascular endothelial growth factor family (reviewed in Rhodes et al., 2011). Angiogenic inhibiting factors include angiostatin, endostatin, interferons, and platelet factor 4 (reviewed in

Rhodes et al., 2011). Only a subset of the extensively investigated angiogenic factors will be further discussed.

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is a family of disulfide-linked homodimeric glycoprotein which primarily act on endothelial cells to function as a mitogen, morphogen, and chemoattractant (Neufield et al., 1994). They are stimulated by a variety of factors including cytokines, hormones, and low oxygen tension, and work in concert with other factors to stimulate branching angiogenesis (reviewed in Rhodes et al., 2011). One of VEGF's regulators includes the transcription factor hypoxia inducible factor-1α, which interacts with the hypoxia response element of VEGF in times of low oxygen to increase expression (Wang et al., 1995). One of the primary VEGF family isoforms frequently investigated for aberrant placental angiogenesis is VEGF-A. Placental growth factor is also a member of this gene family (Cao et al., 1997). Vascular endothelial growth factor potentiates its actions through its kinase domain receptors including VEGFR1 (Flt1- 1) and VEGFR2 (KDR or Flk-1; Thomas, 1996).

Angiopoietins

Angiopoietins are expressed primarily by endothelial cells, potentiating their actions through tyrosine kinase receptor, TEK, which participates in the activation of downstream signaling pathways including both PI3-kinase/AKT and ERK (Fagiani and Christofori, 2013; Thurston and Daly, 2012; Geva et al., 2002). Specifically, ANGPT1 functions as an agonist for TEK, and ANGPT2 as a TEK antagonist (Thurston and Daly,

2012; Geva et al., 2002). During the first trimester, low oxygen tension increases the ANGPT2 expression as well as VEGF-A as previously discussed (Geva et al., 2002; Erickson et al., 2005). Together, these two factors encourage branching angiogenesis by keeping the vessel in a more plastic state, with VEGF-A providing the signal for sprouting and ANGPT2 encouraging vessel destabilization at the leading-edge of the invading vascular sprout (Geva et al., 2002; Erickson et al., 2005). After the second trimester however, ANGPT2 expression begins to decline in favor of ANGPT1 and non-branching angiogenesis (Geva et al., 2002). Functionally, ANGPT1 acts as a weak mitogenic signal for endothelial cells and causing maturation and vascular stabilization (Geva et al., 2002). Fundamentally, the shift in ANGPT1:ANGPT2 ratios later in pregnancy favors non-branching angiogenesis (Geva et al., 2002; Erickson et al., 2005). A discussion of aberrant expression of angiogenic factors and regulators, and their potential role in pregnancy pathologies will be included in the section of this literature review on intrauterine growth restriction.

Adaptations over gestation

To accommodate the ever-increasing fetal oxidative demands, the placenta must adapt over gestation. As gestation progresses, placental weight, diameter, villous volume and surface area, and umbilical cord length all increase (Benirschke et al., 2006). In contrast, the maternofetal diffusion distance and villous trophoblastic thickness both decrease (Benirschke et al., 2006). These decreases in diffusion distance and trophoblastic thickness are driven by several factors. First, there is a shift in the composition of trophoblastic cells as gestation progresses to include only 20%

cytotrophoblasts on villous surfaces at term (reviewed in Frank, 2011). Secondly, the fetal villi diameter continues to decrease as gestation progresses (Benirschke et al., 2006). These factors support more efficient transfer of substrates.

Placentation in the Sheep

The sheep placenta is characterized by the presence of placentomes; discrete sites of interdigitation between fetal (cotyledonary) and maternal tissue (caruncular). The maternal portion of the placentome first develops *in utero* and is comprised of highly vascularized, aglandular uterine epithelium (reviewed in Regnault et al., 2002). The fetal contributions of the placentome, originating from the trophectoderm (chorion), intimately associate with the raised surfaces of the caruncle (reviewed in Regnault et al., 2002). While the sheep placenta structurally differs from the human, there are functional similarities including 1) structural similarities of the fetal villous tree, 2) the relative maturity of the fetus at parturition, and 3) tolerance towards repeated blood samplings via chronic catheterizations of singleton pregnancies (reviewed in Barry and Anthony, 2008). It is for these reasons that the sheep has become a valuable biomedical model for human medicine.

Placentome development

The ruminant placenta, unlike the human placenta, is non-invasive, and forms specialized zones for nutrient exchange where the trophectoderm fuses with the maternal epithelium (Wooding, 1982). At approximately 15 dpc, the highly vascularized allantois expands from the fetal hind gut which is responsible for fusing with the chorion

to develop the fetal placental vasculature (Regnault et al., 2002). By approximately 4 weeks post conception, the primitive fetal villi are being formed in areas of close association between the chorion and caruncle as interdigitation becomes apparent (reviewed in Regnault et al., 2002). Just as in the human, the ovine fetal villi can be divided into stem, intermediate, and terminal villi (Leiser et al., 1997; Kaufman, et al., 1979; Kaufman, 1982). Approximately 15-20% of the trophectoderm contains binucleate cells (BNCs), which form a partial syncytium between maternal and fetal cells (reviewed in Wooding and Burton, 2008). The functions of BNCs are vast and varied, but includes delivering both hormones and effectors to communicate with the maternal system as well as support fetal growth (reviewed in Wooding and Burton, 2008).

Placental angiogenesis in the sheep

The same placental angiogenic regulators discussed previously also play a role in sheep placental angiogenesis. During the first 30 days of gestation, in unperturbed sheep pregnancies, there is a progressive increase in the message of VEGF, VEGFR1, ANGPT1, and ANGPT2 in the cotyledons, with corresponding increases of ANGPT1, ANGPT2, and VEGFR1, VEGFR2 in the caruncles (Grazul-Bilska et al., 2011; Grazul-Bilska et al., 2010). From day 50 to 90 in normal sheep pregnancies, there is an increase in cotyledonary VEGF, as well as a continuous increase in ANGPT1 and ANGPT2 message as gestation progresses (Borowicz et al., 2007). However, in fetal growth restriction pregnancies, placental vascular architecture is already modified by 90 dGA (Regnault et al., 2002) and this translates to altered placental hemodynamics (Regnault et al., 2003; Galan et al., 2005). Near term, fetal growth restricted sheep

pregnancies have documented reductions in VEGF, VEGFR1, ANGPT2, and TEK (Regnault et al., 2003; Erickson et al., 2005) which mirrors the human (reviewed in Barry and Anthony, 2008). Much more work has been done examining placental angiogenic factors in perturbed sheep pregnancies and will be discussed later.

Adaptations over gestation

As gestation progresses, the caruncles continue to develop deeply branched crypts to support cotyledonary villi elongation and branching as it becomes more intimately associated with the caruncle to maximize surface area which is related to uteroplacental size (reviewed in Regnault et al., 2002). In the sheep, the window of maximal placental growth occurs approximately between 40 dGA and 75-80 dGA, with maximal protein and dry matter accretion at 55 dGA (Ehrhardt and Bell, 1995).

Placental perfusion and structure

To accurately assess placental function, it is critical to examine factors that influence the placenta's ability to transfer substrates. This process of substrate transfer is complex and limited by an intricate set of variables including 1) rate of blood flow (maternal and fetal), 2) the pattern of placental perfusion, 3) the physical properties of the placenta including surface area, thickness, and other physiochemical properties, 4) the metabolic demands of the placenta, and 5) mechanism of transfer for a given molecule (reviewed in Meschia and Battaglia, 1986). Factors influencing maternal and fetal control of blood flow will be discussed in depth in a separate section of this literature review and thus, will not be further described here.

Patterns of placental perfusion

The second factor that can influence the ability of the placenta to transfer a given molecule is the efficiency of placental perfusion. This constitutes a categorical attempt to classify placental efficiencies based on the maternal to fetal geometrical arrangement of capillaries or blood flows. With this approach, maximal efficiency of nutrient transfer (especially oxygen) occurs in countercurrent systems; when two capillary streams run in opposite directions (Leiser and Kauffman, 1994; Pardi and Cetin 2006). The least efficient means of capillary arrangement is concurrent, when both capillary streams run the same direction (Leiser and Kauffman, 1994; Pardi and Cetin 2006). Both the human and the sheep have an intermediate capillary arrangement, described as multivillous, a mixture between countercurrent and concurrent (Lesier and Kauffman, 1994).

In the human, maternal blood from uterine spiral arteries is ejected into the intervillous space and drains from the intervillous space via branches of the uterine vein (reviewed Battaglia and Meshia, 1986). This creates fountain-like blood streams that flow across the villous trees (Lesier and Kauffman, 1994). It is difficult to visualize placental perfusion patterns, but it is likely that the human placenta functions as a venous equilibrator (reviewed in Battaglia and Meschia, 1986). On the fetal side of circulation, hairpin-like capillary loops have flow in opposite directions, and the maternal vessels lie approximately 90 degrees to the fetal circulation (Faber and Thornburg, 1983; Kaufman, 1985).

In the sheep, maternal arteries on the maternal surface of the caruncle tend to branch at right angles off the main vessel, whereas the fetal vessels have a central arteriole with fewer branches off the tip of the villi (reviewed in Battaglia and Meschia,

1986). Similar to the human, the sheep placenta has demonstrated the properties of a venous exchanger but cannot be classified as either countercurrent or concurrent (reviewed in Battaglia and Meschia, 1986). This is supported by studies conducted by Battaglia and Meschia, 1986 which found that an ideal venous equilibrator has a flow-limited clearance of inert substrates equal to the product of flows divided by their sum. The sheep in those studies actually had flows 20% less than the estimated ideal venous equilibrator, suggesting that the sheep placenta functions somewhat less effectively than an ideal venous equilibrator, likely due to uneven placental perfusion (reviewed in Battaglia and Meschia, 1986).

Placental morphology

The third variable influencing rate of transfer of molecules is the physical properties of the placenta such as thickness, surface area, etc. From an evolutionary perspective, there is incredible diversity in placental structure and morphology. Thus, a variety of strategies have been employed to morphologically categorize placentas including by types of membranes, shape, maternofetal interdigitation, layers of interhaemal barrier, invasiveness, formation of decidual cell reactions, and maternofetal blood flow (Lesier and Kaufmann, 1994).

The most frequently utilized placental classification strategy was developed by Grosser in 1909 based on interhaemal membrane layers; layers of separation between maternal blood and fetal trophoblast (chorionic) tissue (reviewed in Leiser and Kaufman, 1994). The first of Grosser's classification is the epitheliochorial placenta. In this type, the chorion attaches to the uterine mucosal epithelium and represent the placentas of

the major livestock species (reviewed in Battaglia and Meschia, 1986; Leiser and Kaufmann, 1994). Sheep are described as having a variation of epitheliochorial placentas called synepitheliochorial, where trophoblast cells fuse with the uterine epithelium to form hybrid cells called symplasms (Wooding, 1992). The second of Grosser's categories is the endotheliochorial placenta, which involves close proximity of the chorion to the uterine endometrial capillaries (reviewed in Battaglia and Meschia, 1986; Leiser and Kaufmann, 1994). This describes the placentas of many carnivores including the domestic dog (reviewed in Battaglia and Meschia, 1986; Leiser and Kaufmann, 1994). The last of Grosser's classifications is the hemochorial placenta (human and rodent), in which the chorion comes into direct contact with maternal blood by invading the uterine mucosa and degrading the endometrial capillaries (reviewed in Battaglia and Meschia; 1986, Lesier and Kaufmann, 1994). However, from a functional perspective, Grosser's classification grossly oversimplifies placental transfer.

It would seem intuitive that placentas with fewer tissue layers separating maternal and fetal circulation (hemochorial) would be more efficient than species with more layers (epitheliochorial). This assertion, however, does not hold up *in vivo* as assessed by physiological studies evaluating placental efficiency (placental mass: fetal mass; Dantzer et al.,1988). For instance, some species with epitheliochorial placentas such as the pig (9 grams fetus: 1 gram placenta) or horse (14 grams fetus: 1 gram placenta) produce more grams of fetal mass per unit of placental mass (Dantzer et al., 1988). The human placenta produces 6 grams fetus: 1 gram placenta, which supports the functional complexity of placental physiology as deduced by morphology (Dantzer et al., 1988; reviewed in Leiser and Kaufmann, 1994). Additionally, the shortcomings of

this classification strategy are further illustrated by the relative similarities in placental barrier depth between the epitheliochorioal pig placenta (six-layered barrier) in comparison to the hemochorial placentas (three-layered barrier) of the human (Leiser and Kaufmann, 1994). This is due in part due to Grosser's classification not accounting for additional layers of separation within a placental "layer" such as the trophectoderm. Example prima facie is the human fetal villi consist of five layers of cells including a continuous layer of syncytiotrophoblasts, a layer of cytotrophoblasts, trophoblastic basal lamina, the extraembryonic mesoderm derived connective tissue, and a fetal endothelium (reviewed in Frank, 2011).

Thus, in spite of morphological differences, placentas from livestock species are not necessarily less efficient than human placentas and possess similar placental barrier distances. An astute thinker might question whether additional layers of separation, in spite of similarities in placental barrier depth, would alter the uptakes of readably diffusible gases like oxygen. Battaglia and Meschia (1986) evaluated oxygen uptakes in chronically catheterized, late-gestation sheep and found that umbilical oxygen uptakes were approximately 1.18 mmol/min. To assess this metric in the human, Pardi and Certi (2006) utilized ultrasound and arteriovenous differences in umbilical oxygen content to estimate human umbilical oxygen uptakes. They found that human umbilical oxygen uptake ranged between 0.9 – 1.20 mmol/min, closely rivaling the sheep (Pardi and Certi, 2006). Thus, in spite of morphological differences, it does appear that placental function is quite similar between the human and livestock species.

Transport of Substrates

Another critical factor that determines placental function is the mechanism of transfer of substrates across the placenta. A variety of transport mechanisms exist within the placenta to facilitate the movement of substrates including simple diffusion, restricted diffusion, facilitated diffusion, active transport, and receptor mediated endocytosis (Morris et al., 1994). Transport capacity however is dependent on placental size, perfusion patterns and surface area, blood flow and transporter abundance (Fowden et al., 2006; reviewed in Barry and Anthony, 2008). In order to assess the potential ramifications of placental dysfunction on transport capacity, it is critical to examine the mechanism or transport system by which substrates are transported across the placental tissues.

Diffusion

Diffusional transfer, represented by Fick's Law, is impacted by the maternofetal concentration gradient, the surface area available for transfer, and the permeability of the placenta (reviewed in Jones et al., 2011). Broadly speaking, molecules that diffuse across the placenta have two potential routes; the hydrophilic route and the lipophilic option (reviewed in Jones et al., 2011).

The diffusion of lipophilic molecules such as respiratory gases or anesthetic agents are capable of diffusing through the trophoblast cell surface (Wilkening and Meschia, 1983). Lipophilic diffusion is flow limited as it is dependent on blood flow velocity and the patterns of placental perfusion (Faber and Thornburg, 1983). In contrast, some hydrophilic molecules (sucrose, mannose, etc) lack specific transport

mechanism and cannot permeate the trophoblast (reviewed in Jones et al., 2011). Thus, such molecules are dependent on porous leak pathways to cross the placental barrier, making these substances membrane-limited in their transport potential (reviewed in Jones et al., 2011). The ruminant synepitheliochorial placenta, with smaller paracellular channels, is less permeable than the human hemochorial placentas to hydrophillic molecules that lack transporter systems (Faber and Thornburg, 1983).

The net molar flux of water across the placenta is the greatest out of any substrate including oxygen, which is not surprising as the term human conceptus is comprised of 80% water (Barcroft, 1946; Seeds, 1965). Water flux appears to be driven by hydrostatic and osmotic pressure gradients between maternal and fetal circulation, with transcellular flux likely being facilitated by aquaporins (Faber and Thornburg, 1981; Damiano et al., 2001). There is debate about the relative importance of transcellular vs. paracellular water transport and much work remains to better understand the control of water transfer.

Transporter-mediated transfer

Transporter proteins are responsible for the trafficking of small hydrophilic substrates across the placental plasma membrane. A subset of transporter proteins are carriers, translocating the solute via either electrochemical gradients (cotransport of an ion) or from the hydrolysis of ATP (active transport; reviewed in Jones et al., 2011). Electrochemical gradients are set-up by the diffusion of ions active transport (Stein, 1990). Two mechanisms of transplacental active transport include 1) pump-leak which is the mechanisms for many amino acids or 2) leak-pump mechanisms which is likely

the method for Ca^{2+} flux (reviewed in Jones et al., 2011). The pump-leak mechanism works by creating a higher intercellular concentration in the microvillous membrane than in maternal circulation which allows the substrate to leak across the opposite plasma membrane (reviewed in Jones et al., 2011). The leak-pump mechanism works by pumping the substrate out of the trophoblast at the basal membrane to permit the influx of the substrate from maternal plasma at the syncytium (reviewed in Jones et al., 2011).

Glucose

One of the most critical substrates for fetal and placental growth is glucose. Placental glucose transfer is characterized by saturable, carrier mediated transport that is dependent on the maternofetal concentration gradient (Widdas et al., 1952; reviewed in Hay, 1991). Glucose is primarily transported via sodium-independent transporter proteins via facilitated diffusion (Ehrhardt and Bell, 1997; Wooding et al., 2005). Functionally, glucose must be shuttled from maternal circulation by the glucose transporters on maternal facing microvillous membrane of the trophoblast, then across the cytoplasm, before being transported across the fetal facing basal membrane of the trophoblast to reach fetal circulation (Barry and Anthony, 2008). While a variety of glucose transporters have been identified in various tissues, only SLC2A1 (GLUT1) and SLC2A3 (GLUT3) have been localized to the appropriate locations of the trophectoderm, to participate in the placental glucose transport system in humans and sheep (Wooding et al., 2005; Illsley, 2000).

The most abundant placental glucose transporter SLC2A1, has been localized to both the basal (fetal facing) and microvillous (maternal-facing) membranes in the

syncytiotrophoblast (Jansson et al., 1993; Hauguel-de Mouzon et all 1994; Farrell et al., 1992; Barros et al., 1995; Jansson et al., 1993). The abundance of SLC2A1 on the microvillous membrane is much greater, possibly to increase glucose extraction from maternal circulation (Barry and Anthony, 2008). Additionally, SLC2A1 increases expression across gestation (Jansson et al., 1993; Hauguel-de Mouzon et all 1994). In the sheep, due to the fusion of binucleate cells and maternal epithelial cells, SLC2A1 has been localized to base of the syncytial layer of the placenta, as well as the basolateral membrane of the trophoblast (Wooding et al., 2005). In spite of its abundance, SLC2A1 has relatively low affinity in comparison to the other class 1 facilitated diffusion glucose transporter, SCL2A3 (Simpson et al., 2008).

In spite of some inconsistencies in the human literature, SLC2A3 message has been found in the human trophoblast (Illsley, 2000; Wooding et al., 2005). SLC2A3 protein has primarily been located in the microvillous membrane of the syncytiotrophoblast (Jansson et al., 1995; Hauguel-de Mouzon et al., 1997; Hahn et al., 2001; Ericsson et al.,2005). In the sheep, SLC2A3 has been localized to the microvillar junction between the maternofetal syncytium and the trophoblast (Wooding et al., 2005). As gestation advances, the concentrations of SLC2A1 decrease and SLC2A3 increase in the sheep (Ehrhardt and Bell, 1997). While SLC2A1's higher abundance has encouraged greater research emphasis, SLC2A3 has a higher affinity for glucose and a 5 to 6-fold greater transport capacity, which potentially offsets its lower relative abundance (Simpson et al., 2008). While there is some variation in reporting relative abundance of SLC2A3 across gestation, some evidence supports that SLC2A3 is most abundant during the first half of gestation in humans (Brown et al., 2011). Another

glucose transporter (class 3), SLC2A8 (GLUT8) is speculated to play a role in intercellular glucose transport and more work needs to be conducted to determine its role during pregnancy.

Endocrine factors can alter the expression of glucose transporters. Insulin like growth factor 1 has been demonstrated to increase SLC2A1 in human trophoblasts (Baumann et al., 2014), and intraamniotic infusions of insulin-like growth factor 1 (IGF1) increased placental expression of SLC2A1 in the sheep (Wali et al., 2012). Additionally, deficiency of the placental hormone chorionic somatomammotropin (CSH) resulted in suppressed SLC2A1 and SLC2A3 message, and SLC2A3 protein in the sheep (Jeckel et al., 2018).

Amino acids

Amino acids also function as a critical oxidative substrate in supporting appropriate fetoplacental growth and development. They are transported across the syncytiotrophoblast via active transport by a variety of different mechanisms (reviewed in Jones et al., 2011). The complexity of amino acid transport is supported by the redundancy of multiple transport systems for neutral, anionic, and cationic amino acids resulting in at least 15 different transporter proteins (Regnault et al., 2005). However, categorization of amino acid transporter systems can be conducted on the bases of substrate specificity, ion-dependence, light and heavy chain interactions, transport kinetics, and regulation of activity (Regnault et al., 2005).

Active transport of amino acids occurs in three steps: 1) amino acids are extracted from maternal circulation across the microvillous membrane, 2) amino acids are shuttled

through the cytoplasm of the trophoblast, and 3) amino acids are transported across the basal membrane of the trophoblast (Regnault et al., 2005). Examples of neutral amino acid transporters include: System A, System B^0, System b^{0+} and System L (Regnault et al., 2005). Examples of anionic transporters include System ASC and System X_{AG}^-, and cationic transporters such as System y^+L and System y^+ (Regnault et al., 2005).

Out of these transport systems, System A and System L have been most heavily investigated (Regnault et al., 2005). System A is a sodium-dependent transport system for small neutral amino acids (SNATs; SLC38A1-6; Mackenzie and Erickson, 2004) and is stimulated by insulin, leptin, IGF1 and interleukin 6 (Fang et al., 2006; Jansson et al., 2003; Jones et al., 2009; Roos et al., 2009). System L shuttles large neutral amino acids (LATs; SLC7A5-13 and SLC7A15; Fotiadis et al., 2013), taking up branch chain amino acids by exchanging non-essential amino acids (Regnault et al., 2005). System L is a sodium-independent transport system stimulated by glucose and insulin as well as the availability of nonessential amino acids to exchange (Roos et al., 2009; Verrey, 2003). Another transport system impacted by IUGR include the CATs (System y+; SLC7A1–4 and SLC7A14; Fotiadis et al., 2013) which are also stimulated by IGF1 in the sheep (Wali et al.,2012).

Carrier-mediated transfer

Molecules can also be transported bidirectionally across the placenta via endocytosis or exocytosis. The endocytotic pathways of phagocytosis (larger particles) and pinocytosis (smaller fluid phase particles) in the human can include substrates like gamma immunoglobulin G, the transferrin-iron complex and low-density lipoprotein

(reviewed in Jones et al., 2011). These processes are complicated by the fusion of vesicles with lysosomes as well as asymmetric transport (reviewed in Jones et al., 2011). Less is known about this process in the epitheliochorial placenta.

To transport lipids across the placenta, three primary mechanisms exist: 1) direct transfer by carrier-mediated transport, 2) placental synthesis and secretion of lipids into fetal circulation, and 3) hydrolysis of lipid sources from either maternal or fetal circulation (reviewed in Hay, 1991). While many fatty acids are transported via direct carrier, some studies have suggested that the trophoblastic peroxisomes may metabolically alter the fatty acid chain length to increase the transfer of medium-chain fatty acids to the fetus (reviewed in Hay, 1991).

Placental Endocrine Function

Another key function of the placenta is as an endocrine organ, capable of secreting a wide variety of factors necessary to signal for maternal recognition of pregnancy, modify maternal physiology to sustain pregnancy, and influence the successful outcome of pregnancy (reviewed in Siler-Khodr, 2011). Fundamentally, placental-produced hormones can be broken down into several functional categories including maternal recognition of pregnancy, growth hormone/prolactin family hormones, hypothalamic-like releasing hormones, steroidogenic hormones, chorionic growth factors, eicosanoids, and other substances like cytokines and enzymes (reviewed in Siler-Khodr, 2011). This discussion is not exhaustive, but rather, an attempt to highlight well documented hormones that play a key role in pregnancy physiology.

Maternal recognition of pregnancy

One key function of the developing trophoblast is to provide the signal for maternal recognition of pregnancy. In the human, the syncytiotrophoblast cells produce chorionic gonadotropin (hCG), a glycoprotein which supports corpora lutea function and later stimulates placental steroidogenesis (reviewed in Siler-Khodr, 2011; reviewed in Napso et al., 2018). By approximately 25 h post implantation, hCG is detectable in maternal circulation with peak secretions 9-10 weeks after the last pre-conception menstruation, then decreases into mid-gestation (reviewed in Siler-Khodr, 2011). Chorionic gonadotropins are not produced by the sheep placenta. Instead, the trophectoderm synthesizes and secretes interferon tau (IFNτ) as the maternal recognition of pregnancy signal which functions as an anti-luteolytic agent from approximately between 10-25 days post conception (Roberts et al., 1999; Bazer, 1992).

Chorionic somatomammotropin

A key category of hormones secreted by the placenta is the growth hormone/prolactin family. One of the most notable members of this family is the hormone chorionic somatomammotropin (CSH; aka placental lactogen). In the human placenta, CSH is synthesized by the syncytiotrophoblast cells and is produced by the binucleate cells in the ruminant placenta (reviewed by Anthony et al., 1995). The contributions of CSH to placental endocrine secretions are not trivial, as it is responsible for approximately 10% of all protein synthesis in the placenta near term (reviewed in Siler-Khodr, 2011). Circulating levels of CSH increase over gestation (Regnault et al., 1999). In spite of the discovery of CSH in 1962, the exact function of CSH remains to be

elucidated but it has been postulated to contribute to fetal and placental growth as decreases in circulating maternal CSH levels were associated with IUGR in both the human and the sheep (Spellacy et al., 1976; Lea et al., 2007; Regnault et al., 1999). It was postulated by Handwerger (1991) that CSH has separate actions on maternal and fetal physiology. He suggested CSH acts to mobilize maternal nutrients for transfer to the fetus, and acts on fetal circulation to increase nutrient uptake (Handwerger, 1991). Recently, the necessity of CSH for fetal and placental growth was directly demonstrated using an *in vivo* model of lentiviral-mediated RNA interference (RNAi) in sheep, with CSH deficient pregnancies resulting in marked decreases in fetal and placental mass (Baker et al., 2016). Chorionic somatomammotropin will be discussed in depth later in this literature review.

Steroidogenic hormones

The placenta is the primary source of sex steroid hormones throughout pregnancy for both the human and sheep, and aberrant production of steroid hormones by the placenta has been implicated in pregnancy complications in the human such as gestational diabetes and preeclampsia (reviewed in Napso et al., 2018). Not surprisingly, steroid hormones are believed to play a role in not only maintaining pregnancy but also in altering maternal physiology to support the growing conceptus (reviewed in Napso et al., 2018).

One of the essential placental-derived hormones is progesterone, which acts to prepare and maintain an appropriate uterine environment capable of sustaining pregnancy. In both the sheep and the human, a luteo-placental shift occurs as the

placenta takes over progesterone production during gestation (Meyer, 1994, Mitchell and Taggart, 2009). This luteo-placental shift is one of the factors that allows sheep to be utilized for chronic cannulations which will be discussed in depth later in this review. The majority (90%) of the progesterone produced by the placenta is secreted into maternal circulation with only about 10% into fetal circulation (reviewed in Siler-Khodr, 2011).

The placenta is also the primary source of estrogens during pregnancy which act in concert with progestins to fundamentally support successful pregnancy outcomes. These estrogens are derived from fetal androgens such as androstenedione, dehydroepiandrosterone (DHEA) or sulfonated DHEA (DHEAS) (reviewed in Schule et al., 2018). Species variation exists regarding the primary active estrogen (estradiol, estriol, etc.) produced by the placenta, but estrogens fundamentally act to stimulate uterine growth, blood flow, vascular function, metabolism, and uterine contractility (reviewed in Siler-Khodr, 2011; Pepe and Albrecht, 1995). The majority of estrogens are secreted into maternal circulation vs. fetal circulation (reviewed in Siler-Khodr, 2011).

Placental steroidogenesis requires contributions from both maternal and fetal circulation. In the human, cholesterol derived from low-density lipoprotein to supplied to the syncytiotrophoblast cells by maternal circulation which convert cholesterol to 1) pregnenolone which is transported to the fetus, and to 2) progesterone which is primarily secreted in maternal circulation (Kallen, 2004). The fetus then produces the androgen DHEA-SO4 which is converted to estrogen by the placenta (Kallen, 2004) and secreted into maternal circulation.

Growth factors

Insulin-like growth factors (IGF) 1 and 2 are produced by the placenta, with IGF1 being localized to the syncytiotrophoblast cells (Han and Carter, 2000; Hill et al., 1993). Placental IGF1 and IGF2 are also present in the sheep placenta (Jeckel et al., 2018). The IGF axis is complex and regulated at many steps, which will be more completely discussed later in this literature review. Among its many functions in the placenta, IGFs enhance the placental transport of glucose and amino acids and dysfunction is thought to contribute to the development of the IUGR phenotype (Kniss et al., 1994; Zumkeller 2000; Gluckman and Harding, 1997). Both syncytiotrophoblast and binucleate cells produce IGF binding proteins (IGFBPs) which are responsible for regulating the actions of IGFs. They act as carrier molecules to transport, reduce the bioavailability of the IGFs, and protect them from degradation (Han and Carter, 2000; Hill et al., 1993; Jeckel et al., 2018). The placental expression of IGF1, IGF2, and IGFBP2 and 3 are reduced in CSH deficient sheep placenta (Jeckel et al., 2018).

The placenta is also a site of relaxin production, with relaxin receptors being localized to the syncytiotrophoblast (Sakbun et al., 1987). Relaxin can act as a potent vasodilator, as well as stimulate collagenase activity and prostaglandin release for parturition (reviewed in Napso et al., 2018, reviewed in Siler-Khodr, 2011).

Eicosanoids

The roles of eicosanoids across pregnancy are vast and varied, ranging from assisting with implantation (thromboxane, prostaglandins and prostacyclins) to supporting fetal growth to supporting parturition (reviewed in Siler-Khodr, 2011).

Prostacyclins are potent uterine vasodilators and thus can play a role in perfusion of the uteroplacental unit (Ylikorkala et al., 1983).

Placental Metabolism and Nutrient Transfer

In the teleological sense, the main purpose of the placenta is to supply adequate nutrients to meet fetal growth potential while still satisfying its own exacting metabolic needs. The ability of the placenta to achieve this purpose is dependent on appropriate placentation and perfusion, endocrine function, availability of substrates, transport capacity, and the oxidative demands of the placenta. Due to understandable ethical limitations associated with invasive investigations of human placental-fetal physiology, much of our understanding of placental transport capacity and metabolism has been derived from the pregnant sheep (Barry and Anthony, 2008). Thus, the following section, primarily based on data collected from the pregnant sheep, will discuss important factors necessary for the functional uptake and transfer of nutrients by the placenta. The equations necessary to calculate uterine and umbilical substrate uptakes and uteroplacental utilization are summarized in Table 1.

Table 1.1 Calculations[1] nutrient uptake and utilization by the transplacental diffusion technique.

Nutrient Uptake and Utilization Rates	
Umbilical Oxygen Uptake (fetal oxygen utilization; UOU; mmol/min)	$UBF * ([O_2]_{\gamma(WB)} - [O_2]_{\alpha(WB)})$
Uterine Oxygen Uptake (UtOU; mmol/min)	$UtBF * ([O_2]_{A(WB)} - [O_2]_{V(WB)})$
Uteroplacental Oxygen Utilization (mmol/min)	UtOU – UOU
Plasma to WB Glucose Conversion	$[G]_{pl} * [1 - (0.24 * Hct)] - (3.3 * Hct)$
Umbilical Glucose Uptake (UGU; µmol/min)	$UBF * ([G]_{\gamma(WB)} - [G]_{\alpha(WB)})$
Uterine Glucose Uptake (UtGU; µmol/min)	$UtBF * ([G]_{A(WB)} - [G]_{V(WB)})$
Uteroplacental Glucose Utilization (µmol/min)	UtGU – UGU
Umbilical Lactate Uptake (µmol/min; ULU)	$UBF * ([L]_{\gamma(pl)} - [L]_{\alpha(pl)})$
Uterine Lactate Secretion (µmol/min; UtLS)	$UtBF * ([L]_{V(pl)} - [L]_{A(pl)})$
Uteroplacental Lactate Production (µmol/min)	ULU + UtLS
Umbilical AA Uptake (µmol/min; UAAU)	$UPF * ([AA]_{\gamma(pl)} - [AA]_{\alpha(pl)})$
Uterine AA Uptake (µmol/min; UtAAU)	$UtPF * ([AA]_{A(pl)} - [AA]_{V(pl)})$
Umbilical AA Carbon Uptake (µmol/min; UCU)	(#AA carbons) * UAAU
Uterine AA Carbon Uptake (µmol/min; UtCU)	(#AA carbons) * UtAAU
Umbilical AA Nitrogen Uptake (µmol/min; UNU)	(#AA nitrogens) * UAAU
Uterine AA Nitrogen Uptake (µmol/min; (UtNU)	(#AA nitrogens) * UtAAU

[1]Calculations are derived from Cilvik et al., 2021.

Oxygen

Placental oxygen transport (diffusion) capacity across the placenta is incumbent upon rates of blood flow (uterine and umbilical), the oxygen carrying capacity of maternal and fetal blood, hemoglobin oxygen binding efficiency, placental permeability, placental surface area, and oxygen consumption by the placenta (Carter 1989; Barry and Anthony, 2008). When any of these factors are perturbed, deficits in uteroplacental oxygen delivery to the fetus can occur and lead to fetal hypoxemia and subsequent IUGR.

In both the sheep and the human, uterine and umbilical blood flows rise by 3-fold and 2.5-fold respectively over gestation which increases the perfusion of the uteroplacental unit and drives the delivery of oxygen to the fetus (Konje et al., 2003; Meschia, 1984). Factors controlling blood flow will be covered in depth later in this literature review. However, it is worth noting that reductions in uterine blood flow can result in reduced fetal oxygen delivery below the critical threshold necessary to preserve oxygen consumption in the fetus, leading to fetal hypoxemia. Reductions in either fetal or maternal blood oxygen carrying capacity, or changes in fetal hemoglobin oxygen affinity can also lead to fetal hypoxemia (Wilkening et al., 1983; Barry and Anthony, 2008).

Meschia et al. (1965) investigated factors that could influence the uptake and transfer of oxygen by the placenta including the 1) transplacental difference in oxygen tension, 2) the rate at which oxygen crosses the placenta relative to fetal weight, and the 3) diffusing capacity of the placenta for oxygen. Using chronically catheterized sheep, blood oxygen tension was measured by determining the arteriovenous

differences in the partial pressure of oxygen (pO_2) based on equations documented in Meschia et al. (1961). The rate of oxygen transfer across the placenta was calculated by the Fick principle requiring the rate of blood flow for a specific vessel (umbilical, uterine, etc.) and the arteriovenous difference of oxygen (Meschia et al., 1965). The mathematical principles necessary to utilize the transplacental diffusion technique will be discussed in depth, later in this dissertation. Meschia et al. (1965) determined the transplacental oxygen gradient was relatively constant over gestation. However, the placental uptake of oxygen (rate of transfer) increased with fetal weight (Meschia et al., 1965).

To better understand how gestation impacted placental oxygen permeability and uptake, Meschia et al. (1965) set out to determine if O_2 permeability peaks near 100-110 dGA in the sheep or whether it continues to increase with fetal nutritional requirements after 100 dGA. To this end, they determined the oxygen dissociation curves for each vessel (umbilical artery, umbilical vein, and uterine vein) based on the oxygen content, oxygen capacity and saturation, and the type of hemoglobin (Meschia et al., 1965). Furthermore, they calculated maternal and fetal "effective oxygen pressures (pO_2 mm. Hg) to determine the change in pO_2 which is necessary to calculate the diffusing capacity of the placenta (O_2 uptake/ change in pO_2) (Meschia et al., 1965). The results of this study revealed that placental oxygen permeability does not peak at 100-110 dGA but increases as gestation progresses (increases 10-fold) in proportion to fetal weight, which appears to be the main driver of increased oxygen transfer (Meschia et al., 1965).

Changes over gestation

In early gestation, oxygen diffusion across the placenta is nominal as the requisite uteroplacental vascular and fetal villi architecture has yet to mature (reviewed in Barry and Anthony, 2008). In fact, in the human, the placental pO_2 in early gestation (8 weeks) is less than 10 mmHg but rises to 60 mmHg by the end of the first trimester (Jauniaux et al., 2001; Rodesch et al., 1992). Low oxygen tensions during early pregnancy are present in the sheep as well. Barry and Anthony (2008) report that at 55 dGA, umbilical pO_2 was approximately 17 mmHg.

During mid-gestation, the placenta consumes approximately 83% of the oxygen it takes up from uterine circulation (Bell et al., 1986). From mid-to late gestation, there is a proportional decrease in oxygen demands by the placenta as it only consumes approximately 45% of the oxygen it takes up from maternal circulation (Meschia et al., 1980). However, on a total oxygen volume basis, the late gestation placenta actually consumes approximately 75% more oxygen compared to the mid-gestation placenta (Meschia et al., 1980). On a weight-specific basis, the oxygen consumption by the placenta is four to five times greater than the oxygen consumption of the fetus (Hay, 1991) with approximately 90% of the oxygen utilized by the placenta going to oxidative phosphorylation (Meschia et al., 1980; Bonds et al., 1986). From a comparative perspective, the metabolic demands of the placenta rival those of the highly metabolic brain, liver, and kidney (Hay, 1991). Placental oxygen consumption rates have been reported in the human (37 mL/kg/min) and the sheep (34 mL/kg/min) which are fairly close and support the similarities in oxygen consumption between species (Meschia et al., 1980; Bonds et al., 1986).

Complications

Failure of the placenta to successfully extract sufficient oxygen for adequate transfer and consumption can result in pathologies such as preterm delivery, intrauterine growth restriction or preeclampsia (Kingdom and Kaufmann, 1997). In intrauterine growth restricted pregnancies in the human, there is a marked decrease in the placental transfer of oxygen as identified by higher uterine pO_2 with lower uterine oxygen extraction rates (Pardi et al., 1992). These results are mirrored in the fetal growth restricted sheep which also exhibits a 65% elevation in the uterine to umbilical pO_2 differences (Regnault et al., 2003; de Vrijer et al., 2004; Regnault et al., 2007)

Kingdom and Kaufmann (1997) proposed several modalities of examining pregnancy hypoxia, their causes and ramifications. The first category of pregnancy hypoxia is preplacental hypoxia. This category is characterized by reduced oxygen content within maternal blood which leads both the placenta and the fetus to become hypoxic (Kingdom and Kaufmann, 1997). Examples of pre-placental hypoxia include high altitude and maternal anemia (Kingdom and Kaufmann, 1997). High-altitude pregnancies are characterized by increased capillary volume fraction and branching which is accompanied by increased villous cytotrophoblast density and syncytial knotting (Kingdom and Kaufmann, 1997). Similarly, maternal anemia can induce excessive branching angiogenesis with decreased mean capillary diameter but increased capillary volume fraction which causes the placenta to reduce the placental barrier depth in an attempt to maintain oxygen transfer (Kingdom and Kaufmann, 1997)

The second category is uteroplacental hypoxia where maternal blood is adequately oxygenated but cannot appropriately diffuse across placental tissues due to

restricted entry (Kingdom and Kaufmann, 1997). Examples of uteroplacental hypoxia includes occlusion of uteroplacental arterioles or failed trophoblast invasion (Kingdom and Kaufmann, 1997). Similar structural perturbations to preplacental hypoxia are observed in cases of uteroplacental hypoxia in the pathology of preeclampsia, with the increased capillary volume fraction as well as increased villous cytotrophoblastic density and syncytial knotting (Kingdom and Kauffman, 1997). Additionally, IUGR in late pregnancy with preserved end-diastolic flow velocity (PEDFV) with shared histological alterations as preeclampsia, and thus is also considered a type of uteroplacental hypoxia (Kingdom and Kaufmann, 1997).

The last proposed modality is postplacental hypoxia, in which adequately oxygenated maternal blood is able to enter the intervillous space (either normally or at a reduced rate) but a defect exists that precludes appropriate perfusion of the fetal villi and the fetus becomes hypoxic (Kingdom and Kaufman, 1997). An excellent example of postplacental hypoxia are pathologies that result in compromised umbilical blood flow such as IUGR pregnancies with absent or reverse end diastolic flow velocity (AEDFV) which are characterized by reduced proliferation of villous cytotrophoblasts and increased extracellular matrix (Kingdom and Kaufmann, 1997). Increased trophoblastic knotting also occurs with IUGR with AEDFV but is most likely the result of placental senescence (Kingdom and Kaufmann, 1997).

Glucose

Glucose has long been considered the key substrate for placental and fetal oxidative metabolism in both the human and the sheep. Glucose transport is dependent

on 1) the availability to transporters to shuttle glucose across the placenta, 2) the maternofetal glucose concentration gradient as well as 3) the placenta's inordinate metabolic demands. Glucose utilized by the placenta can meet several fates including oxidation, glycogen formation, and conversion to lactate or fructose (Hay and Meznarich, 1989). In spite of its vast metabolic demands, the placenta remains quite agile at shifting oxidative substrates in times of maternal hypoglycemia to reduce the drain on the limited maternal glucose pools by the uteroplacental unit and divert glucose to the fetus to sustain fetal viability (Hay, 1991). This shift in oxidative substrate utilization is critical or the highly metabolic placenta would almost entirely block the net transport of glucose to the fetus (Hay, 1991). Under normal conditions, the fetus has a limited ability to produce glucose and thus, glucose supply is primarily from maternal circulation (reviewed in Barry and Anthony, 2008)

To better understand the role of maternal hypoglycemia on the partitioning of glucose to the uteroplacental unit Hay et al. (1983) utilized catheterized, late gestation pregnant ewes that were either fasted or fed. They found that the proportion of maternal glucose supplying the uteroplacental unit and the fetus was constant in spite of the drastic reductions in maternal glucose levels (Hay, 1983). Next, to understand how maternal glucose concentrations alter glucose consumption by the placenta, Hay and Meznarich (1989) generated a range of maternal glucose gradients by maternal glucose clamps and applied the Fick Principle to directly assess uteroplacental glucose consumption. They found that uteroplacental glucose transport and uptake/consumption were directly related to maternal arterial glucose concentrations and reach maximal transport and uptake at the same threshold (Hay and Meznarich, 1989). Next, they

wanted to investigate the relative importance of fetal glucose concentrations by altering them with glucose clamps (and thereby, testing the other half of the maternofetal glucose concentration gradient) on placental glucose consumption and transfer (Hay et al., 1990). They found that ability of the placenta to transfer glucose was dependent on both the uterine and umbilical glucose concentrations, and thus, the maternofetal glucose concentration gradient (Hay et al., 1990). This has been demonstrated *in vitro* in the human as well. This was tested by utilizing placental perfusion to examine the relative glucose uptakes from the maternal vs. fetal vasculature when perfused with equal concentrations of glucose (Schneider et al., 2003). This study revealed the uptake of glucose from maternal circulation was double that extracted from fetal circulation and thus, supported the importance of the maternal to fetal glucose gradient on fetal glucose concentrations in the human (Schneider et al., 2003). Thus, the principal determinant of fetal glucose concentrations is the maternal-fetal glucose concentration gradient. One crucial concept about placental glucose utilization and transfer that frequently gets overlooked is placental glucose consumption which constitutes a significant portion of the transplacental glucose gradient (Hay, 1991).

Changes over gestation

During mid-gestation, the placenta consumes approximately 83% of the glucose it takes up from uterine circulation (Bell et al., 1986). Between mid-to late gestation, the placenta increases its glucose demands 6-fold to support a 10-fold increase in fetal mass (Bell et al., 1986). During late gestation, the placenta utilizes 72% of the glucose taken up from uterine circulation (Meschia et al., 1980). While this a proportionally

lower degree of glucose consumption compared to mid-gestation, the absolute glucose consumption by the placenta increases by 86% compared to mid-gestation (Meschia et al., 1980). This means that changes to uteroplacental glucose consumption directly impacts the concentration of glucose in the umbilical vein (Hay and Meznarich, 1989).

Now, in spite of the placenta's vast metabolic demands, it is somehow able to facilitate an increase in placental glucose transport as gestation progresses to meet fetal (11-fold increase) needs (Molina et al., 1991). This increase in placental glucose transport is facilitated by two mechanisms: 1) an increase in the maternofetal glucose concentration gradient and 2) an increase in the placental transport capacity of glucose (Hay, 2006; Molina et al., 1991). The transplacental glucose gradient increases as a result of the decrease in fetal glucose concentrations (Hay, 1991). Placental transport capacity increases by 8-fold over the last half of gestation, while placental weight in the pregnant sheep declines approximately 20% (Hay, 1991). Thus, it is possible to see how perturbations to factors influencing the placental glucose transport capacity could result in fetal hypoglycemia and potentially, IUGR.

Perturbations

Fetal hypoglycemia characterizes fetal growth restricted pregnancies in both the human and the sheep (reviewed in Barry and Anthony, 2008). In sheep models of IUGR, absolute glucose uptake by both the fetus and placenta is reduced, but this effect is diminished when normalized to fetal weights (Thureen et al., 1992; Wallace et al., 2005). However, in IUGR pregnancies, glucose clamp induced hyperglycemia increases the flux of glucose across the placenta in spite of reduced placental glucose transport

capacity under normal conditions (Thureen et al., 1992). The etiology, characteristics and implications of IUGR will be discussed in depth later in this literature review.

Another complication with investigating the specific role of glucose transport in IUGR is the documented global reductions in nutrient uptake. One of the difficulties with directly assessing the role glucose plays in the development of IUGR is that until recently, there was no way to specifically prevent the transfer of glucose without also impairing the uptake of oxygen or other carbon sources (generally by inducing placental insufficiency, or blood flow reductions). With the use of lentiviral-mediated RNA interference to target the sheep trophectoderm, this question is actively be assessed with studies ongoing in our lab to examine this question, but the data will not be presented in this dissertation.

Amino Acids

One of the key functions of amino acids during pregnancy is regulating placental and fetal development (Regnault et al., 2005). Amino acids are also considered an important carbon source for fetal development, and fetal amino acid concentrations are reduced in IUGR pregnancies (reviewed in Barry and Anthony, 2008). Much of placental amino acid uptake is dependent on energy availability as many amino acid transporters require energy to concentrate amino acids within the trophoblast intercellular matrix for transport into fetal circulation (reviewed in Hay, 1991). Thus, the transport of amino acids can interplay with the availability of other nutrients. When glycolysis and aerobic metabolism are inhibited, both *in vivo* and *in vitro*, amino acid uptakes are suppressed (reviewed in Hay, 1991).

The transport of amino acids is dependent on 1) active transport across the placenta based on availability of transporters, polarity, and surface area and density of transport systems, 2) the rate of placental consumption of those amino acids, and 3) amino acid interconversion (Battaglia, 2002; Jansson, 2001; Barry and Anthony, 2008). The transfer of amino acids is complex due to the large number (at least 15) of amino acid transporter systems identified (Regnault et al., 2005) with multiple transporter systems capable of transporting the same amino acid, and competition inhibition between amino acids for available transporters (reviewed in Anthony and Barry, 2008). Another convoluting factor in determining amino acid flux is the ability of the placenta to produce amino acids through interconversion of metabolically related amino acids and transfer those to the fetus. This has been demonstrated *in vivo* in both the human and the sheep (Cetin et al., 2005; Cetin, 2001). Fortunately, the development of tracer-labeled amino acids has permitted the study of bidirectional amino acid flux and improved understanding of the placental amino acid transport capacity. Furthermore, this technique has been used to demonstrate the incorporation of amino acids from transplacental uptake into placental proteins or structural tissue (reviewed in Hay, 1991; reviewed in Barry and Anthony, 2008).

Many different fates exist for placental utilization of amino acids as it possesses a variety of enzymes for either catabolic (gluconeogenic, glycolytic, oxidation, ammoniagenesis) or anabolic (protein synthesis) processes, as well as the ability to produce secreted protein products like hormones (reviewed in Hay, 1991). In the human and the sheep, the concentration of many amino acids is higher in fetal than maternal circulation, including many essential amino acids (Regnault et al., 2005) which is

expected due to active transport of amino acids across the placenta. The relationship between maternal amino acid concentrates and the fetal uptake of amino acids was investigated using a mix of labeled amino acids infused into maternal circulation of the catheterized pregnant sheep (Jozwik et al., 1999; Jozwik et al., 2001). They found that increasing maternal amino acid concentrates lead to varying impacts on umbilical uptakes of that amino acid (reviewed in Battaglia, 2002; Jozwik et al., 1999; Jozwik et al., 2001). In some instances, the umbilical uptakes increased, stayed the same, or decreased, likely due to competitive inhibition (reviewed in Battaglia, 2002; Jozwik et al., 1999; Jozwik et al., 2001). However, the infusion of a single amino acid into maternal circulation positively increases the fetal uptakes of that amino acid (reviewed in Battaglia 2002; reviewed in Regnault et al., 2005). From a clinical standpoint, it is easy to see how this might be relevant as an infusion of mixed amino acids into maternal circulation could result in an imbalance of amino acids in fetal circulation.

The net transfer of amino acids is still from maternal circulation (reviewed in Regnault et al., 2005). However, this does not apply to every amino acid. No placental net transfer of glutamate or aspartate occur in both the sheep and human placenta, as they enter the placenta from fetal circulation (reviewed in Barry and Anthony, 2008). Additionally, in the sheep, the fetus transfers serine to the placenta (Moores et al., 1993).

Changes over gestation

The capacities of amino acid transport systems shift over pregnancy. For instance, first trimester microvillous membrane in the human has enhanced transport

capacity compared to term placental vesicles (reviewed in Regnault et al., 2005). Additionally, the K_m of specific transport systems shift over pregnancy (reviewed in Regnault et al., 2005). As gestation advances, the increase in fetal demand has to be met by an increase in placental amino acid transport capacity. In part, this is met by increases in blood flow and perfusion, as well as an increase in membrane surface area (reviewed in Regnault et al., 2005). However, the increase in placental surface area (9-fold) is less than necessary to support fetal weight increases (20-fold) from mid-gestation to term (Regnault et al., 2005). Thus, to meet this demand, the placenta must change the concentration and affinity characteristics of key placental transporter proteins, as well as alter circulating amino acid concentrations (Regnault et al., 2005).

Perturbations

In the human, fetal growth restriction is characterized by suppressed fetal amino acid concentrations, which have been linked to reduced placental transport (Cetin et al., 1988; Cetin et al., 1996; Godfrey et al., 1998). Based on ultrasound data, this is thought to be caused by increased vascular resistance leading to poorer placental perfusion and a subsequent decrease in placental amino acid transfer (reviewed in Barry and Anthony, 2008). Additionally, in IUGR pregnancies, maternal concentration of amino acids is elevated, coupled with lower fetal concentrations of amino acids (Pardi et al., 2002) which certainly stresses dysfunction in the placental transfer of amino acids. In contrast to the IUGR phenotype, in gestational diabetes and fetal overgrowth, there is a documented increase of System A amino acid transporters (Jansson and Powell, 2000).

In the sheep, the impacts on fetal amino acid concentrations are more variable, and likely dependent on severity of fetal growth restriction (de Vrijer et al., 2004). In instances of moderate growth restriction, fetal amino acid concentrations were reduced but conversely, in instances of severe growth restriction, fetal amino acid concentrations were elevated compared to healthy pregnancies (de Vrijer et al., 2004). They attribute this to less severe reductions in glucose and oxygen concentrations in moderate growth restricted sheep and more severe reductions in severely growth restricted sheep (de Vrijer et al., 2004). It is possible however, that this relationship might be different depending on the specific cause of the placental insufficiency. Using tracer labeled leucine and threonine, IUGR pregnancies had marked reductions in the fluxes of both amino acids (Battaglia, 2002).

Another factor that has recently been demonstrated to regulate placental amino acid transfer is fetal glucagon concentrations. Fetal glucagon concentrations are elevated in cases of IUGR or fetal stress (Limesand et al., 2006; Hubinont et al., 1991). However, chronically elevated glucagon has been directly demonstrated to reduce fetal amino acid concentrations in a model of chronic fetal hyperglucagonemia in the late gestation ewe (Cilvik et al., 2021).

Lipids

Great species-specific variations exist in placental transport and permeability to fatty acids with fetal adiposity at birth being directly related to maternal free-fatty acid concentrations (reviewed in Hay, 1991). Lipids are not considered a major fetal nutrient for oxidative metabolism which are glucose, lactate, and amino acids (Hay and Sparks,

1985; Regnault et al., 2002), but all species require essential fatty acids for membrane production. Thus, the placenta likely plays an important role in synthesis, modification, and functional transfer of fatty acids to fetal circulation (Hay, 1995).

The placenta can utilize mono & diglycerides from maternal circulation and produce triglycerides to supply to fetal circulation (reviewed in Hay, 1995). Thus, both maternal diet and placental lipid transport capacities and metabolism influence fetal lipid concentrations (reviewed in Hay, 1995). The fates of lipids entering the placenta include oxidation by the placenta, synthesis of glycerolipid, esterification of cholesterols, membrane biosynthesis, or direct transfer to the fetus (reviewed in Hay, 1991).

The placenta supplies long-chain fatty acids by direct carrier mediated transfer to the fetus, although some evidence exists that intratrophoblast peroxisomes lead to a greater transfer of medium-chain fatty acids (reviewed in Hay, 1995). Increased placental triglyceride storage has been documented in women with gestational diabetes, preterm delivery, and maternal fasting (reviewed in Hay, 1991). However, Pardi et al. (2002) reported no differences in fatty acid concentration between normal and IUGR pregnancies. However, they did show changes in essential fatty acid composition in IUGR pregnancies including elevated linoleic acid and reduced docosahexaenoic acid (DHA; Pardi et al., 2002).

Summary

Placental development is crucial for a successful pregnancy outcome, and placental dysfunction can lead to pregnancy complications. In order to assess the level at which the dysfunction occurs, it is important to consider factors impacting

placentation and fetal villi development, placental perfusion, and placental endocrine function and how these factors ultimately alter placental transfer capacity and/or metabolism. Due to ethical limitations, these factors are difficult to directly assess in the human and thus, the pregnant sheep has proved instrumental in investigating research questions relating to fetal and placental physiology. As hopefully evident by this review, placental physiology is a complex field of study, influenced by an intricate interplay of factors. One aspect of placental physiology that was not discussed in this review due to scientific knowledge gaps is the role that under-investigated placental hormones play in regulating nutrient transfer and placental metabolism. This is the critical knowledge gap that this dissertation will in part, attempt to address.

FETAL PHYSIOLOGY

From a fetal growth and development perspective, pregnancy can be broadly divided into two periods: the embryonic period and the fetal period. In human pregnancies, the embryonic period constitutes the first 8-weeks of gestation, which can be further subdivided into 23 developmental stages (Bolender and Kaplan, 2011) which are beyond the scope of this review to discuss. It is during this embryonic period that initial morphogenesis of organ systems occurs (Bolender and Kaplan, 2011). In the sheep, the embryonic period lasts until 33 days post conception (dpc; Green and Winters, 1945). The period of fetal development begins after the 8th week of gestation in the human (Bolender and Kaplan, 2011) or ≥ 34 dpc in the sheep (Green and Winters, 1945). This review aims to 1) discuss the development of key organs that impact perinatal and postnatal metabolism, 2) define fetal circulation, 3) examine fetal

endocrinology and the interplay between hormones, and 4) describe fetal nutrient requirements for growth.

Development of Key Organs

In the human embryo, organ morphogenesis peaks between 3-8 weeks post conception (Bolender and Kaplan, 2011). Logically, this is the period that the developing organs are most sensitive to toxin-based (alcohol, teratogens, etc.) perturbations (Bolender and Kaplan, 2011) although organs are certainly capable of damage outside this key window. For instance, the brain, the organ with the longest developmental period, remains susceptible outside the embryonic period (Bolender and Kaplan, 2011). However, most organs systems complete morphogenesis by the end of the embryonic period (Bolender and Kaplan, 2011).

Less has been documented regarding organ development in the sheep. In their technical bulletin in 1945, Green and Winters carefully documented through the histological development of the sheep embryo and fetus. They found by the end of the third week of gestation, most of the key organs had been formed in the sheep embryo (Green and Winters, 1945). While most organ differentiation was completed before 34 days of gestation, each organ still underwent dynamic growth and maturation across gestation (Green and Winters, 1945). While this review will not describe organogenesis for every fetal organ, it will attempt to highlight a few key organs critical for fetal metabolic function and survival.

Liver

In the human, the liver bud arises from the lumen of the distal foregut during the fourth week of development (Bolender and Kaplan, 2011). The liver bud forms the parenchyma of the liver, the bile ducts and the gall bladder (Bolendar and Kaplan, 2011). Many components of the liver are formed from the endoderm including the hepatocytes, the bile canaliculi, and the epithelium of the hepatic biliary ducts (Bolendary and Kaplan, 2011). In the sheep, Green and Winters (1945) document the presence of the fetal liver by 18 dpc, but do not specify exactly when it formed. Bazer et al. (2012) document that sheep fetal liver increases from 0.06 g at 30 dGA to 96.4 g near term (140 dGA). The fetal liver works in tandem with the placenta to produce insulin-like growth factors (IGF1 and IGF2) which potently stimulate fetal growth and respond to circulating glucose and/or insulin concentrations (Limesand and Davis, 2018). Fetal IGF2 production begins around 6 weeks of gestation, followed by IGF1 production (Limesand and Davis, 2018).

As the fetal liver is critical for many metabolic and endocrine processes within the fetus, much consideration has been placed on measuring its growth and function. By using 3D ultrasound, Pardi and Cetin (2006) measured fetal liver volume and determined the fetal liver grew exponentially from 18 weeks to term (Pardi and Cetin, 2006). Fetal liver function and maturation can also be assessed by measuring the distribution of umbilical blood flow between the fetal liver and the ductus venosus (Pardi and Cetin, 2006). As gestation progresses, umbilical blood shunted through the ductus venosus decreases from ~40% to ~15% (Pardin and Cetin, 2006). This increases the proportion of umbilical blood supply and therefore, nutrients, to the fetal liver (Pardi and

Cetin, 2006). Blood flow distribution to the right liver lobe also shifts across gestation, increasing from 20% to 40% (Pardi and Cetin, 2006). Aberrations in fetal liver function in IUGR pregnancies result in increased ductus venosus shunting and reduced right liver lobe blood flow (Pardi and Cetin, 2006) which would indicate perturbed liver function. Unsurprisingly, pathological pregnancies can result in reduced liver volume. The fetal brain to liver weight ratio which usually decreases over gestation in healthy pregnancies, is altered in growth restricted pregnancies (Pardi and Cetin, 2006)

Heart

In the human, mesoderm-derived cells form the primitive heart on either side of the midline of the embryo, between ectodermal and endodermal layers (Bolender and Kaplan, 2011). The cell clusters develop into two parallel cords of cells which canalize and form the endocardial tubes, which fuse together to form the primitive heart tube (Bolender and Kaplan, 2011). After additional growth and morphogenesis, the primitive heart chambers are formed and the heart begins beating at approximately 22 days after fertilization (Bolender and Kaplan, 2011). At approximately 23 days after fertilization, a weak movement of fetal fluids is established and cardiovascular development continues (Bolender and Kaplan, 2011). The heart is the first organ to become functional in the embryo (Bolender and Kaplan, 2011). The atria of the heart separate by approximately 5 weeks post conception (Bolender and Kaplan, 2011).

In the sheep, by 15 dpc, the heart bulge has formed and continued to develop and mature (Green and Winters, 1945). By 18 dpc, the chambers of the fetal heart are visible (Green and Winters, 1945). Across gestation, the sheep heart increases from

0.02 g at 30 dGA to 24.4 g near term (Bazer et al., 2012). In the fetal lamb, cardiac output is estimated at 315 mL/min/kg which closely rivals estimates (300 mL/min/kg) in the human fetus (Pardi and Cetin, 2006). Proportionally, 45% of this blood volume originates from the right ventricle and 55% from the left ventricle (Pardi and Cetin, 2006). Glucose is the primary oxidative substrate for the fetal and neonatal heart, which provides some resistance to hypoxia through increased glycogen stores (Battaglia and Meschia, 1978). The dependence on glucose shifts to free fatty acids in adulthood (Battaglia and Meschia, 1978).

Pancreas

The primary transition of the endoderm into the fetal pancreas occurs prior to 24 dpc in the sheep and around 25-26 dpc in the human (reviewed in Boehmer et al., 2017). By 8 weeks into gestation, β-cells begin to proliferate, and around 11 weeks post conception, immature pancreatic islets begin forming (Limesand and Davis, 2018). The fetal β-cells first become responsive to glucose during mid-gestation, acting as the sole fetal source for insulin as insulin cannot cross the placenta (Pictet and Rutter, 1972). While less is known about the developmental timeline of β-cells in the sheep pancreas, the fetal sheep also becomes responsive to both insulin and arginine around mid-gestation (reviewed in Boehmer et al., 2017; Limesand and Davis, 2018). Thus, the pancreatic developmental timelines are likely similar between the sheep and the human. In mature pancreatic islets, α-cells secrete glucagon; β-cells secrete insulin, δ-cells secrete somatostatin, and F-cells secrete pancreatic polypeptides (Limesand and Davis, 2018). Glucagon is present by 6 weeks of gestation in the fetal pancreas and is

secreted into fetal circulation by 15 weeks (Bassett, 1977; Girard and Sperling, 1983; Gittes, 2009; Cilvik et al., 2021). Fetal glucagon concentrations can increase in response to stress and are elevated 2-fold in response to IUGR in sheep (Limesand et al., 2006). Fetal growth restriction, especially severe growth restriction, has been also associated with diminished insulin concentrations. In some cases of severe IUGR, pancreatic islets had fewer β-cells (Van Assche et al., 1977). This is important as individuals impacted by IUGR *in utero* are more likely to have increased insulin resistance in adulthood and perturbed β-cell function.

Thyroid

The thyroid is also important for fetal growth and development, which is evidenced by its formation by 7 weeks of gestation (Limesand and Davis, 2018), making it one of the earliest endocrine organs. It is formed from the primitive pharynx, giving rise to the median thyroid and the neural crest which develops into the lateral thyroid (reviewed in Limesand and Davis, 2018). Prior to the development of a functional fetal thyroid gland, the fetus is dependent on placental transport of maternal thyroid hormone (Limesand and Davis, 2018). After undergoing three distinctive phases of growth and differentiation, the thyroid gland is functional by 12 weeks of gestation, with full function achieved by 16-18 post conception (Limesand and Davis, 2018). This is critical to fetal neural development as well as fetal metabolism (Limesand and Davis, 2018). In late gestation, fetal concentrations of the prohormone thyroxine (T_4) and the biologically active form triiodothyronine (T_3) become elevated.

Adrenal gland

Fetal adrenal glands, responsible for cortisol and catecholamine production, begin developing by week 4 of gestation in the human (Limesand and Davis, 2017). In the sheep, the fetal adrenal glands are measurable by 60 dpc and weigh 0.38 g by term (Bazer et al., 2012). The fetal adrenal cortex produces 3 categories of steroid hormones including 1) androgens (fetal zone), 2) glucocorticoids (transitional zone), and 3) mineralocorticoids (definitive zone; Limesand and Davis, 2017). By the end of early gestation (9-12 weeks), the adrenal cortex is steroidogenically active, mostly producing the fetal precursor to estradiol (dehydroepiandrosterone sulfate; DHEA-S; Limesand and Davis, 2017), which has previously been discussed in the placenta review and will not be further discussed here. The fetal hypothalamic-pituitary-adrenal axis matures after mid-gestation, as evidenced by the ACTH stimulation of cortisol developing a negative feedback regulation (Ng, 2000). In spite of the critical nature of glucocorticoids for fetal brain and lung development and maturation, excessive glucocorticoids can have lasting programming consequences (Fowden and Forhead, 2004). The placenta and fetal tissues convert cortisol to its inert form, cortisone, but extreme maternal stress can elevate cortisol concentrations beyond what can be inactivated (Fowden and Forhead, 2004).

Brain

The human nervous system begins to form at 18 days post conception (Bolender and Kaplan, 2011) with the diencephalon and telencephalon distinguishable by 5 weeks post conception (reviewed in Katugampola et al., 2020). The hypothalamus, pituitary

stalk and posterior pituitary are largely developed by 7 weeks of gestation (reviewed in Katugampola et al., 2020). From a functional perspective, hypothalamic neurons contain neuropeptides by 10-14 weeks post conception and all pituitary hormones can be identified between 10-17 weeks of gestation (reviewed in Katugampola et al., 2020). Brain growth also provides insight into whether a fetus is growing appropriately. When the brain is measured *in utero* through 3D ultrasound, it is estimated to contribute to 15% of fetal body weight (Pardi and Cetin, 2006). The fetal brain grows rapidly, increased 10-fold in mass over the second half of gestation (Pardi and Cetin, 2006). The importance of brain development and growth is emphasized by the relationship between brain and fetal body weight in IUGR pregnancies. While umbilical blood flow is often reduced in IUGR pregnancies, brain growth is preserved especially over abdominal organs such as the liver (Pardi and Cetin, 2006).

In the sheep, development of the brain begins early in gestation, with key components in place by 15 dpc (Green and Winters, 1945). By 35 dGA, the sheep fetal brain weighed 0.14 g and increased to 49.97 g near term (Bazer et al., 2012). Less is documented about brain function and development in the sheep, but sheep IUGR pregnancies do share the “brain sparing” effect documented in humans. The key oxidative fuel for the sheep fetal brain is glucose, and no uptake of ketoacids have been demonstrated in the sheep *in utero* (Battaglia and Meschia, 1978). In the human neonate, ketoacid uptake is present at birth, representing 10-15% of oxidative substrate utilized by the neonatal brain (Battaglia and Meschia, 1978).

Fetal Circulation

Circulation in the fetus differs from the adult in several ways including 1) gas exchange occurs in the placenta, not the lungs and 2) a variety of extracardiac and intracardiac shunts to ensure the brain and heart receive sufficient oxygen to function (Kiserud et al., 2000). The fetal sheep has been paramount to the study of fetal circulation. The ductus venosus was discovered in 1939 by Barclay et al., using rapid serial radiography. Dawes et al., (1954) used fetal catheters to measure blood oxygen and mathematically modeled blood flow using oxygen content differences in the sedated sheep fetus. The Dawes et al. (1954) study informed the basic principles of fetal circulation which has been followed up with steady-state instrumentation (Edelstone et al., 1977) of fetal lambs to examine ductus venosus flow, and through Doppler ultrasonography in the human (reviewed in Battaglia, 2011).

Fetal circulation starts as oxygenated, nutrient rich blood enters the fetal liver via the umbilical vein, where a portion bypasses hepatic circulation and enters the inferior vena cava via the ductus venosus shunt (Edelstone et al., 1977; Kieserud et al., 2000). In animal models like the sheep, approximately 53% of umbilical venous flow and <9% of portal vein blood is shunted through the ductus venosus in late gestation (116-136 dGA; Edelstone et al., 1977). In the human, around 20 weeks of gestation, approximately 30-40% of umbilical venous flow (Keiserud et al., 2000; Battaglia, 2011) is shunted through the ductus venosus which decreases to 18% by 31 weeks of gestation (Keiserud et al., 2000) and to 15% by term (Battaglia, 2011).

Oxygenated blood shunted to the inferior vena cava is directed into the left atrium via the foramen ovale (46%; Dawes et al., 1954) through the left ventricle towards the

ascending aorta where it can supply the developing brain with oxygen rich blood (Murphy, 2005). Blood transported from the vena cava into the right ventricle is then ejected into the pulmonary artery (Murphy, 2005). As vascular resistance at this time is high, only a small amount of the right ventricular output (10-12%; Dawes et al., 1954; Murphy 2005) enters pulmonary circulation, and large volumes of blood are shunted across the ductus arteriosus (estimated at 35% by Dawes et al., 1954 and 88% by Murphy, 2005) to the descending aorta.

Unlike adult circulation, the ventricles do not exhibit equal stroke volume in the fetus. The right ventricle receives 65% of the venous return and the left ventricle only receives 35% (Murphy, 2005). In the human, approximately 45% of the combined ventricular output is directed towards placental circulation (Murphy, 2005) whereas approximately 57% in the fetal sheep is directed towards the placenta (Dawes et al., 1954). When the fetus transitions to postnatal life, these shunts must close, and left ventricular output must increase for the neonate to successfully adapt to postnatal life (Murphy, 2005).

Fetal growth restricted pregnancies often have reduced umbilical blood flow and fetal hypoxia, and thus, compensatory mechanisms are initiated. One of these mechanisms is increased shunting through the ductus venosus to the inferior vena cava to maintain brain and myocardial oxygenation (Battaglia, 2011). This combination between reduced umbilical blood flow and increased shunting by the ductus venosus leads to severe reductions in hepatic blood flow (Battaglia, 2011). This may be why fetal liver weights are often reduced in growth restricted pregnancies.

Fetal Endocrinology

Fetal endocrine signaling begins early in gestation, augmenting fetal growth and metabolism. The fetal endocrine milieu is mostly distinctive from maternal as the placenta neutralizes the biological activity of many maternal hormones during maternal-fetal transfer. Additionally, the placenta itself is a major endocrine organ and facilitates maternal-fetal crosstalk and physiological adaptions via the secretion of hormones. This crosstalk, although poorly understood, is capable of eliciting altered maternal and fetal physiological responses. In some instances, fetal endocrine hormones acting through the placenta modify maternal physiology. For instance, in the sheep, sustained fetal hyperglucagonemia reduces maternal (but not fetal) CSH secretions and uterine blood flow (Cilvik et al., 2021). Thus, to appreciate fetal endocrinology, it is necessary to understand 1) where key hormones originate, 2) what receptors they act through, 3) which factors impact their secretion or bioavailability, and 4) how these hormones are altered by nutrient availability from placental transfer.

Insulin

Insulin is a significant fetal growth promoting hormone, playing a crucial role in glucose homeostasis (Hay et al., 1988; Boehmer et al., 2017). Insulin is produced by the β-cells as previously discussed and potentiates its actions through binding its various receptors. The primary receptor type for insulin is insulin receptor (IR) which contains two isoforms, IR-A or IR-B (Belfiore et al., 2009). While insulin has limited affinity for the main IGF receptor, IGF-1R, IGF-1R can form a hybrid receptor with each of the IR isoforms (Oh et al., 1993). Insulin does have affinity for the Hybrid-A receptor

(IR-A/IGF-1R) but has limited affinity for Hybrid-B (IR-B/IGF-1R) and no affinity for IGF-2R (Oh et al., 1993). Thus, it is difficult to discuss insulin without also discussing the insulin like growth factors (IGF1 & IGF2). IGF2 acts on both IR and IGF-1R, whereas IGF1 primarily acts through IGF-1R (reviewed in Belfiore et al., 2009). Both IR and IGF-1R receptors are expressed early in fetal development, with IR-A as the predominant isoform until postnatal life (reviewed in Belfiore et al., 2009). The necessity of IR is emphasized by gene mutations in the human resulting in severe IUGR (Liu et al., 1993; Psiachou et al., 1993; Krook et al., 1993; Jospe et al., 1996; Belfiore et al., 2009). This growth restriction is not as evident in IR null mice (10-20% growth restriction; Louvi et al., 1997) but is true for IGF-1R null mice (65% growth restriction; reviewed in Belfiore et al., 2009) which may suggest species differences in insulin/IGF signaling for fetal growth. If IR and IGF-1R are both ablated in rodents, the embryos are 70% growth restricted (30% of normal size), which suggests multi-receptor signaling has additive effects on fetal growth (Efstratiadis, 1998). Thus, it is difficult to individually parse out the effects of insulin vs. the IGF's on fetal growth due to reciprocal interaction on their receptors. Therefore, both insulin and the IGFs must be considered together when evaluating the physiological impacts of each hormone, receptor, and phenotype.

While the liver, adipose tissue, and skeletal muscle are well established major targets of insulin, other tissues such as the brain, heart, kidney, lung, the vascular endothelium of the placenta, monocytes, granulocytes, erythrocytes, and fibroblasts also possess IR (Kaplan, 1984; Belfiore et al., 2009). Insulin directly effects systemic fetal growth as pancreatectomized fetal lambs supplemented with exogenous insulin had rescued fetal growth (Fowden et al., 1989). In the fetal lamb, it appears the key role of insulin is to

increase the permeability of insulin-responsive cells to glucose flux vs. increasing cellular glucose utilization (Hay, 1991). The pancreas contributes to fetal metabolism, nutrient uptake and anabolic growth via the β-cell (Boehmer et al., 2017). Both IR and IGF-1R receptors have been identified in the β-cell (Van Schravendijk et al., 1985; Schravendijk et al., 1987; Belfiore et al., 2009). The role of IR in the β-cell is difficult to parse out, especially because IGF-1R/hybrid-A activation by insulin may interfere with IR signaling, and IGF1 acts as a negative regulator of insulin secretion (Schravendijk et al., 1985; Schravendijk et al., 1987; Marincola et al., 1983; Stagner et al., 1986; Belfiore et al., 2009).

Fetal insulin deficiency is associated with fetal growth restriction in both the human and the sheep (Hill, 1982; Gluckman & Liggins, 1984; Fowden, 1987; Fowden et al., 1989). Experimentally, this relationship has been demonstrated through the administration of diabetogenic drugs or by fetal pancreatectomy, both of which result in smaller fetuses with altered organ weights and growth of the appendicular skeleton (reviewed in Fowden et al., 1989). Reduced fetal weights can also be induced by chronic hyperinsulinemia which cause fetal hypoglycemia, and disrupts nutrient stimulated insulin secretions (Rozance et al., 2006). In animal models (lamb and rat), fetuses from growth restricted pregnancies often have reduced pancreatic mass with smaller islets and fewer β-cells that secrete less insulin (Green et al., 2010; Abuzgaia et al., 2015; Boehmer et al., 2017). Functionally, this results in lower basal insulin concentrations and glucose-stimulated insulin concentrations in the PI-IUGR fetal sheep across late gestation (Limesand et al., 2006; Limesand et al., 2013; reviewed in Boehmer et al., 2017). However, islets from IUGR fetuses secrete a higher proportion of

their insulin in response to glucose and other secretagogues (Limesand et al., 2006; Kelly et al., 2017; reviewed in Boehmer et al., 2017). Alterations in β-cell replication and altered expression of genes involved in β-cell proliferation and apoptosis are likely contributors to reduced β-cell density and function (Limesand et al., 2006; reviewed in Boehmer et al., 2017).

Insulin-like growth factors

The insulin-like growth factor (IGFs) system contains two ligands (IGF1 and IGF2) which are of paramount importance for fetal growth and placental function, as well as four receptors (IGF-1R, IGF-2R, Hybrid A, Hybrid B), and six binding proteins (IGFBP1-IGFBP6). Both ligands are synthesized and secreted by the fetal liver and the placenta, with placental IGF2 production beginning as early as 6 weeks of gestation (Limesand and Davis, 2017). Early in gestation, IGF2 is the predominant factor modulating fetal growth, and is stimulated by glucose (Limesand and Davis, 2017). Later in gestation and throughout postnatal life, IGF1 becomes more critical, and can be stimulated by both insulin and glucose (Limesand and Davis, 2017). *In utero*, IGF1 secretion is independent of pituitary GH, and while the exact reason is not known, it is postulated that IGF1 and likely IGF2 is stimulated by CSH (Limesand and Davis, 2017). Other growth factors can also potentiate the actions of IGFs including platelet-derived growth factor, epidermal growth factor, and fibroblast growth factor (Hakuno and Takashi, 2018). The importance of each IGF on fetal growth was demonstrated in the rodent through a series of gene knockout experiments. If dual knockouts of IGF1/IGF2 are generated, severe fetal growth restriction (70% restriction) occurs with mice dying after

birth, emphasizing the critical role of the IGFs in fetal growth and development (Liu et al., 1993). However, a single IGF1 or IGF2 mutation results in a 40% weight reduction, with some IGF1 null mice dying after birth (Liu et al., 1993). This could indicate that deficiency of one IGF can be partially compensated by the other IGF. However, adequate fetal growth is not possible without the presence of both IGFs. It is possible for IGF resistance to develop in response to various conditions such as malnutrition or inflammation (Hakuno & Takashi, 2018). This resistance leads to impairment of the anabolic effects of IGF, especially in cases of malnutrition, which may act as a safety measure to prevent additional growth incompatible with survival (Hakuno & Takashi, 2018). Additionally, as previously noted, it is possible that IGF/insulin signaling differs between the rodent and other species.

As previously mentioned, there is overlap between insulin and IGF signaling. Not only can insulin and the IGFs potentiate their actions through their shared receptors, but IGF1 has been shown to regulate β-cell mass and insulin production (reviewed in White et al., 2018). The predominant receptor that both IGFs act through is IGF-1R, however, both can also act through Hybrid-A, and IGF2 also has strong affinity for IR-A (Oh et al., 1993). A second IGF receptor, IGF-2R, exists as a mannose 6-phosphate receptor which is involved in lysosomal enzyme trafficking, and may play role in fetal organogenesis but lacks the intracellular signal transduction capabilities of IGF-1R (Wylie et al., 2003). It has been postulated that IGF-2R is a clearance receptor to dispose of excess IGF2 (Wylie et al., 2003). This is demonstrated by IGF-2R mutations resulting in fetal overgrowth of the rodent as well as fetal lethality (Wylie et al., 2003). To deduce the relative impact of each receptor to fetal growth, a series of gene knockout

experiments were performed in rodents. If IGF-1R alone is knocked out, mice are born with a 55% weight reduction and all died immediately after birth (Liu et al., 1993). Dual IGF2/IGF-1R null mice experience additional growth restriction, as they are born weighing only 30% of wild-type (WT) mice (Liu et al, 1993). However, dual IGF1/IGF-1R null mice do not experience additional growth restriction from the IGF-1R null only mice (55% restriction; Liu et al., 1993). This is somewhat expected as IGF1 primarily acts through IGF1-R, whereas IGF2 can also act through IR.

To further complicate the investigation of IGFs, the bioavailability of each IGF is regulated through at least six binding proteins (IGFBPs). Not only do these IGFBPs alter the bioavailability of the IGFs, but the actions of the binding proteins can also be further regulated through post translational modification, proteolysis, and heparin binding domains. These binding proteins are critical to extend the half-life of IGFs, can regulate their movement into tissues, and IGF activity at the tissue level (Bach, 2018). Up to 99% of IGFs are bound to IGFBPs in circulation (Bach, 2018). The IGFBPs do not interact with insulin (Bach, 2018).

IGFBP1

The first binding protein, IGFBP1 binds both IGF1 and IGF2 with similar affinity and is regulated at the posttranslational level by phosphorylation of serine residues (Bach, 2018). IGFBP1's actions are inhibitory if phosphorylated, and stimulatory if non-phosphorylated (Bach, 2018). IGFBP1 is important in fetal growth, metabolism, and placental function and is expressed highly by many organs including the liver (Bach, 2018). Hypoxia and leucine deprivation can increase IGFBP-1 phosphorylation, which

can further inhibit IGF availability (Bach, 2018). IGFBP1 transcription can be directly inhibited by insulin (Bach, 2018).

IGFBP2

The second binding protein, IGFBP2 binds both IGFs, but has increased affinity for IGF2 (Bach, 2018). IGFBP2 is not post-translationally modified but can undergo proteolysis to regulate its activity (Bach, 2018). IGFBP2 is expressed in the liver, adipocytes, reproductive tract, and central nervous system, and is sensitive to GH (Bach, 2018). If knocked out, IGFBP2 has been associated with angiogenic defects (Wood et al., 2000; Bach, 2018).

IGFBP3

The third and most abundant binding protein, IGFBP3 has equal affinitiy to IGF1 and IGF2, is sensitive to GH, and may either augment or inhibit the actions of IGF (Bach, 2018). IGFBP3 is glycosylated (N-linked) and may also be phosphorylated (Bach, 2018). If IGFBP3 is overexpressed (in the rodent), animals had reduced growth both *in utero* and postnatally, along with insulin resistance and impaired glucose tolerance (Bach, 2018). Thus, while IGFBP3 does appear to be metabolically important, IGFBP3 knockouts do not produce a phenotype (Bach, 2018). IGFBP3 also is likely involved with inhibiting angiogenesis through prevention of IGF1 and VEGF induced cell proliferation (Bach, 2018).

IGFBP4

The fourth IGFBP, is glycosylated (N-linked) and binds both IGF1 and IGF2 with equal affinity, but has predominantly inhibitory effects on IGF (Bach, 2018). IGFBP4 plays a biological role in adipogenesis and IGF signaling in the adipocyte (Bach, 2018). A key factor regulating IGFBP4 is proteolysis, which increases the bioavailability of IGF (Bach, 2018). Like several other binding proteins, IGFBP4 also plays an inhibitory role in angiogenesis (Bach, 2018).

IGFBP5

The fifth IGFBP, can also be glycosylated (O-linked) and phosphorylated, and has a higher affinity for IGF2 (Bach, 2018). Interestingly, IGFBP5 can also bind other biomolecules including glycosaminoglycans, fibronectin, and vitronectin (Bach, 2018). These additional factors, mostly interacting with IGFBP5's C-terminal, heparin binding sequence and can alter IGFBP5's binding affinity to IGF and its actions (Bach, 2018). For instance, if IGFBP5 is bound to glycosaminoglycans, IGF2 is activity stimulated due to decreased affinity to IGFBP5 (Bach, 2018). IGFBP5 is expressed in the placenta, uterus, ovaries, testis, lung and bone (Bach, 2018). From a functional standpoint, an overexpression of IGFBP5 in the rodent increased neonatal demise, decreased growth *in utero* and postnatally, and reduces female fertility (Bach, 2018).

IGFBP6

The last of the IGF binding proteins is IGFBP6 which has a pronounced affinity preference for IGF2, and thus, primarily inhibits IGF2 (Bach, 2018). IGFBP6 regulates

cell proliferation, differentiation, migration, and survival (*in vitro*), primarily through inhibition (Bach, 2018). It can be post-translationally modified through glycosylation (O-linked) which extends its half-life but does not impact binding affinity (Bach, 2018). Like other of the binding proteins, IGFBP6 can also inhibit angiogenesis, but uniquely, IGFBP6 is involved with immune regulation (Bach, 2018). IGFBP6 knockout mice do not have a phenotype, but overexpression inhibits embryonic growth in zebrafish (Bach, 2018). Up to 4 more IGFBP's have been identified, but less is known about their function, regulation, or expression (Kim et al., 1997).

While the importance of IGFs have been clearly demonstrated in the rodent using gene knockout, reduced IGF is associated with impaired fetal growth in both the human and the sheep (Tzschoppe et al., 2015; Fowden et al., 2003; Rozance et al., 2018; Wai et al., 2018). Due to the relationship between fetal growth and IGF concentrations, some investigations have tried to supplement IUGR pregnancies with exogenous IGF1. Short-term (4h) IGF1 infusions were conducted in the late-gestation sheep fetus, and while limited changes were observed during acute infusions, some studies revealed reduced nutrient uptakes (reviewed in Sferruzzi-Perri et al., 2017; Jensen et al., 1999). Bloomfield and colleagues (2002) used intraamniotic infusions of IGF1 as means to administer IGF1 to the late-gestation sheep fetus. The effectiveness of this approach to treat IUGR was tested *in vivo* during late gestation, in a sheep model of placental embolization (Eremia et al., 2007). Using this approach, modest improvements (but not full growth rescue) of fetal growth occurred, including the preservation of organ weights such as the fetal liver (Eremia et al., 2007). To try to ascertain the mechanism by which IGF1 intraamniotic infusions contributed to fetal growth improvements, Wali et al. (2012)

conducted *in vivo* steady-state metabolic studies and saw no changes to uterine or umbilical blood flow or umbilical glucose uptake but did see an increase in mRNA concentrations of various amino acid transporters.

Additional studies have aimed to investigate the short term (1 week) impacts of fetal IGF1 infusions on fetal physiology. White et al. (2018) reported decreased fetal arterial insulin concentrations in response to one week of IGF1 analogue (LR3) infusions, as well as reduced fetal glucose concentrations, PaO_2, and O_2 content. However, pancreatic islet vessel density and pancreatic insulin protein content both increased, as did fetal weight (White et al., 2018). When glucose sensitivity of LR3 -infused fetuses was assessed with hyperglycemic clamps, insulin secretions were reduced as were plasma insulin and glucose, however, pancreatic insulin content was not impacted (White et al., 2021). From a nutrient flux perspective, in response to LR3 infusions, uterine and umbilical blood flows were not impacted, nor were the uptakes of glucose, oxygen and lactate (Stremming et al., 2021). Umbilical amino acid uptakes were reduced in LR3 infused fetuses, but fetal heart, adrenal and spleen weights were increased (Stremming et al., 2021). Investigators have now hypothesized that IGF1 aids in nutrient utilization in an organ specific manner rather than through targeting blood flow or transplacental nutrient uptake (Stremming et al., 2021).

Cortisol

Cortisol is a steroid hormone critical for maturation of the fetal organs and can also influence fetal growth (Limesand and Davis, 2017). Approximately 2/3 of circulating cortisol in the fetus is produced by the fetal adrenal glands with the remainder

transferred from maternal circulation by the placenta (Kota et al., 2013). Adrenal hormones act through their two nuclear receptors, glucocorticoid (GR) and mineralocorticoid (MR) receptors (Kota et al., 2013). The primary cortisol receptor, GR, is present by mid-gestation in the placenta, lung, brain, liver, and gut (Kota et al., 2013). A substantial proportion of fetal cortisol (~80%) is converted to the inactive cortisone or other metabolites by the enzyme 11β HSD-II expressed by placental and fetal tissues in both the human and the sheep (Kota et al., 2013; Wood and Keller-Wood, 2016). This inactivation is critical to insulate the fetus from maternal stress. The activity of 11β HSD increases as gestation progresses through the increase in placental estrogen biosynthesis (Kota et al., 2013). As the fetus matures and prepares for parturition, there is a cortisol surge to mature fetal organs (Limesand and Davis, 2017). During this time, the fetal liver and lung express 11-ketosteroid reductase to promote the conversion of cortisone to cortisol (Kota et al., 2013). Disruption of the hypothalamic-pituitary-adrenal axis in the sheep prevents spontaneous parturition, but not in humans (reviewed in Wood and Keller-Wood, 2016). Glucocorticoids are critical to accelerate lung development and surfactant production, as well as mature the liver, heart, and GI tract (Wood and Keller-Wood, 2016). However, excessive cortisol can result in permanent adaptations from maternal stress. Elevated glucocorticoids have been associated with some IUGR pregnancies, and can directly alter receptor expression, enzymes, ion channels, transporters, growth factors, and signaling cascades or indirectly alter the bioavailability of other hormones (Fowden and Forhead, 2004). The degree of reprogramming caused by cortisol is dependent on timing and duration of insult

(Limesand and Davis, 2017). Some rodent and porcine studies indicate that female fetuses are more susceptible to elevated cortisol (Limesand and Davis, 2017).

Glucagon

Glucagon plays an important role in glucose homeostasis through its actions on glycogenolysis, gluconeogenesis, and hepatic glucose uptake (Unger and Orci, 1976; Stevenson et al., 1987; Unger and Cherrington, 2012; Cilvik et al., 2021). Produced by the α-cells of the pancreas, glucagon acts through its receptor, GCGR, which is expressed in the pancreas, liver, lung, kidney, placenta, and GI tract in the rodent (Campos et al., 1994; Ouhilal et al., 2012). GCGR has also been identified in first trimester human placenta (Perlman et al., 1988) and its mRNA has been reported in the near-term sheep placenta (Cilvik et al., 2021). Functionally, glucagon stimulates gluconeogenesis, which in the fetal sheep, has been induced through maternal starvation or by the fetal administration of glucagon (Limesand and Davis, 2018). Glucagon also impacts amino acid metabolism (reviewed in Cilvik et al., 2021), with short-term, acute infusions (20 h) in conjunction with somatostatin infusions resulting in suppressed amino acid concentrations (Teng et al., 2020; Cilvik et al., 2021). This is relevant as glucagon can be elevated in response to IUGR in both sheep and human pregnancies (Limesand et al., 2006; Hubinont et al., 1991; Cilvik et al., 2021). Recently, the effects of chronic hyperglucagonemia on fetal physiology and placental function were assessed through infusions of glucagon into fetal circulation (8-10 days; Cilvik et al., 2021). Elevated fetal glucagon concentrations resulted in reduced fetal weights and uterine blood flow, suppressed amino acid concentrations and umbilical uptakes, lower

protein synthesis and accretion, and decreased maternal CSH secretions (Cilvik et al., 2021). Not only does this emphasize the negative impacts of hyperglucagonmeia on fetal physiology, but also stresses the distinction between maternal and fetal responses to hormones as well as the negative regulatory impacts a fetal origin hormone can have on placental function and maternal physiology (Cilvik et al., 2021).

Chorionic somatomammotropin

While the actions of CSH on fetal physiology remain to be fully elucidated, it is clear that CSH plays a role in fetal growth. Handwerger postulated that CSH may directly act on fetal tissues to alter polyamine production by the fetal liver, nutrient uptake (amino acids), DNA synthesis, and IGF1 production (Handwerger, 1991). This hypothesis is supported by a strong association between low maternal CSH and fetal growth restriction in both the human and the sheep (Spellacy et al., 1976; Daikoku et al., 1979; Lea et al 2007). The hypothesized functions of CSH, as well as recent insights into the role of CSH in fetal growth will be thoroughly discussed later in this literature review.

CSH is produced by the binucleate cells of the ruminant placenta and the syncytiotrophoblast cells in the human placenta. The secretion of CSH can differ between maternal and fetal physiology as evident in the Cilvik et al. (2021) study, however, the mechanisms responsible for this difference have yet to be elucidated. CSH lacks circadian rhythms for secretion and can rapidly and spontaneously fluctuate (Taylor et al., 1980; Butler et al., 1987; Bauer et al., 1995). One factor that controls placental secretion of CSH into fetal circulation was reported in Freemark et al. (1992). In response to fetal hypoglycemia (due to maternal fasting) the placenta secreted

increased CSH into fetal circulation. The specific affinity of CSH with growth hormone receptor, prolactin receptor, and placental lactogen receptor will be further expounded on later in this review in the section pertaining to CSH. Major research gaps still exist in fully understanding the factors that influence the placental production and secretion of CSH, as well as the specific biological function of CSH on placental and fetal physiology.

Thyroid hormone

In the fetus, thyroid hormones are not only critical for neural development but also modulate fetal metabolism, tissue accretion and differentiation (Fowden and Forehead, 2014). The thyroid gland synthesizes and secretes the prohormone T_4 which is activated through tissue specific deiodinases (reviewed in Limesand and Davis, 2017). Placental deiodinases convert maternal T_4 to rT_3, although some transfer of biologically active T_3 can occur, which is critical for early embryonic brain development from ~12-20 weeks of gestation (Kota et al., 2013). Due to the expression of type 3 deiodinases by the placenta, rT_3 is abundant in fetal circulations throughout the second and third trimester (Kota et al., 2013). At approximately 30 weeks of gestation, type 1 and 2 deiodinases begin to be produced by the fetus and the concentration of T3 increases in fetal circulation (Kota et al., 2013). Thyroid hormone acts through its two nuclear receptors, thyroid hormone receptor (TR) α and TR β, each receptor with 2 additional isoforms (Kota et al., 2013). The fetal brain has TR expression by 8-10 weeks of gestation, and other tissues such as the liver, heart, and kidney by 13-18 weeks post conception (Kota et al., 2013).

The placenta is impermeable to thyroid releasing hormone (TRH), thyroid stimulating hormone (TSH), and thyroid-binding globulins (Kota et al., 2013). Around 10 weeks of gestation, fetal TSH begins to be secreted and reaches adult concentrations by late gestation (26-36 weeks), where it acts to promote thyroid follicular growth as well as thyroid hormone production (reviewed in Limesand and Davis, 2017). By 0.25 gestation in the human, fetal thyroglobulin production begins (Kota et al., 2013). As T_3 and T_4 levels drop, TSH increases and vice versa (reviewed in Limesand and Davis, 2017). After parturition, TSH, T_3, and T_4 all rise dramatically, especially as the hypothalamic-pituitary-thyroid axis matures postnatally (Forhead and Fowden, 2014). The impacts of *in utero* fetal hypothyroidism differ by species. In the human, fetal development does not appear greatly altered by fetal hypothyroidism, possibly because of adequate transfer of maternal thyroid hormone by the placenta (reviewed in Limesand and Davis, 2017). Hypothyroidism in the fetal sheep however, results in asymmetric fetal growth restriction with reduced muscle mass (Forhead and Fowden, 2014). This is because thyroid hormone directly stimulates fetal oxygen consumption and promotes anabolic effects on fetal metabolism (Forhead and Fowden, 2014). Thyroid hormone also indirectly promotes fetal growth by controlling the bioavailability of IGFs and catecholamines (Forhead and Fowden, 2014). Other consequences of hypothyroidism in the sheep are deficiencies in bone, lung, and cardiovascular maturation (Fowden and Forhead, 2014; reviewed in Limesand and Davis, 2017). This is because thyroid hormones modulate the effects of glucocorticoids in maturing fetal organs prepartum to promote neonatal viability (Forhead and Fowden, 2014)

Catecholamines

The fetal adrenal medulla first becomes innervated by the sympathetic preganglionic nerves at 0.8 gestation in the sheep, facilitating the secretion of catecholamines (Kota et al., 2013). Prior to innervation, the main driver of fetal catecholamine release is fetal hypoxia. Fetal hypoxemia stimulates the release of norepinephrine and epinephrine from adrenal chromaffin cells (Limesand and Rozance, 2017). This is relationship has been demonstrated in IUGR pregnancies, as hypoxemia accompanies hypercatecholemia (reviewed in Limesand and Rozance, 2017). This can be experimentally induced as well, as fetal infusions of norepinephrine results in elevated fetal oxygen (Basset and Hanson, 1998; Chen et al., 2014; reviewed in Limesand and Rozance, 2017). Additionally, pharmacological inhibition of adrenergic receptors in sheep leads to diminished blood oxygen content (Leos et al., 2010; Macko et al., 2013; reviewed in Limesand and Rozance, 2017). Catecholamines in conjunction with fetal hypoxia and hypoglycemia inhibit insulin secretions (reviewed in Davis et al., 2021). Elevated catecholamines have been postulated to inhibit insulin secretion from β-cell via the α_2-adrenergic receptor (reviewed in Limesand and Rozance, 2017).

In an experimental model of fetal sheep catecholamine inhibition through the ablation of medullary fetal chromaffin cells, fetal glucose-stimulated insulin secretion was suppressed (Yates et al., 2012). However, if the fetuses are already growth restricted (PI-IUGR) then undergo a bilateral adrenal demedullation (IAD), fetal glucose stimulated insulin secretions are augmented (Davis et al., 2021). This enhanced insulin secretion in IAD – IUGR fetuses does not alter fetal glucose production, utilization, or oxidation in contrast to IUGR fetuses with intact adrenal medullas (Davis et al., 2021). This might

suggest that this elevated glucose utilization and production is incumbent on fetal growth restriction, not catecholamines. Catecholamines however, do provide many other biological functions in the fetus and will be discussed later in this review, in the section pertaining to regulation of blood flow.

Fetal Nutrient Requirements & Uptake

Because factors influencing the transfer of substrates to the fetus have already been extensively discussed in the section on the placenta, as have the ramifications on fetal growth, this section will focus mostly on substrate specific fetal requirements for: 1) the maintenance of fetal oxidative metabolism and 2) fetal tissue accretion and growth. To understand the distinction between nutrients available for maintenance and tissue accretion, the fetus must be examined under “steady-state” where the flow of oxidative substrates can be defined as the fetal exogenous uptake of substrates (Battaglia and Meschia, 1978). This steady state occurs when the concentration of substrates in the blood and other body fluids are relatively constant despite the growth of the organism and storage of substrates in tissues (Battaglia and Meschia, 1978). The ability to quantitively compare both aspects (maintenance and growth) of fetal metabolism is dependent on the application of the Fick principle and the transplacental diffusion technique which will be discussed later in this review in the section detailing the transplacental diffusion technique. The majority of the literature to be discussed regarding fetal physiology and metabolism has been derived from sheep, due to tolerance towards chronic canulations and the understandable ethical limitations of performing these experiments in the pregnant human.

To estimate the caloric requirement of the fetal lamb for tissue accretion, bomb calorimetry was utilized to calculate the caloric value of the 130 dGA (~0.9 term) fetal sheep carcass which was 0.895 kcal/g (Battaglia and Meschia, 1986). The healthy fetal lamb at 130 dGA gains approximately 36 g/day/kg, thus, the caloric needs to facilitate tissue accretion is 32 kcal/day/kg (Battaglia and Meschia, 1978). Thus, the estimated caloric requirement of the 130 dGA fetal lamb is 88 kcal/day/kg (56 + 32 kcal/day/kg) for the fetus to maintain its metabolic function and grow (Battaglia and Meschia, 1978) although serial measurements of oxidative uptake and fetal growth measurements have yet to be recorded in the same animal due to the terminal nature of the latter.

Oxygen

As previously discussed, oxygen is a flow limited substrate transported across the placenta by simple diffusion. The near-term fetal lamb consumes approximately 75% of the uterine O_2 uptake which is approximately 8 mL/min of O_2 per kg of body weight (Battaglia and Meschia, 1978). Other species fetal O_2 uptake have also been documented in the same range (6-9.4 mL/min/kg fetal weight) as the fetal lamb while differing greatly in fetal size and gestational length (horse, cow, rhesus monkey, guinea pig, human; Battaglia and Meschia, 1978). While stressing the need for follow up studies using common methods and fetal preparations, Battaglia and Meschia (1978) describe that if oxygen consumption really is constant across mammals, this could explain, in part the allometric relationship between maternal and offspring weight. Because the fetus cannot appreciably store O_2, umbilical uptake rates for O_2 are equivalent to the rates of fetal O_2 utilization (Battaglia and Meschia, 1986). Fetal CO_2

excretion is comparable to the rate of fetal oxygen consumption, with a mean fetal respiratory quotient (RQ) of close to 1 (RQ = 0.94) (Battaglia and Meschia, 1978).

Maintenance & Growth:

Approximately 4.7-5.0 kcal per liter of oxygen is required to maintain metabolic processes, depending on substrate being oxidized (Battaglia and Meschia, 1978). Thus, the fetal lamb oxidizes approximately 56 kcal/day/kg fetus with 8 mL/min O_2 uptake (Battaglia and Meschia, 1978). Absolute fetal oxygen consumption increases over gestation, but not on a wet-weight basis (reviewed in Hay, 1991). However, on a dry-weight basis, fetal oxygen consumption decreases over gestation likely due to a fractional decrease in protein synthesis and the shift in tissue specific oxygen demands (Hay, 1991). In contrast to the fetal lamb, the human fetus grows at a rate approximately 3x slower, thus, requiring a greater supply of oxidative substrates (Battaglia and Meschia, 1978).

To understand the relative importance of any specific oxidative substrate in supporting fetal metabolism, nutrient quotients can be calculated to explain how the finite amount of oxygen being taken up by the fetus is being utilized (substrate/O_2 quotient = #C in substrate x change in substrate/ change in O_2). The closer the quotient gets to 1, the greater the role of the substrate in fetal oxidative metabolism (Battaglia and Meschia, 1978). This relationship aids in our understanding of what nutrients are primarily used to fuel fetal oxidative processes and growth. The best way to understand the relative importance of a substrate in context to fetal growth (not just maintenance) is to sum up all nutrient to oxygen quotients (glucose: oxygen, amino acid: oxygen, and

lactate: oxygen) to calculate a total nutrient quotient. If the number is equal to 1.0, the fetus is maintaining metabolic processes but not growing. If the number is >1.0, the fetus is growing. Any additional oxidative substrates over 1.0 fuel fetal growth, with higher values indicating a greater proportion of oxidative substrates available for growth.

Glucose

Umbilical glucose uptake is dependent on several factors including 1) the availability of transporters for facilitated diffusion, 2) rates of blood flow, 3) the maternal-fetal glucose gradient, and 4) the metabolic demands of the placenta. Unlike fetal oxygen uptake, the uptake of glucose by the fetus is not necessarily equivalent to fetal glucose utilization, as the fetus is capable of both producing and storing glucose (Battaglia and Meschia, 1986). Umbilical glucose uptake does however represent the exogenous supply of glucose to the fetus (Battaglia and Meschia, 1986). To accurately assess the impacts of exogenous glucose in fetal oxidative metabolism, umbilical glucose uptake must be considered against umbilical oxygen consumption (Battaglia and Meschia 1978). The closer the ratio is to 1.0, the more important glucose is in meeting fetal oxidative demands.

The rates of fetal glucose utilization and oxidation depend directly on the additive effects of plasma glucose and insulin, as well as the size of the fetal glucose pool, all of which can be disrupted in instances of IUGR. The duration of decreased glucose supply to fetal circulation can alter fetal glucose utilization. With short-term, abrupt decreases in available glucose, fetal utilization of glucose proportionately decreases (reviewed in Hay, 1991). If fetal glucose utilization decreases, the fetal demand for oxidative

substrates is replaced by other carbon sources such as glycogen, lactate, or amino acids as evidenced by constant O_2 utilization (reviewed in Hay, 1991). Longer periods of reduced fetal glucose supply decrease fetal oxygen utilization by 25-30% (reviewed in Hay, 1991).

Maintenance & growth

Under normal conditions, the singleton-bearing gravid uterus takes up 50 mg/min of glucose from uterine circulation, which is 3x as high as the concentration of glucose transferred from the placenta to the fetus (Battaglia and Meschia, 1986). To assess the relative importance of glucose to fetal oxidative processes, the umbilical glucose: oxygen quotients were assessed, and averaged 0.65 in well-fed pregnancies (Battaglia and Meschia, 1978). Under conditions of prolonged maternal fasting, umbilical glucose: oxygen quotients drop, and normalize to a steady-state fasted ratio of 0.30 (Battaglia and Meschia, 1978). This means that glucose is a significant oxidative substrate for fetal maintenance and growth. However, even under normal conditions, the quantity of glucose supplied to the fetus is less than what would be necessary for glucose to be the sole fetal oxidative substrate (Battaglia and Meschia, 1986). The other two key fetal oxidative substrates are amino acids and lactate (Battaglia and Meschia, 1986).

In the human, the umbilical glucose: oxygen quotient has been estimated at 0.80, thus a higher proportion of fetal oxidative substrate is supplied by glucose (Battaglia and Meschia, 1986). It is worth noting that these measurements were recorded at parturition and thus, may not necessarily be representative of steady-state nutrient quotients (Battaglia and Meschia, 1986). However, the increased brain: fetal weight ratio in the

human fetus vs. the sheep, may partially explain this increase in the glucose: oxygen quotient as the brain is the major site of glucose consumption in the fetus (Battaglia and Meschia, 1986; Sparks et al., 1980). Additionally, in humans and primates, the maternal to fetal glucose gradient is smaller than in sheep, which may indicate increased placental permeability to glucose in the human (Battaglia and Meschia, 1986).

Fetal glucose utilization and production can be calculated under steady state conditions where fetal glucose utilization = exogenous glucose uptake + endogenous glucose production (Battaglia and Meschia, 1986). Under normal conditions, fetal glucose utilization is approximately equivalent to exogenous glucose supply and thus, fetal gluconeogenesis is not a major supply (3.3%) of glucose (Battaglia and Meschia, 1986; Hay et al., 1981; Sparks et al., 1982). However, under conditions of maternal fasting, exogenous glucose uptake and glucose utilization are both decreased, but the decrease in utilization is less than the decrease in glucose uptake thus, the fetus must increase its endogenous (gluconeogenic) glucose production to meet this demand (Battaglia and Meschia, 1986).

Role of Insulin

The endogenous levels of circulating fetal insulin are fundamental to regulating glucose metabolism in the fetus. In fact, in pregnant ewes that undergo cycles of feeding, fasting, and refeeding, maternal and fetal plasma glucose and insulin are positively correlated (Battaglia and Meschia, 1986). Because the sheep placenta is impermeable to maternal insulin, fetal insulin concentrations are determined by the fetus (Battaglia and Meschia, 1986). The direct relationship between insulin and glucose

concentrations was demonstrated by a study designed to test selective hyperinsulinemia on glucose utilization, oxidation, and production (Fowden and Hay, 1988). Using chronically catheterized, late-gestation pregnant ewes with fetal pancreatectomy Fowden and Hay (1988) found that fetal pancreatectomy not only resulted in the predicted hypoinsulinemia, but also reduced the rate of fetal glucose uptake and utilization (Fowden and Hay, 1988). Interestingly, they found no difference in the glucose oxidation fraction in the pancreatectomized fetuses, but the rate of oxidation was still reduced due to the lower glucose utilization (Fowden and Hay, 1988). Next, they infused insulin into the pancreatectomized fetuses and were able to partially rescue (40-50%) the umbilical uptake and utilization rates of glucose (Fowden and Hay, 1988). As gestation progresses, glucose-stimulated insulin secretion increases (Hay 2006).

Other hexoses

Fructose

Fructose, a hexose present in high concentrations in fetal and neonatal circulation, possess a ketone group on the C_2 position (Battaglia and Meschia, 1986; Hugget et al., 1951). *In vitro* studies revealed placental origin fructose was likely the product of the reduction of glucose into sorbitol by the placenta and the conversion of sorbitol into fructose by the fetal liver (Alexander et al., 1970; Hers, 1961). Despite its high concentrations in fetal circulation, the role of fructose in fetal metabolism (if any) has yet to be demonstrated. Warnes et al (1977a & 1977b) infused [^{14}C] fructose into the fetal lamb and documented low fetal utilization of fructose (0.12mg/kg/min) in

contrast to glucose (4-5mg/kg/min). This is further evidenced by no significant umbilical arteriovenous difference in fructose concentrations (Battaglia and Meschia, 1986). While little metabolism of [^{14}C] fructose or turnover was documented by Alexander et al. (1970) in the fetal lamb, the quantity of $^{14}CO_2$ increased when glucose concentrations decreased. This may suggest that fructose plays a larger role as a fetal oxidative substrate in cases of prolonged fetal hypoglycemia. This is corroborated by low maternal and fetal glucose during maternal fasting, where the concentrations of fructose decreased slowly during this period which indicates some utilization of fructose by the fetus in these conditions (Battaglia and Meschia, 1986), or that hypoglycemia results in less glucose for the placenta to convert into sorbitol. Fructose levels do appear to be responsive to insulin and hypoglycemia. In response to two-week maternal hypoglycemia (induced through insulin infusions), fetal fructose concentrations fell (Brown et al., 2014). Additionally, during a longer duration (8 week) sustained hypoglycemia or hypoglycemia + insulin, fructose concentrations decreased (Brown et al., 2014). The role of fructose in IUGR pregnancies has yet to be directly assessed.

Galactose

Galactose is an aldose, critical for neonatal oxidative metabolism but has little function in the fetus (Battaglia and Meschia, 1986). Galactose is phosphorylated in the liver by galactokinase, eventually being converted into glucose (Battaglia and Meschia, 1986). The enzymes required to transform galactose into glucose are present during fetal life in the human and in other species such as the rat and rhesus monkey (Battaglia and Meschia, 1986). Under normal conditions (no dietary manipulation), little

galactose is present in fetal or maternal blood (Battaglia and Meschia, 1986). This changes after parturition, as the primary disaccharide in the neonatal diet, lactose, contains equal proportions of glucose and galactose (Battaglia and Meschia, 1986). Thus, the neonatal liver must be equipped and prepared to produce glucose from galactose (Battaglia and Meschia, 1986). During early neonatal life, in contrast to glucose, the concentrations of galactose in circulation are low due to rapid uptake and conversion by the liver (Battaglia and Meschia, 1986). Galactose clearance rates in the neonate are approximately 6.9% per minute vs 1.4% for glucose (Battaglia and Sparks 1983; Battaglia and Meschia, 1986). Thus, while galactose plays little documented role in fetal metabolism, the fetus undergoes preparation *in utero* for a postnatal life dependent on galactose uptake and conversion.

Lactate

Lactate is second only to glucose as a major carbohydrate source for the fetus (Battaglia and Meschia, 1986). Concentrations of lactate are higher in fetal blood compared to maternal blood, which was first demonstrated in the 1930's (Eastman and McLane, 1931). Historically, lactate concentrations were misinterpreted as an indicator of fetal hypoxia and the end product of anaerobic metabolism (Battaglia and Meschia, 1986). However, it has since been understood that lactate is a significant carbon source for the fetus, representing 25% of oxygen consumption or 0.25 metabolic quotient (Battaglia and Meschia, 1986). This was first evidenced by investigating late-gestation, chronically catheterized pregnant sheep where umbilical vein lactate concentrations were determined to be persistently higher than umbilical artery concentrations (Battaglia

and Meschia, 1986; Burd et al., 1975). The placenta is a net producer of lactate, secreting into both fetal and maternal circulation. Despite higher lactate concentrations in fetal circulation, the placenta secretes higher concentrations of lactate into fetal circulation compared to maternal (Battaglia and Meschia, 1986). It is still unclear why placental lactate production occurs in aerobic conditions and why lactate is important for fetal metabolism. Some of the fetal organs are net consumers of lactate like the heart (Fisher et al., 1980) and liver (Sparks et al., 1982) whereas other organs such as skeletal muscle are net producers (Battaglia and Meschia, 1986). Interestingly, only a small fraction of the lactate the fetal liver uses can be accounted for by glucogenesis (Battaglia and Meschia, 1986).

Maintenance & growth

The placental transfer/production of lactate is 2 mg/kg/min, constituting a sizeable supply of fetal carbon (Battaglia and Meschia, 1986). Fetal utilization of lactate has been calculated using [U-^{14}C] lactate (Battaglia and Meschia, 1986; Sparks et al., 1982). In late gestation, fetal utilization (5.9 mg/kg/min) of lactate is 3x higher than umbilical lactate uptake (1.9 mg/kg/min; Battaglia and Meschia, 1986; Sparks et al., 1982). This indicates that the fetus, even under normal conditions, produces most of the lactate (4 mg/kg/min) for the fetal lactate pool (Battaglia and Meschia, 1986). The majority (70%) of the lactate pool is oxidized (4.2 mg/kg/min) and the rest (1.8 mg/kg/min) is transferred into the fetal carbon pool (Battaglia and Meschia, 1986; Burd et al., 1975; Hay et al., 1983; Sparks et al., 1982). Lactate tracer lost into the placenta was only ~8% vs 53% for glucose, which supports high utilization by the fetus (Battaglia

and Meschia, 1986; Sparks et al., 1982). Functionally, lactate may be important to preserve the other carbon sources such as amino acids from oxidation so they can be available for tissue accretion and protein synthesis (Battaglia and Meschia, 1986).

Amino acids

Amino acids constitute a significant source of carbon (50%) for fetal growth and metabolism as well as placental function (reviewed in Hay 1991; Battaglia and Meschia, 1986; Meier et al., 1981b). Not only are amino acids an oxidative fuel, they also function as building blocks for proteins and can alter metabolic pathways in the fetus and placenta (Regnault et al., 2005). Amino acids are actively and competitively transported across the placenta, and thus, are dependent on both energy and transporter availability. To support appropriate fetal growth, the fetus must receive all the essential amino acids (Lys, His, Thr, Val, Met, Ile, Leu, Phe, and Trp) from maternal circulation, as well as the amino acid cysteine (Battaglia and Meschia, 1978). This is because the fetal liver lacks the transulferase, cystathionase, necessary to produce cysteine from methionine (Battaglia and Meschia, 1978). The nonessential amino acids (Orn, Arg, Tau, Asp, Asn, Ser, Glu, Gln, Gly, Ala, Tyr, Pro) are also critical for fetal growth but can come from either maternal circulation or fetal *de novo* synthesis (Battaglia and Meschia, 1986). Through the combination of the transplacental diffusion technique, the Fick principle, and HPLC to analyze amino acids, it is possible to examine the specific uptakes of individual amino acids.

Maintenance & growth

During the last 20% of pregnancy in the fetal sheep, the net daily uptake of amino acids provides the fetal lamb with 3-4 g of carbon per kg of fetal weight (Battaglia and Meschia, 1978; Hay, 1991). During this period, the fetus also takes up 0.9 g of nitrogen per kg of fetal weight, of which, the fetus can store 0.65g of nitrogen per day in the form of new tissue (Battaglia and Meschia, 1978; Hay, 1991). Due to the shifts in individual amino acids taken up by the fetus, the amino acid carbon: nitrogen ratio in the sheep fetus decreases from 3.42 at 66 dGA to 3.06 at term (Battaglia and Meschia, 1986).

Individual amino acids cannot be stored in the fetus, thus, the fate of the amino acids can either be as an oxidative fuel or tissue accretion/ protein synthesis (Battaglia and Meschia, 1986). The best way to document the utilization of specific amino acids is through labeling them with tracer (reviewed in Hay, 1991). The fate of amino acids can be shifted from anabolic pathways to oxidative catabolism during fetal hypoglycemia (Hay, 1991). This indicates the potential role of non-protein energy sources play in regulating amino acid fates (Hay, 1991). In some instances, the fetus supplies the placenta with amino acids. For example, glutamine taken up by the fetus and transferred back to the placenta as glutamate (Hay, 1991). This is also true for glycine, which is returned to the placenta as serine, and asparagine returned as aspartate (Hay, 1991).

The abundance of individual amino acids differs dramatically. Glutamine is the most abundant amino acid taken up by fetal circulation (Battaglia and Meschia, 1986). The fetal lamb also takes up neutral amino acids at a higher rate than their rates of accretion in protein in late gestation (Battaglia and Meschia, 1986). However, the amino acids

lysine, histidine, and glycine are only taken up at levels approximating their rate of accretion, thus perturbations in their concentrations can quickly impact net protein accretion rates (Battaglia and Meschia, 1986). The oxidation rates of individual amino acids also vary. Leucine has high oxidation rates (determined with tracers) and thus, must be taken up at approximately double the rate of accretion (4.0 vs 2.0 μmol/min/kg; van Veen et al., 1987). This high leucine oxidation rate is detected even at mid-gestation and continues through late gestation in the sheep (Kennaugh et al., 1985). These high oxidation rates can be increased further by maternal fasting (Battaglia and Meschia, 1986; van Veen et al., 1987).

One way to assess protein synthesis is by assessing protein accretion rate and degradation (Battaglia and Meschia, 1986). These rates can be calculated through the infusion of tracer labeled amino acids. This has been conducted with [^{14}C] lysine and [1-^{14}C] leucine labeled amino acids infused into the fetal lamb (Meier et al., 1981a; van Veen et al., 1987; Battaglia and Meschia, 1986). As defined by Battaglia and Meschia (1986), the rate of flux by which tracer labeled amino acids are incorporated into proteins = [tracer infused] – [^{14}CO2 excreted] – [tracer placental loss]. The quantity of tracer escaping through the placenta can be substantial (upwards of 60% based on a leucine tracer in a small fetus during mid-gestation) and the protein synthesis rates can vary from organ to organ (Battaglia and Meschia, 1986). From a tissue growth standpoint, fractional protein synthetic rate is much greater than the fractional growth rate (Battaglia and Meschia, 1986). The rate of protein synthesis accounts for approximately 25% of the oxygen consumption by the fetus (Battaglia and Meschia, 1986). A shortcoming of this approach is the inability to calculate total unidirectional

amino acid flux, as some amino acids can be released by intracellular protein degradation and then are utilized by that same cell without entering circulation (Battaglia and Meschia, 1986). While investigating amino acid metabolism is of great value to understanding fetal physiology, it remains a daunting subject due to the complexities of amino acid transporter systems, competitive inhibition of maternal amino acids on fetal uptake (Jozwik et al., 2003), and the necessity of tracer-labeling amino acids to understand how a single amino acid is utilized and incorporated into tissues or proteins.

Role of Glucagon

Glucagon regulates amino acid metabolism, stimulating hepatic uptake of amino acids and inhibition of protein synthesis (reviewed in Cilvik et al., 2021). Elevated glucagon levels (caused by glucagonomas) result in hypoaminoacidemia and decreased lean mass (reviewed in Cilvik et al., 2021). In contrast, glucagon deficiency (pancreatectomy-induced) or glucagon receptor mutations lead to hyperaminoacidemia without hypoglycemia (reviewed in Cilvik et al., 2021). During pregnancy, glucagon is elevated in IUGR sheep pregnancies induced by maternal hyperthermia (Limesand et al., 2006), elevated catecholamines, and fetal hypoxia (Sperling et al., 1980; Schreiner et al., 1981; Shelly & Girard, 1981). Elevated glucagon has also been documented in the human fetus during instances of fetal distress and hypoxia at parturition (Hubinont et al., 1991).

To investigate the impacts of chronic hyperglucagonemia on fetal amino metabolism, Cilvik et al. (2021) infused glucagon into fetal circulation for 8-10 days after placing surgical catheters into fetal and maternal circulation. Using L-[1-^{13}C] Leu tracer,

they assessed leucine flux into the placenta, fetal tissues, and plasma (Cilvik et al., 2021). They also examined fetal leucine disposal, oxidation, protein accretion and synthesis (Cilvik et al., 2021). Chronic hyperglucagonemia reduced concentrations of 16 fetal amino acids and decreased umbilical uptakes of 7 amino acids, ultimately diminishing umbilical carbon and nitrogen uptakes (Cilvik et al., 2021). Using the leucine tracer, Cilvik et al. (2021) found that high levels of glucagon reduced leucine disposal rate as well as reduced leucine flux into fetal tissue, protein accretion and protein synthesis. Clearly, glucagon can impact amino acid uptakes and utilization, as well as protein accretion and synthesis. In addition to regulation by glucagon, amino acids also regulate protein synthesis by interacting with insulin and IGF signaling to regulate mammalian target of rapamycin pathway (mTOR; Regnault et al., 2005).

Lipids

In contrast to the other oxidative substrates, fetal fat metabolism has not been as heavily investigated. This is largely due to difficulties in accurately measuring the flux of free fatty acids (FFAs) as well as the large quantity of oxygen necessary to fully oxidize each fatty acid. There appear to be species specific differences in maternal lipid concentrations, and thus, fetal concentrations (Battaglia and Meschia, 1986). In the human, it appears that plasma free fatty acids (FFAs) increase during late gestation (Battaglia and Meschia, 1986). This is in contrast to the sheep, guinea pig and rabbit which show no appreciable changes in FFAs or ketoacids over gestation (Battaglia and Meschia, 1986; Gilbert et al.,1984; Sparks et al., 1981). Maternal fat deposition does increase across many species in early gestation, and often, those fat stores are

mobilized later in gestation (Battaglia and Meschia, 1986). Another common response across many species including the human and the sheep are that fasting increases blood glycerol, FFAs, and ketoacids during pregnancy (Freinkel and Metzger., 1975; Battaglia and Meschia, 1986). In the sheep, the shift to fat mobilization occurs around 135 dGA, with the degree of mobilization depending on fetal number and maternal nutritional status (Battaglia and Meschia, 1986; Robinson et al., 1978; Vernon et al., 1981). It is difficult to accurately assess the flux of free fatty acids across the placenta, since a relatively small uptake can profoundly impact the density of carbon sources available for catabolism. Additionally, the methods to measure the flux (barring a lipidomic approach) are not sensitive enough to discriminate between different FFAs to determine exact fluxes and how they impact the fetal carbon pool if fully oxidized. Powell et al. (2021) assessed placental fatty acid oxidation, esterification, and transfer capacity in cases of maternal obesity using lipidomic approaches. They observed sex-specific decreases in umbilical plasma docosahexaenoic acid (DHA) in male fetuses from obese mothers at term caesarean-section, and decreased MUFA-carnitine concentrations in female placentas.

Another complication is the substantial quantity of oxygen required to fully oxidize a lipid. For instance, in comparison to lactate which requires 3 mmol of oxygen to be fully oxidized, palmitate requires 23 mmol of oxygen (Battalgia and Meschia, 1986). Additionally, due to the low concentrations of FFAs, it is difficult to measure arteriovenous gradients and thus, obfuscates the application of the Fick principle (Battaglia and Meschia, 1986).

Maintenance & growth

From a fetal perspective, lipids are essential for fetal brain development. The human has one of the higher newborn lipid concentrations with approximately 18% body fat at birth (reviewed in Hay, 1991). In contrast, the fetal lamb has approximately 3% body fat or 30g fat per kg (reviewed in Hay, 1991). Metabolically, the fates of fatty acids in the fetus include 1) β-oxidation, 2) the incorporation of phospholipids into membranes, 3) esterification of fatty acids into triglycerides and 4) lipid deposition into brown and white adipose tissue (reviewed in Hay, 1991). The potential sources of umbilical FFA uptakes come from maternal circulation and placental transfer and *de novo* synthesis (Battaglia and Meschia, 1986). The sheep placenta is relatively impermeable to maternal FFAs but is quite metabolically active at synthesizing and desaturating long chain FFAs (Battaglia and Meschia, 1986). The human placenta has been demonstrated to synthesize arachidonic acid from acetate (Zimmerman et al., 1979). Additionally, *de novo* synthesis of fatty acids from non-lipid precursors (lactate, amino acids, glucose, etc.) has been demonstrated in many species by the fetal liver, as well as from short-chain fatty acids (reviewed in Hay, 1991).

Summary

Fetal organogenesis occurs early in gestation, however much of organ growth and function develops throughout gestation. Thus, factors that prevent adequate transfer of nutrients to the fetus to support appropriate organ development can set the stage for postnatal health complications in endocrine function, as well as reduced fetal growth. The placenta can directly impact fetal endocrine function through 1) the

stimulation of hormones due to nutrient transfer, 2) placental production or transfer to fetal circulation, or 3) placental modifications of hormone bioavailability (either neutralizing or supplying binding proteins). Fetal endocrine status also plays an important role in the regulation of nutrient utilization by the fetus which ultimately controls fetal growth. While the appropriate balance of fetal hormones is necessary for normal development, fetal IGF production is especially critical. Because the placenta is a source of both IGF1 and IGF2 as well as many of the IGFBPs, factors that impact placental and fetal IGF production and regulation are crucial to investigate. The placental hormone CSH has been hypothesized to impact fetal IGF production, and CSH deficiency have already been associated with IUGR. Therefore, it is vital to examine the direct and indirect relationships that CSH has on IGF production and fetal nutrient uptake to address this research gap, and how these interactions may modulate fetal growth.

REGULTATION OF BLOOD FLOW

Adequate perfusion of the uteroplacental unit is critical for maintaining placental function and fetal growth. This review will explore factors regulating uterine and umbilical vasoactivity, changes in perfusion over gestation, and experimental methodologies to assess blood flow. As defined in Guyton and Hall (2006), the rate of blood flow to a tissue is dependent on tissue-specific nutrient demands. Ultimately, two key factors control blood flow in a vessel; the pressure gradient between blood entering and exiting the vessel, and vessel resistance (reviewed in Guyton and Hall, 2006). To understand the regulation of blood flow, it is necessary to take into account factors that

control vascular tone and magnitude of blood flow for both the maternal and fetal aspects of circulation. Fundamentally, vascular resistance can be understood by the relationship between arterial characteristics (length, size, etc.) and blood viscosity (reviewed in Poston, 1997). This relationship is derived from Poiseuille's Law.

$$R = \frac{(\pi r^4)}{8\eta l}$$

As defined by the above equation, R = vessel resistance, r = vessel radius, η = blood viscosity, and l = vessel length (reviewed in Poston, 1997). While many factors contribute to vascular resistance, vessel radius is the most significant factor. This is because impacts on vessel radius produce exponential changes to resistance, known as the "4th power law" (reviewed in Guyton and Hall, 2006). For instance, a 4-fold increase in vessel diameter would cause a 256-fold decrease in vessel resistance, producing profound impacts on blood flow. Thus, it is logical to examine what factors can influence vessel radius through vasodilation or vasoconstriction.

Vasoactivity and Pregnancy

Over gestation, uterine blood flow increases 40-fold over gestation and maternal cardiac output doubles in the pregnant woman (reviewed in Rosenfeld, 2011). The increase in cardiac output is supported by elevating maternal heart rate and stroke volume (Rosenfeld, 1977), and the rise in blood flow is facilitated by decreasing uterine (Rosenfeld, 1977) and umbilical arterial resistance (reviewed in Poston 1997; Battaglia, 2011). More specifically, the reductions in arterial resistance are supported by an attenuation in responsiveness to vasoconstrictors. This pregnancy-specific attenuation has been observed in women (Chesley et al., 1965, Gant et al., 1973) and sheep

(Rosenfeld and Gant, 1981, Rosenfeld, 2001). If this attenuation is perturbed, hypertension during pregnancy can occur (Chesley et al., 1965, Gant et al., 1973), resulting in placental hypoperfusion (Erkkola and Pirhonen 1992). On the fetal side of circulation, increased vascular resistance can reduce umbilical blood flow (Battaglia, 2011). Thus, it is valuable to examine some of the endocrine and paracrine factors impacting both vasoconstriction and vasodilation.

Vasoconstrictors

Angiotensin II

Angiotensin is a member of the Renin-Angiotensin-Aldosterone system (RAAS) and its biologically active form, angiotensin II (ANG II), is a potent vasoconstrictor (Giebisch and Windhager, 2009). ANG II has a short half-life (~2 min) and renin secretions regulate its plasma concentrations (Giebisch and Windhager, 2009). Women with healthy pregnancies experience an attenuated responsiveness to ANG II starting in mid-gestation, evidenced by measuring changes in vascular resistance (Chesley et al., 1965, Gant et al., 1973). Sheep also experience similar attenuations in responsive to ANG II during pregnancy (reviewed in Rosenfeld, 2011), supporting the utility of investigating sheep to better understand uterine vasoactivity.

A key factor in regulating uterine artery responsiveness is proportional density of ANG II type 1 (vasoconstriction) vs. type 2 receptors (does not mediate vasoconstriction). The most predominant uterine artery ANG II receptor in both humans and sheep is type 2 (McMullen et al., 1999). The type 2 receptor may, in fact, directly attenuate type 1 receptor induced vasoconstriction (McMullen et al., 1999). Umbilical

circulation is also highly responsive to ANG II. This could, in part, explain why the umbilical vasculature is more sensitive to ANG II than the uterine vasculature as there is a greater proportion of type 1 receptors in umbilical vasculature (reviewed in Rosenfeld, 2011).

Much like the complex and sophisticated balance of angiogenic vs angiostatic factors responsible for mediating the shape of the fetal villi discussed in the placenta section of this literature review, uterine artery vessel tone is dependent on the balance of vasoconstrictive vs. vasodilatory factors. Angiotensin is an excellent example of this balance. In response to elevated ANG II, a 3-fold increase in prostacyclin was observed in the ewe (Rosenfeld, 2001). Prostacyclin is a potent vasodilator and ANG II antagonist (Rosenfeld, 2001). Furthermore, the uterine endothelial synthesis of nitric oxide (NO) is also stimulated by elevated ANG II concentrations (Rosenfeld, 2001).

Catecholamines

Adrenal derived catecholamines such as epinephrine and norepinephrine act through their α- receptor to induce vasoconstriction (reviewed in Rosenfeld, 2011). In healthy pregnancies, pressor responses to catecholamines are somewhat attenuated (Magness and Rosenfeld, 1986). Even so, the uterine vasculature remains more sensitive to catecholamines compared to systemic circulation (reviewed in Rosenfeld, 2011). This means that in comparison to vasoconstrictors like ANG II, catecholamines can more potently induce reductions in uterine blood flow, even at low concentrations (reviewed in Rosenfeld, 2011).

There is likely an evolutionary reason behind this phenomenon. Factors that rapidly alter ANG II concentrations such as rising from a supine position do not pose a threat to the mother's life, thus, it is advantageous to preserve fetal wellbeing by attenuating uterine vascular sensitivity (reviewed in Rosenfeld, 2011). However, factors that rapidly elevate catecholamines can be life-threatening (hemorrhage, etc.) and thus, the risk of maintaining a reasonable level of vascular responsiveness is evolutionarily advantageous (reviewed in Rosenfeld, 2011). In contrast to maternal circulation, umbilical responses to catecholamines are muted (reviewed in Rosenfeld, 2011; Poston, 1997). In fact, fetal catecholamine concentrations are associated with the maintenance of appropriate umbilical perfusion (reviewed in Rosenfeld, 2011).

Other Vasoconstricting Factors

Thromboxane is a potent umbilical artery vasoconstrictor and is thought to contribute to its vasoconstriction at parturition (reviewed in Poston, 1997). It is elevated in some cases of pregnancy induced hypertension, and the placenta is a possible source for thromboxane (reviewed in Rosenfeld, 2011). Endothelin is another well documented fetal vasoconstrictor and was found to be elevated in cases of fetal hypoxia (reviewed in Poston 1997). Concentrations of endothelin are higher in fetal vs. maternal circulation (reviewed in Poston, 1997). In comparison to both thromboxane and endothelin, prostaglandins ($PGF_2\alpha$ and prostaglandin E_2; PGE) have only mild vasoconstricting effects in fetal circulation (reviewed in Poston, 1997). Conversely, in the uterine artery, PGE acts as a vasodilator which may assist in increasing uterine perfusion of the placenta in response to low umbilical blood flow (reviewed in Rosenfeld, 2011). Arginine

vasopressin (AVP), also known as antidiuretic hormone, is another vasoconstricting factor. AVP is secreted in response to intrauterine hypoxia or fetal asphyxia (Iwamoto et al., 1979; reviewed in Rosenfeld, 2011), however, appears to act mostly on uterine vessels as fetal umbilical vascular responses are attenuated (reviewed in Rosenfeld, 2011). Lastly, glucocorticoids act by potentiating the actions of ANG II, AVP, and catecholamines to induce vasoconstriction (Yagil and Krakoff, 1988; Xiao et al., 2002). Additionally, glucocorticoids reduce vasodilators like NOS3 but the mechanisms driving this reduction remains poorly understood (Malek et al, 1999; Li et al., 2007).

Vasodilators

Steroid hormones

The placenta is a major source of steroid hormones for both the human and the sheep. Estrogen and progesterone are not only requisite for a successful pregnancy outcome but are also necessary for promoting cardiovascular adaptations and uterine blood flow (Paria et al., 2002). Estradiol 17β is a potent vasodilator which promotes increased blood flow in two ways: first, by increasing maternal heart rate and cardiac output, and second, by decreasing vascular resistance through vasodilation (Ford et al., 1977; Magness and Rosenfeld, 1989). This is supported by studies in the sheep which saw a 40-50% increases in uterine blood flow in response to estrogen infusion (reviewed in Rosenfeld, 2011). Estrogen-mediated vasodilation is largely through the activation of endothelial nitric oxide synthase (NOS3) which drives the production of nitric oxide (reviewed in Rosenfeld, 2011). This is evidenced by a 60-70% reduction in

estradiol-mediated vasodilation when NOS3 is inhibited (reviewed in Chang and Zhang, 2008).

Progesterone is thought to maintain phasic contractility in the uterine artery vascular endothelium by increasing the density of α1 receptors (Ford, 1995). While progesterone alone possesses no vasodilatory effects, it functions as an estrogen antagonist when administered in conjunction with estrogen (Ford et al., 1977). In contrast to its antagonistic functions, progesterone has also been shown to increase NOS3 in the uterine artery epithelium, but not as robustly as estradiol (reviewed in Chang and Zhang, 2008). Clearly, the regulation of vascular tone by steroid hormones is complex and multifaceted.

Nitric oxide

Nitric oxide (NO) is also a potent vasodilator which works by activating smooth muscle guanyl cyclase to generate cGMP which induces vasorelaxation (Rosenfeld, 2011). Under normal conditions, NO production increases over gestation in both the pregnant woman and sheep (Sladek et al., 1997, Rosenfeld et al., 1996). This increase in NO may contribute to cardiovascular modifications necessary to sustain pregnancy and promote uterine blood flow. This is evidenced by 25% reductions in uterine blood flow with inhibition of NOS (Rosenfeld et al., 1996, Miller et al., 1999).

In fetal circulation, NO also appears to be an important vasodilator. On a relative basis, perfused umbilical arteries and veins release greater amounts of NO compared to PGI_2 (Chaudhuri et al., 1993), and the inhibition of NOS3 increases placental perfusion pressure *ex vivo* (reviewed in Poston, 1997). A study in sheep provides the most

compelling evidence for the importance of NOS3 in fetal circulation as its inhibition reduced umbilical blood flow by 50% (Chang et al., 1992). Overall, NO is thought to contribute to the complex equation of vasoactive factors regulating blood flow and may help in attenuating constrictor responses.

PGI_2 & PGE

In uterine circulation, increased vessel perfusion resulting in sheer stress can increase the secretion of the PGI_2, a vasodilator (Frangos et al., 1985). However, the direct effects of the vasodilatory prostanoids are complex to investigate as inhibition of their production (cyclooxygenase inhibitors) does not alter uterine blood flow but does increase vascular responsiveness to vasoconstrictors (Magness et al., 1992, Naden et al., 1985). As previously mentioned, PGE can either act as a weak vasoconstrictor (umbilical circulation) or as a vasodilator (uterine circulation).

Kinins

Another vasodilatory factor are the kinins. Kinins are produced from the catabolism of kininogens by kallikreins (Boulpaep, 2009). Bradykinin, a common nonapeptide kinin, is inversely linked to the angiotensins as angiotensin converting enzyme (ACE) promotes the disposal of bradykinin while synthesizing ANG II (Boulpaep, 2009). Bradykinin acts through the B2 receptor on endothelial cells to increase production of both NO and prostaglandins, causing vasodilation (Boulpaep, 2009). While this is not a comprehensive list of all vasodilatory factors, it is an attempt to highlight key factors involved in regulating blood flow to the placenta.

Uterine vs. Umbilical Changes over Gestation

To achieve a wholistic understanding of placental perfusion, it is also necessary to an examine how blood flow is distributed and what must occur to support hemodynamic shifts across pregnancy. This section also aims to provide insights about key timepoints that are more sensitive to aberrant production of vasoactive factors.

Changes in distribution of blood flow

Major changes in uterine mass and perfusion occur over gestation to support successful pregnancy outcomes. Due to the invasiveness of the techniques required to determine distribution of blood flow, the studies reviewed below were conducted in pregnant sheep. Prior to conception, uterine blood flow is equally distributed between the myometrium, the endometrium, and the caruncles (Rosenfeld et al. 1974). During early pregnancy (prior to 50 dGA), 50% of the uterine blood flow perfuses the endometrium and only 27% is directed towards the site of implantation (Rosenfeld et al., 1974). As gestation progresses, more uterine blood flow is directed towards the placenta, which receives approximately 64% of uterine blood flow by 57-85 dGA and 82-90% between 100- 140 dGA (Rosenfeld et al., 1974). The proportion of cardiac output supplying the uterus also shifts from less than 1% with the nongravid uterus to upwards of 25% near term (Rosenfeld, 1977).

Relative umbilical blood flow per gram of cotyledon increases over gestation and is highest in late gestation (Rosenfeld et al., 1974). Umbilical blood flow and fetal growth follow similar growth curves; however, fetal growth occurs at a greater rate than umbilcal blood flow (Rosenfeld et al., 1974). Thus, on a relative basis, umbilical blood

flow relative to fetal weight decreases over gestation (Rosenfeld et al., 1974). Gestational increases in umbilical blood flow are related to placental maturation which involves concomitant increases in fetoplacental perfusion and permeability (Meschia et al., 1967).

A hallmark of a healthy pregnancy is excess umbilical blood flow relative to fetal growth which serves to insulate the fetus against sudden drops in blood flow as a result of increased concentrations of vasoconstrictors (reviewed in Rosenfeld, 2011). Not surprisingly, factors that sustain vasoconstrictor concentrations or perturb fetoplacental perfusion can lead to inadequate blood flow and poor nutrient transport. Because the fetal growth curve is dependent on adequate perfusion of the uteroplacental unit, reductions in uterine blood flow likely have an amplifying effect on pregnancy perturbations.

Maternal hemodynamic shifts

Uterine blood flow is characterized by three hemodynamic shifts: 1) early pregnancy vasodilation, 2) maximal growth of placental vascular volume, 3) exponential increase in uterine blood flow and perfusion of the uteroplacental unit (Rosenfeld, 2011). In contrast to the human, the pregnant ewe has two hemodynamic shifts; the first from 40-70 days and the second from 120-140 days (Rosenfeld et al., 1974).

The first hemodynamic shift is characterized by vasodilation of the uterine artery as a result of ovarian hormones (estrogen, progesterone, etc.) and possibly, some placental hormonal contributions (reviewed in Rosenfeld, 2011; Paria et al., 2002). This first phase in the woman occurs early in pregnancy, generally in the first few days or

weeks (reviewed in Rosenfeld, 2011). The second phase is marked by placentome development in the ruminant or development of the intervillous space in the human, followed by peak growth of the fetal placenta (reviewed in Rosenfeld, 2011). During this period in the sheep, uterine blood flow plateaus and relative uterine blood flow decreases as a function of increasing placental and fetal mass (reviewed in Rosenfeld, 2011). The last phase of hemodynamic shift is exponential, increasing fetal weight three-fold (>110 dGA in the sheep or >30 weeks in the human; reviewed in Rosenfeld, 2011). This exponential increase is currently thought to be driven by vasodilation, as neither placentome nor spiral artery count increase during this period (Paria et al., 2002, reviewed in Rosenfeld, 2011). Due to the reliance of many uterine hemodynamic shifts on vasodilation, it is not surprising that factors that cause vasoconstriction or prevent the attenuation of responsiveness of the uterine artery to vasoconstrictors may perturb these key shifts.

Umbilical hemodynamics

Fetal blood flow is controlled by a combination of factors including fetal cardiac output, placental resistance, and structural organization of placental arteries (Poston, 1997). Near term, umbilical blood flow represents approximately 20% of fetal cardiac output (Poston, 1997). The major site of placental vascular resistance is likely the main stem villi and their intermediate branches as evidenced by their substantial smooth muscle content (Kaufman, 1982). In healthy pregnancies, there is little vascular resistance to umbilical perfusion to facilitate nutrient exchange (Poston, 1997). Unlike the uterine vasculature, umbilical blood flow is thought to be primarily driven by vascular

growth, with some contributions of vasoactivity (reviewed in Rosenfeld, 2011). This is supported by relatively stable blood flow across gestation (reviewed in Rosenfeld, 2011). However, secretion of vasoactive factors remains vital for fetal health. In response to fetal hypoxia, the fetus produces catecholamines, angiotensin II and vasopressin to alter vascular resistance, but does not impact fetal brain or myocardial blood flow (Poston, 1997).

Methods for Tracking Blood Flow

In order to assess perturbations in uterine and umbilical blood flow during key timepoints *in vivo*, a variety of techniques have been developed. Each technique has relative strengths and weaknesses, and the methods are worth describing to interpret their use throughout the literature. Each technique varies in terms of invasiveness, duration of use, and potential safety considerations for the fetus.

Transplacental diffusion technique & the Fick principle

The transplacental diffusion technique involves the surgical preparation of pregnancies with indwelling catheters followed by a recovery period to allow the animal to return to steady state conditions (non-stressed, non-anesthetized, non-fasted), and a subsequent metabolic study. The transplacental diffusion technique involves the infusion of a tracer molecule until steady-state concentrations are achieved, followed by blood sampling from maternal and fetal catheters simultaneously (Meschia et al., 1965; Battaglia and Meschia 1986). While often more complex, a minimal surgical prep for measuring both uterine and umbilical blood flow includes the placement of vascular

catheters into the umbilical vein, fetal descending aorta (proxy umbilical artery), the uterine vein, and the maternal femoral artery (proxy uterine artery; Meschia et al., 1965; Battaglia and Meschia 1986). By infusing a tracer molecule which cannot be metabolized or produced within the exchanger, the transplacental diffusion rate is calculated by the difference between the infusion and escape rates of the tracer (Battaglia and Meschia, 1986). The tracer is infused into the fetal umbilical artery and thus, concentrations of the tracer molecule are highest in that vessel (**Figure 1**), followed by the umbilical vein, uterine vein, and uterine artery. Blood flow is calculated by dividing the transplacental diffusion rate by the arteriovenous differences in tracer concentrations (Battaglia and Meschia, 1986). This allows the application of the Fick principle to calculate substrate specific uptakes.

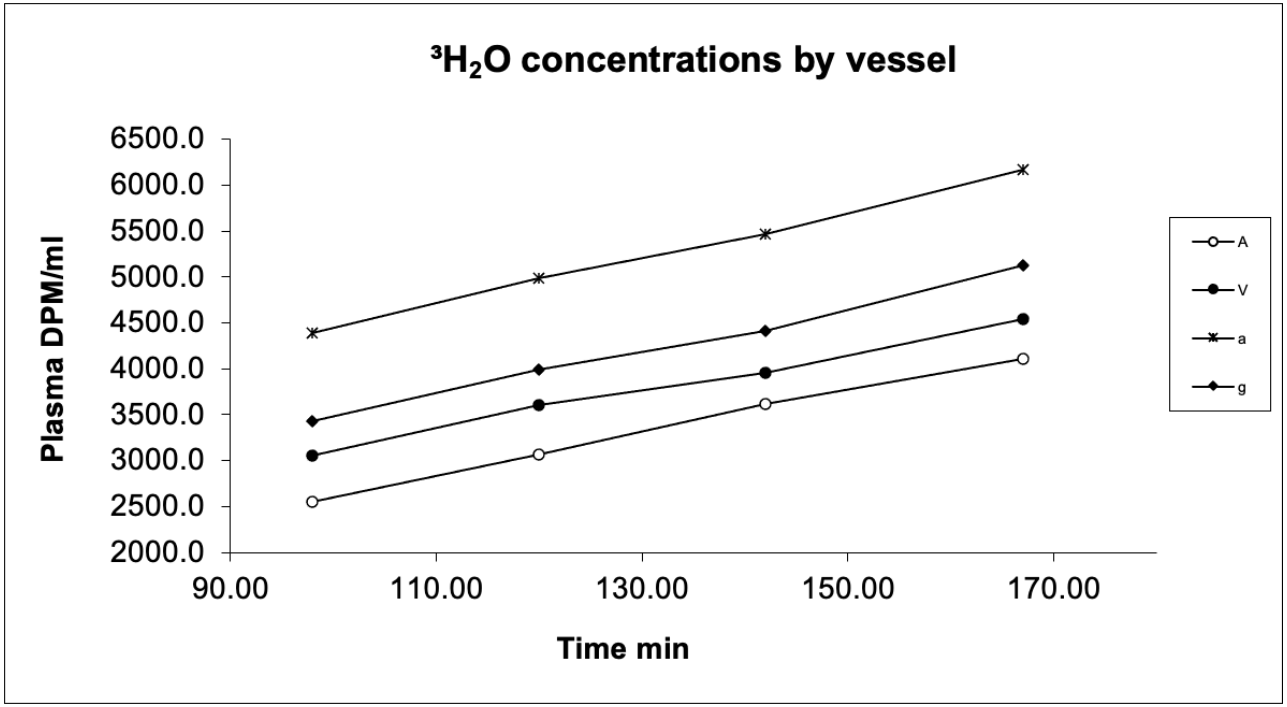


Figure 1.1: Tracer ($^3H_20$) concentrations in the umbilical vein (g), umbilical artery (a), uterine vein (V), and uterine artery (A) after reaching steady-state tracer concentrations from 90-170 minutes of study.

The Fick Principle is defined as the quantity of a substrate entering an organ (arterial blood) must equal the quantity of the substrate being extracted from the blood by the organ plus the quantity of substrate leaving an organ (venous blood; Battaglia and Meschia, 1986). As summarized by **Table 1**, to calculate uptake rate of a substrate by the uterus (uteroplacental unit) or by umbilical circulation (fetal), the rate of blood flow is multiplied by the arteriovenous differences in the substrate (Battaglia and Meschia, 1986). To calculate what the uteroplacental unit is utilizing, the difference between uterine and umbilical uptakes is calculated (Battaglia and Meschia, 1986).

From a research perspective, the Fick principle is the gold standard for investigating placental and fetal physiology as it not only addresses rates of blood flow but the transplacental transport of nutrients. Some shortcomings of this technique are the technical expertise necessary to surgically place the catheters as well as the possibility of catheters becoming dislodged or failing to draw. Additionally, this technique is only useful in a research setting due to its invasiveness and risk to the pregnancy, thus, other methods should be employed when screening for IUGR.

Table 1.2. Calculations[1] for blood flow by the transplacental diffusion technique.

Blood Flow (^3H_2O tracer)	
R_{inf} ^3H_2O (dpm/min)	Pump rate * [infusate]
$R_{acc(f)}$ (dpm/min)	α_{pl} slope * (0.8 * fetal weight)
$R_{acc(m)}$ (dpm/min)	$R_{acc(f)}$ + [α_{pl} slope * 0.8(uterine weight)]
Umbilical Blood Flow (UBF; mL/min)	$(R_{inf} - R_{acc(f)}) / ([^3H_2O]_{\alpha(WB)} - [^3H_2O]_{\gamma(WB)})$
Umbilical Plasma Flow (UPF; mL/min)	UBF * [1 – $Hct_{f(avg)}$]
Uterine Blood Flow (UtBF; mL/min)	$(R_{inf} - R_{acc(m)}) / ([^3H_2O]_{V(WB)} - [^3H_2O]_{A(WB)})$
Uterine Plasma Flow (UtPF; mL/min)	UtBF * [1 – $Hct_{m(avg)}$]

[1]Calculations are derived from Cilvik et al., 2021.

Electromagnetic and Doppler flow probes

Both electromagnetic and Doppler flow probes have been heavily utilized throughout the literature to examine blood flow through a vessel. From a research perspective, indwelling flow probes offer tremendous advantages as they can directly evaluate blood flow over time to examine the regulation of uterine blood flow (Battaglia and Meschia, 1986). The flow probe is surgically placed around the middle uterine artery (Battaglia and Meschia, 1986) with the poles of a strong magnet and electrodes opposite to each other, perpendicular to the magnetic line of force (Guyton and Hall, 2006). This allows electric voltage to be generated and recorded using a voltmeter as blood passes through the vessel (Guyton and Hall, 2006). Electromagnetic flow probes utilize Faraday's Law of Induction, generating a magnetic field that captures the electromotive force (voltage) from the iron in the red blood cells passing through the vessel. For Doppler flow probes, in place of magnets, energized piezoelectric crystals transmit ultrasound waves at a desired frequency (Guyton and Hall, 2006).

Electromagnetic flow probes are advantageous due to their sensitivity as they can document changes in flow in less than 1/100 of a second (Guyton and Hall, 2006). From a research perspective, a few weaknesses exist. First, the rigid nature of the probe does not allow the vessel to undergo the necessary vasodilation to support pregnancy (Meschia, 1989). Secondly, the uterus is fed by multiple arteries, thus measuring a single vessel is not necessarily representative of total perfusion of the uteroplacental unit (Battaglia and Meschia, 1986). On its own, this technique cannot be utilized for the Fick principle without the placement of additional catheters, as the flow

probes cannot sample the blood. From a clinical perspective, this technique cannot be readily applied due to the necessity of surgery and risks that procedure can pose to

Microspheres

Microspheres have been useful in conjunction with other methods such as indwelling vascular catheters and/or electromagnetic flow probes to determine blood flow distribution. Microspheres are labeled, with either radioactivity or fluorescence, and injected into circulation to determine the distribution of blood flow (Rosenfeld, 1977). However, while microspheres can be a useful technique, these measurements are limited to a few points in time and do not necessarily represent blood flow over the period of time that substrates are measured. Furthermore, there is no way to ensure the measurements are recorded under steady-state conditions (Battaglia and Meschia, 1986). These confounding issues preclude the application of the Fick principle.

Doppler ultrasound

One of the best ways to non-invasively detect changes in uterine and umbilical vascular resistance is by Doppler velocimetry (Galan et al., 1998, Galan et al., 2005). An example image of Doppler calculated measures of vascular resistance is displayed on **Figure 2**. Clinically, this technique has been used extensively to assess appropriate umbilical perfusion of the placenta and monitor high risk pregnancies. Doppler ultrasound works by using the change in frequency of energy waves caused by motion between the source of the wave (blood) and the ultrasound transducer (Abramowicz and Sheriner, 2008). Essentially, the reflected ultrasound waves transmit a lower

frequency back to the piezoelectric crystals after they rebound off red blood cells in comparison to the initial frequency of the wave being transmitted by the transducer (Guyton and Hall, 2006). This change in frequency is referred to as the Doppler frequency shift.

The advantage of Doppler ultrasound is it is non-invasive (Poston, 1997) and can be conducted repeatedly without posing risk to the fetus. A shortcoming of Doppler ultrasound is that calculations of blood flow are highly dependent on the angle of insonation, which must be accurate, or the calculations of flow are not accurate (Poston, 1997). Fortunately, great advances in Doppler technology from its first use in fetal velocimetry in the 1980's (Galan et al., 2005) have permitted more accurate measurements of blood flow, angle of insonation, and measures of vessel resistance.

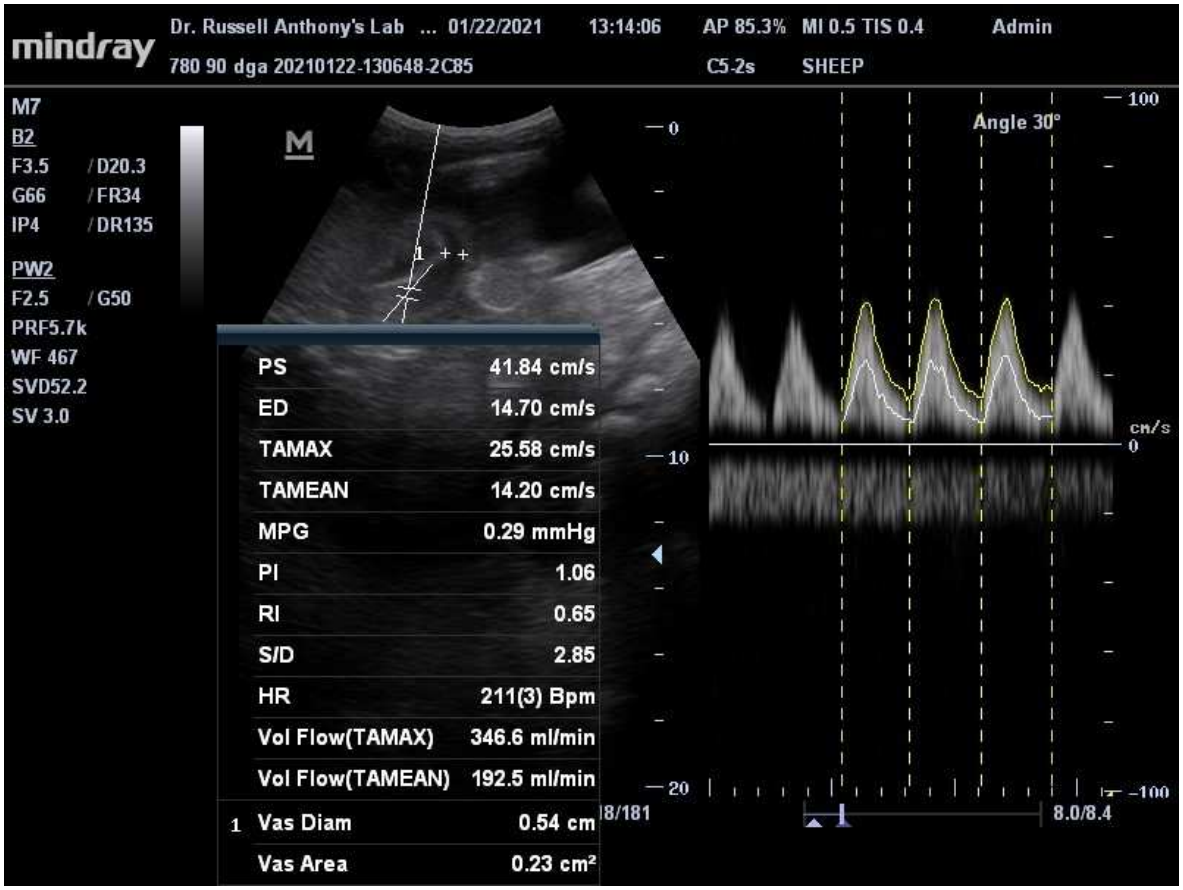


Figure 1.2: Doppler velocimetry measures the umbilical artery of a 90 dGA fetal lamb.

Summary

Dramatic vascular modifications must occur to prepare maternal physiology to support pregnancy. Maternal heart rate, stroke volume and cardiac output all increase while vascular resistance decreases. One of the key factors that is responsible for decreased vascular resistance, especially in the uterine and umbilical vascular beds are the attenuation in response to vasoconstricting factors. This attenuation is complex, likely driven by a dynamic and sophisticated balance between vasoconstriction and vasodilatory factors. While aberrant production of these vasoactive factors has been associated with pregnancy complications, substantial research gaps still exist for determining physiological causes of these perturbations. Through use of the transplacental diffusion technique and the Fick principle, it is possible to accurately assess uterine and umbilical blood flow as well as nutrient uptake. Thus, it is worth investigating the role less studied factors such as placental hormones may play in regulating uterine and umbilical vascular responses and therefore, the perfusion of the uteroplacental unit.

INTRAUTERINE GROWTH RESTRICTION

Intrauterine growth restriction (IUGR) is a leading cause of infant morbidity and mortality, impacting 6-8% of pregnancies worldwide (Brar and Rutherford, 1988; Pollack and Divon, 1992; Gagnon 2003). Furthermore, there is compelling epidemiological evidence that IUGR predisposes individuals to metabolic diseases in adulthood, including hypertension, stroke, diabetes, and coronary heart disease (Regnault et al., 2002). This review aims to 1) describe the etiology and global health consequences of

IUGR, 2) examine the link between IUGR and metabolic dysfunction, and 3) detail the utility of sheep models for interrogating the progression of IUGR.

Etiology of IUGR

Often, the literature discussing fetal growth restriction appears conflicting due to the conflation of terms such as small for gestational age (SGA), IUGR, and low birth weight (LBW). These terms broadly describe similar pathologies but vary in their specificity. The rationale behind the development of these categories was to clinically identify pregnancies that may suffer complications due to a failure for the fetus to grow properly. However, the heterogenicity of babies born within each of those groups makes determining the exact etiology a challenge. Thus, it is necessary to define all of the above terms used to describe fetal growth restriction, where they overlap and where they differ.

Definitions of fetal growth restriction

Part of the aforementioned term conflation is historical, with the categories evolving and becoming more specific over time (Battaglia, 2011). The first definition, small for gestational age (SGA), originated from an attempt to use gestational age and birth weight to identify infants failing to grow appropriately, and potentially requiring more intensive monitoring and/or postnatal care (Battaglia, 2011). Infants are classified as SGA if their birth weights fall below the 10% percentile (Battaglia, 2011). Another iteration of SGA is small for dates (SFD) which is assigned based on standard deviations from the gestational age matched birth weight of infants (Battaglia, 2011).

Both SGA and SFD classifications are dependent on population statistics. One shortcoming of this approach is the lack of antenatal diagnostics, thus missing a key window for monitoring. Additionally, this approach is inadequate at differentiating between small but properly grown infants and infants with pathological conditions preventing them from reaching their growth potential *in utero*.

The category of LBW is based on a World Health Organization defined weight threshold (<2500 grams or 5.5 lbs.) to facilitate international comparisons. This measure is especially susceptible to factors such as genetics and nutrition and is even less effective than SGA at differentiating between small but properly grown infants and poorly grown infants. However, neonates that fit into this category have been epidemiologically linked with an increased risk of coronary heart disease in adulthood, thus, this category is not useless (reviewed in Gagnon, 2003).

The last definition, IUGR is defined as a failure of the fetus to reach its growth potential *in utero* (Gagnon et al., 2003). This category uses clinical diagnostics to determine if a fetus is growth restricted. The ability to distinguish between small, normally grown fetuses from growth restricted fetuses was revolutionized by Doppler ultrasound (Battaglia, 2011). A diagnosis of IUGR is generally based on serial Doppler ultrasound assessment of fetal growth and/or abnormal umbilical artery velocimetry (Battaglia, 2011). Fetal growth and biometrics are assessed by measuring fetal head circumference, abdominal circumference, and femur length while accounting for parental size to assess whether the baby is small but in line with its growth potential or pathologically small (Battaglia, 2011). Umbilical velocimetry can be used to detect increased vascular impedance and/or a redistribution of fetal blood flow in response to

hypoxia (Battaglia, 2011). As proposed by Pardi et al. (2003), Doppler ultrasound can be used to classify IUGR into three subgroups by severity. The first and least severe group (IUGR-1), is characterized by normal umbilical artery resistance (pulsatility index) and normal fetal heart rate, which generally means unperturbed oxygenation and acid-base balance (Pardi et al., 2003). The second group (IUGR-2) has normal heart rates but abnormal umbilical artery resistance which results in an increased incidence of hypoxia and acidosis (Pardi et al., 2003). The most severe group (IUGR-3) is characterized by abnormal fetal heart rate in addition to elevated umbilical arterial resistance, and most of the fetuses in IUGR-3 are acidotic and hypoxic (Pardi et al., 2003). Another hallmark of IUGR pregnancies is absent or reversed end-diastolic blood flow as evidenced by the umbilical artery waveforms (Laurin et al., 1987; Trudinger, 1993). Uterine artery blood flow may also be assessed to detect fetal growth restriction. Because of the use of clinical assessments, the classification of IUGR is the most accurate at identifying pregnancies that will require additional monitoring and potentially postnatal care.

Causes of IUGR

The causes of IUGR are vast and varied, and many remain idiopathic. Broadly speaking, these causes can be divided into three categories based on origin: maternal, fetal, and placental. Based on the source of perturbation (maternal, fetal, placental), timing of insult, and duration of insult, two distinctive IUGR phenotypes arise: symmetric and asymmetric. Symmetric growth restriction is a rarer, earlier onset form of IUGR and is primarily caused by fetal factors such as intrauterine infection or genetic

abnormalities, resulting in symmetrical reductions in fetal and organ size (Anthony et al., 2003). The majority of IUGR cases however, present as asymmetric, driven by mostly maternal or placental abnormalities during late gestation which reduce nutrient delivery to the fetus (Regnault et al., 2002; Anthony et al., 2003). Asymmetric cases of IUGR have reductions in the size of many abdominal organs, especially the liver, while head size is maintained (Anthony et al., 2003). This leads to an effect known as “brain sparing”, the redistribution of nutrients from organs such as the liver to protect the brain and heart. At birth, this pattern of IUGR can be inferred from a low ponderal index which takes into account birth weight and crown-rump length or height (ponderal index= weight (g)/crown-rump length or height (cm^3) x 100; Anthony et al., 2003). While asymmetric growth restriction can result from a variety of maternal and placental factors, the majority of cases result from placental structural and functional abnormalities (Anthony et al., 2003).

Maternal origin IUGR

A variety of maternal factors have been associated with the development of fetal growth restriction. Among these factors are maternal pathologies, physical characteristics, socioeconomic status, and behavioral risks. Some of the most commonly discussed pathologies that elevate the risk of IUGR are maternal preeclampsia, chronic renal insufficiency, chronic hypertension, heart disease, and uncontrolled asthma (Hendrix and Berghella, 2008; reviewed in Suhag and Berghella, 2013). Additionally, diseases such as systemic lupus erythematosus, antiphospholipid syndrome, chronic obstructive pulmonary disease, maternal periodontal disease,

Crohn's disease, ulcerative colitis, gastric bypass surgery and gene mutations also carry an increased risk of IUGR complications (Khader and Ta'ani, 2005; Suhag and Berghella, 2013).

Physical factors that predispose individuals to a higher risk of IUGR include maternal age, especially the extremes of reproductive age (Lee et al., 1988; Aldous and Edmonson, 1993; Strobino et al., 1995; Suhag and Berghella, 2013) as well as the utilization of assisted reproductive technologies (Suhag and Berghella, 2013). Other physical factors include age at menarche, height, uterine abnormalities, and net pregnancy weight gain (Strobino et al 1995; Wen et al., 1990; reviewed in Suhag and Berghella, 2013).

Socioeconomic factors such as poverty, smoking or substance use and ethnicity can also contribute (Strobino et al 1995; Wen et al., 1990; reviewed in Suhag and Berghella, 2013). Additionally, women in less developed countries can experience a wide variety of factors leading to an increased likelihood of IUGR such as poor nutrition, anemia, substance abuse, and poor prenatal care (Suhag and Berghella, 2013). It is difficult to parse out the relative importance of each factor. For instance, data from the "Dutch Hunger Winter" during WWII documents maternal intake must be reduced below 1500 kcal per day to have a measurable impact of birth weight (Anatov, 1947; Smith, 1947; Suhag and Berghella, 2013). This would actually support the resiliency and adaptability of maternal and placental physiology to support fetal growth even in arduous circumstances.

Substance abuse during pregnancy, however, is a substantial contributor to fetal growth restriction (Lieberman et al., 1994; Shu et al., 1995; MacArther and Knox, 1988;

Naeye et al, 1973; Fulroth et al., 1989; Suhag and Berghella, 2013). Maternal use of heroin and cocaine have been associated with 50% and 30% higher incidence of fetal growth restriction, respectively (Shu et al., 1995; Naeye et al, 1973; Fulroth et al., 1989; Suhag and Berghella, 2013). Maternal alcohol use across gestation and smoking, especially heavy smoking during late gestation increases the risk of fetal growth restriction (Lieberman et al., 1994; Shu et al., 1995; Suhag and Berghella, 2013). In fact, smoking cessation during pregnancy has been shown to reduce the incidence of LBW by 17% (Suhag and Berghella, 2013). Other prescribed, nonaddictive substances ranging from warfarin to anticonvulsants, to antineoplastic agents have also been associated with IUGR (reviewed in Suhag and Berghella, 2013).

Fetal origin IUGR

The fetal factors contributing to growth restriction include gene mutations, congenital malformations, infection and multifetation. Genetic origins for IUGR are substantial, accounting for between 5-20% of IUGR cases, especially for early onset growth restricted fetuses (Suhag and Berghella, 2013). These include chromosomal abnormalities (especially trisomy 18), autosomal abnormalities, and mutations in genes such as IGF1 (Hendrix and Berghella, 2008; reviewed in Suhag and Berghella, 2013). Examples of congenital malformations associated with IUGR include congenital heart disease, diaphragmatic hernia, abdominal wall defects, renal dysplasia, anencephaly, and a single umbilical artery (Lin and Santolaya-Forgas, 1998; Khoury et al., 1988; Suhag and Berghella, 2013). Complicating fetal infections can be of viral, bacterial, or parasitic origin but only contribute to less than 5% of IUGR cases (reviewed in Suhag

and Berghella, 2013). The most common infectious causes of IUGR are Cytomegalovirus (viral) and malaria (parasitic) (Wendel, 2010; Adanu, 2010; Suhag and Berghella, 2013).

Multifetation also increases the likelihood (five to ten-fold) of developing IUGR, which accounts for approximately 3% of all IUGR cases (reviewed in Suhag and Berghella, 2013). This is the reason that twin pregnancies are avoided in sheep models of IUGR as it would be difficult to ascertain whether the perturbation causally resulted in IUGR or whether it was brought about by multifetation.

Placental origin IUGR

Placental factors driving IUGR include placental insufficiency (PI), placental previa, placental abruption, placental accreta, placental infarction, fetal villous obliteration, and circumvallate placenta (Hendrix and Berghella, 2008; Wen et al., 1990; Suhag and Berghella, 2013). Umbilical complications such as a single umbilical artery and velamentous cord insertion can also increase the risk of IUGR (Wilkins-Haug et al., 1995; reviewed in Suhag and Berghella, 2013).

The primary cause of IUGR is PI, affecting up to 60% of fetal growth restricted pregnancies with normally formed fetuses (Ghidini, 1996). This pathology leads to a progressive deterioration in the ability of the placenta to transfer substrates (Gagnon, 2003). Some of the characteristics of PI-IUGR are uneven placental perfusion, perturbations in the diffusion of substrates like O_2, decreased surface area for nutrient and gas exchange, and reduced placental mass (Battaglia, 2011). Unsurprisingly, blood flow is a key factor in the growth and function of the placenta which is why an entire

section of this literature review examined this topic. Some causes of PI-IUGR have been documented including maternal smoking, maternal hypertensive disorders, primiparity, as well as previous IUGR (Salafia et al., 1998). However, the majority of the causes of PI-IUGR remain idiopathic. Because PI-IUGR is the most significant driver of fetal growth restriction, and the majority of the PI-IUGR causes are idiopathic, a substantial knowledge gap exists and should be investigated.

Perinatal complications

Intrauterine complications that can afflict IUGR pregnancies include intrauterine demise, intrapartum fetal distress, and perinatal asphyxia (Regnault et al., 2002). From a statistical perspective, IUGR pregnancies have 5 to 10-fold higher risk of perinatal death and a 3-fold increase in preterm birth (reviewed in Gagnon 2003; Suhag and Berghella, 2013). The severity of *in utero* complications is often assessed by the etiology of IUGR, gestational age at diagnosis, and fetal umbilical velocimetry (Alfirevic et al., 2010; Serhag and Berghella, 2013).

Perinatal complications of IUGR can include meconium aspirations, metabolic and hematological perturbations, necrotizing enterocolitis, cerebral palsy and cognitive impairments (Regnault et al., 2002; von Beckerath et al., 2013; Bernstein et al., 2000; Serhag and Berghella, 2013). These infants also may require more intensive care for seizures, sepsis, hypoglycemia, hypothermia, have poor Apgar scores and require intubation (von Beckerath et al., 2013; McIntire et al., 1999; Serhag and Berghella, 2013). During childhood, infants from IUGR pregnancies have elevated risk of growth delay and neurodevelopment impairments (von Beckerath et al., 2013; Pallotto and

Kilbride, 2006; Serhag and Berghella, 2013). The risk of these outcomes can be detected *in utero* as infants from pregnancies with abnormal Doppler flow velocimetry waveforms, had lower IQ scores at 5 years of age (Gagnon, 2003).

Developmental Origins of Health and Disease

The long-term consequences from fetal growth restriction do not end in childhood. The theory of the developmental origins of health and disease (DoHAD) has gained tremendous support over the last 30 years, being spurred by the epidemiological studies of David Barker. While Barker rightfully receives credit for his significant contributions to the idea that adult-onset diseases such as cardiovascular disease, diabetes, and hypertension are programmed *in utero* due to adverse conditions, this relationship was first examined in farm animals back in the 1930's and 1940's. The fetal programming literature is expansive and cannot be adequately reviewed in the scope of this literature review. Thus, the intent of this section is to review the origins and evolution of the fetal programming hypothesis.

Origins of DoHAD

The first study to implicate uterine environment on postnatal outcomes was conducted in horses. In 1938, Walton and Hammond performed an experiment using reciprocal cross between Shire and Shetland horses. They found that Shetland-Shire crosses from Shire dams weighed and grew considerably larger than Shetland-Shire crosses from Shetland dams (Walton and Hammond, 1938). Not only was uterine capacity demonstrated to influence in long-term growth, but maternal nutrition was also

implicated. In 1939, a study was conducted by Thomson and Fraser using 3 planes of nutrition during pregnancy. Ewes were assigned to one of three dietary treatments including 1) adequately fed (3.6 kg of maternal weight gain over gestation), 2) *ad lib* fed (22.7 kg of maternal weight gain), and 3) restricted diet until the last month of gestation when ewes were realimented using an *ad lib* diet (Thomson & Fraser, 1939). In spite of realimentation, lambs born to nutrient restricted ewes were ~1kg smaller at birth and "lacked vitality" (Thomson and Fraser, 1939). In 1948, Wallace conducted more intensive investigations on the postnatal effects of maternal nutrition in lambs where he demonstrated postnatal growth was dependent on maternal plane of nutrition (Wallace, 1948).

Around the time these animal experiments were being conducted, the world was engaged in WWII. While a war might seem like an unexpected development for research into fetal programming, it provided a unique opportunity to investigate the atrocities of war including extreme maternal malnutrition on offspring long-term health. The infamous "Dutch Hunger Winter" is a historical cohort study of the Dutch famine during 1944-1945. In response to a failed strike on a Nazi occupied bridge, the Germans retaliated by restricting Holland's food supply which resulted in a perfect storm of environmental conditions including frigid temperatures, extreme caloric restriction (as few as 670 kcal per day at peak restriction), and the psychological stresses of war (Ravelli et al., 1976; Stein and Susser, 1975; Nathanielsz, 2006). Women subjected to these conditions in the second-to-third trimester had babies with smaller birth weights and ponderal indexes (Stein and Susser, 1975; Stein et al., 1995). Interestingly, infants from women subjected to these conditions during early gestation had normal weights at

birth but postnatally, had a much higher incidence of obesity rates when compared to second and third- trimester nutrient restriction and unexposed babies (Schulz, 2010).

Another frequently overlooked contribution to the fetal programming hypothesis came from James Neel (1962) who first proposed the “thrifty hypothesis” with the intent of examining why children from diabetic parents had a higher probability of developing diabetes. He postulated that the incidence of diabetes was increasing because of the shift in eating habits as a result of the Western diet (Neel, 1962). Essentially, he predicted a shift from an ancestral hunter-gatherer “feast or famine” modality for eating to modern “overalimentation” of food that was somewhat heritable as it appeared to be passed onto children from diabetic parents (Neel 1962). While diabetes certainly proved more complex than Neel originally recognized, his central notion of mismatch between “feast or famine” realities provided a strong basis for fetal programming literature. The “thrifty hypothesis” evolved to represent how maternal physiology was preparing her offspring for the postnatal environment they would face (either feast or famine), and that developmental programming consequences originated from the mismatch between prenatal and postnatal environment.

This idea was further developed by epidemiologist David Barker in the 1980’s and 90’s as he postulated that harsh *in utero* environments followed by plentiful food in adulthood predisposed individuals to adult-onset diseases. Barker was quite prolific, but some notable studies include the link between low birth weight and high blood pressure (Barker and Osmond, 1988; Barker et al., 1989), his formulation of the fetal origins of adult disease hypothesis (Barker, 1990), the association of not only fetal but placental size to the risk of hypertension (Barker et al., 1990), the link between reduced early life

growth and glucose intolerance at 64 (Hales et al., 1991), and the relationship between IUGR and diabetes, coronary heart disease and stroke (Barker, 1998).

Since the formulation of Barker's hypothesis on "fetal programming" or DoHAD, both biomedical and animal sciences have interrogated the relationships between fetal growth, placental function and adult health. In agriculture, emphasis has been placed on how intrauterine environment influences postnatal weight gain and carcass quality, and to a lesser extent, fertility. In biomedical sciences, emphasis has been placed on the progression of chronic diseases such as cardiovascular disease, diabetes, and hypertension among many other non-communicable diseases. Because fetal programming occurs in both livestock and humans, emphasis has been placed on finding animal models for human medicine. Not only do livestock models such as sheep provide a more ethical option for invasive instrumentation, but also may provide valuable insight into the progression of adult-onset diseases.

Sheep Models of IUGR

While no animal model can fully recapitulate human pregnancy, the sheep placenta possesses many structural and functional similarities to the human placenta. Sheep also possess the unique capacity to tolerate surgical instrumentation of their pregnancies for repeated sampling of maternal and fetal blood to study the transport and uptake of substrates. It is for these reasons that sheep has been extensively used by investigators for over 50 years to interrogate questions about the progression of both normal and pathological pregnancies. In the sheep, fetal growth restriction can be induced by a variety of techniques including 1) elevated ambient temperatures, 2)

maternal nutrient manipulation (under or overnutrition), 3) administration of glucocorticoids, 4) uteroplacental embolization or ligation (uterine or umbilical), and 5) limiting area for maternofetal nutrient exchange (carunclectomies). The following section will attempt to highlight similarities and differences between each model and human IUGR pregnancies.

Hyperthermia

One of the most robustly investigated sheep models is maternal hyperthermia. This approach was developed by Alexander and Williams in 1971 and works by exposing pregnant sheep to heat stress (35-40°C with 35% RH; Beede et al., 2019) across specific timepoints in pregnancy. The degree of growth restriction varies based on the duration of maternal hyperthermia. If pregnant sheep are exposed from early to late gestation, ~60% reductions in placental weight and ~50-60% reductions in fetal weight ensue (Anthony et al., 2003). If applied from mid-to late gestation, placental weight reductions are similar to those documented in the early to late gestation animals, but fetal growth restriction is less severe (~20-30%; Anthony et al., 2003). Not only does this model mimic the asymmetric IUGR observed in human pregnancies, but also closely resembles the metabolic and vascular changes of PI-IUGR human pregnancies (Regnault et al., 2002). This would indicate perturbations in both placental structure and function, hallmarks of placental insufficiency.

From a metabolic standpoint, hyperthermic pregnancies result in fetal hypoxia and hypoglycemia as well as altered amino acid transport (Thureen et al., 1992). These changes are also reported in human PI-IUGR pregnancies (reviewed Regnault et al.,

2002). From a vascular perspective, hyperthermic pregnancies have elevated vascular resistance and reduced umbilical blood flow which also mirror responses in human IUGR pregnancies (Galan et al., 1998; Thureen et al., 1992; Trudinger et al., 1987). Fetal vessels from hyperthermic pregnancies have increased coiling and appear more random in orientation, with increased sinusoidal dilations (Regnault et al., 2002). The most likely explanation for this lack of appropriate vessel organization and structure is the dramatic shift in a variety of placental angiogenic regulators (Regnault et al., 2002). Fundamentally, this model not only mirrors human PI-IUGR pregnancies, but also yields a fairly consistent phenotype (in contrast to nutritional manipulation, placental embolism, carunclectomies, etc.) which is why it has been so heavily utilized to investigate the pathophysiology of PI-IUGR.

Nutritional manipulation

Another heavily investigated sheep model of growth restriction is maternal nutritional manipulation. As previously discussed, there is great historical precedent for examining the impacts of maternal malnutrition on fetal and postnatal growth as evidenced by Wallace (1948). The human health relevance of this model was demonstrated by the “Dutch Hunger Winter” as individuals subject to those conditions *in utero* faced long-term health consequences as a result of poor maternal nutrition.

Maternal nutrient restriction

While this model was the first to investigate the relationship between maternal diet and postnatal growth (Wallace, 1948), some attempts have been made to more

specifically address dietary deficiencies (protein, vitamins and minerals, etc.) and their impacts on fetal growth (Lekatz et al., 2015; Lekatz et al., 2011). The timing, degree of nutrient restriction and duration of restriction all influence the severity of the phenotype.

Early to mid-gestation or mid-to late gestation

Maternal nutrient restriction from early to mid-gestation produces mixed results, with some studies seeing no differences fetal or placental parameters near-term (reviewed in Anthony et al., 2003). However, a study that collected fetal weights after nutrient restriction at mid-gestation documented modest reductions in fetal but not placental weight (Vonnahme et al., 2003). This might indicate that re-feeding pregnant ewes to control levels from mid-gestation to term supports catch up growth. No steady-state nutrient uptake studies in this timeframe have been conducted.

Late gestation

Nutrient restriction from mid-to late gestation results in slight reductions (10-18%) in fetal body weight but not placental mass (Lemley et al., 2012; Eifert et al., 2015). If nutrient restriction is confined to late gestation only, the results are also mixed with some studies seeing both fetal and placental growth restriction and some studies observing no changes (reviewed in Anthony et al., 2003). Furthermore, late gestation nutrient restriction reduces uterine blood flow but does not change umbilical blood flow (reviewed in Anthony et al., 2003). Fetuses from late gestation nutrient restricted sheep have reduced umbilical glucose uptake but experience no changes to umbilical oxygen uptake (reviewed in Anthony et al., 2003).

Undernutrition across gestation

If maternal nutrient restriction is applied from early to late gestation, placental mass is reduced by 15-25 % and fetal mass is reduced between 22-45% (reviewed in Anthony et al., 2003). This is not always the case however, as a subset of undernourished ewes demonstrate resiliency to nutrient restriction and produce normal weight pregnancies (Edwards et al., 2020). There is no way to anticipate which animals will be susceptible to nutrient restriction induced IUGR (Edwards et al., 2020). While pre-breeding body condition could certainly contribute to resiliency from fetal growth restriction, no differential body condition scores across gestation were reported. Furthermore, no fetal differences in umbilical glucose concentrations exist at necropsy between growth restricted and non-growth restricted undernourished ewes (Edwards et al., 2020). Hemodynamic and nutrient uptake changes have not been assessed in nutrient restricted ewes across gestation.

Overall, the model of maternal nutrient restriction indicates that timing and duration of insult are critical to the emergence of a phenotype, however inconsistent. Intriguingly, a subset of animals appears to be resilient to nutrient restriction, which may indicate a useful compensatory mechanism that merits investigation. In spite of variable and sometimes limited reductions in fetal growth, this model is valuable for examining “fetal programming” consequences of altered maternal diet during pregnancy which may occur regardless of fetal birth weights (Hoet and Hanson, 1999; Ford and Long, 2012).

Overnutrition

While maternal overnutrition seems like a paradoxical approach, consistent maternal overnutrition of adolescent, singleton-bearing ewe lambs can result in fetal growth restriction (Wallace et al. 1999a). This is thought to be due to the prioritization of maternal growth over supplying adequate nutrients to the gravid uterus (reviewed in Anthony et al., 2003). To generate this model, embryos derived from mature ewes are transferred into adolescent ewes immediately following puberty, then the pregnant ewes are fed at 200% of their daily NRC requirements (Carr et al., 2012). This model results in ~40% reductions in placental mass and ~28-38% reductions in fetal mass which are in line with human IUGR pregnancies (reviewed in Anthony et al., 2003). These weight decreases are accompanied by reductions in uterine and umbilical blood flow and umbilical oxygen and glucose uptake which unsurprisingly results in hypoglycemia and hypoxia (reviewed in Anthony et al., 2003).

Interestingly, if maternal overnutrition is halted by the end of the first third of pregnancy, and ewes are returned to a control diet, fetal and placental growth is enhanced (Wallace et al., 1999b). In contrast, if maternal overnutrition is initiated at mid-gestation onward, fetal and placental growth is suppressed (Wallace et al., 1999b). If maternal overnutrition is continuous over gestation, both uterine and umbilical blood flow are reduced as well as umbilical oxygen and glucose uptakes (Wallace et al., 2002a). This effect however, appears to be a function of reduced placental mass opposed to altered placental glucose transport capacity as indicated by glucose clamp experiments (Wallace et al., 2002b). Maternal placental hormones such as CSH and progesterone are also suppressed in this model (Lea et al., 2007). However, similar to

other sheep models of IUGR, a subset of overfed adolescent ewes with pregnancies created by embryo-transfer produce fetuses with normal weights.

Furthermore, the fetal growth restriction observed in this model may be due to an interaction between maternal diet x embryo transfer. Peel et al. (2012) overfed (2.72 Mcal/kg metabolizable energy) naturally mated, singleton-bearing, adolescent ewe lambs across gestation and observed no differences in lamb birth weight or growth measures. Additionally, they documented no changes in postnatal body weight, plasma insulin or glucose concentrations, or carcass characteristics (Peel et al., 2012). This strongly supports the idea that the growth restriction observed in this model is due to a diet x embryo transfer interaction.

Overnutrition vs. Undernutrition

An interesting finding by Ford and Long (2012) is that disparate management of maternal diets (undernourished vs. overnourished) resulted in lambs that have similar growth trajectories and metabolic dysfunction. This overlap was determined by studying cohorts of overnourished, undernourished and control fed pregnancies. When pregnancies undergo necropsy at 78 dGA, undernourished fetal lambs were substantially smaller (30%), and overnourished fetal lambs were much larger (30%) compared to control fetuses (Ford and Long, 2012). However, following realimentation of nutrient restricted ewes to 100% of their NRC requirements, previously undernourished fetuses underwent rapid catch-up growth from mid-to late gestation (Ford and Long, 2012). In contrast, overnourished (150% NRC) fetuses had rapid reductions in growth rates during the same period (Ford and Long, 2012). By parturition,

fetuses from all dietary treatments had similar birth weights (Ford and Long, 2012). However, both the overnourished and undernourished lambs had reduced plasma insulin and elevated glucose at birth (Ford and Long, 2012). Postnatally, lambs from both over and underfed ewes had accelerated growth rates and adiposity, as well as increased insulin resistance and elevated plasma leptin concentrations (Ford and Long, 2012). This would seem to agree with the increased incidence of metabolic dysfunction of individuals from both growth restricted and gestational diabetes pregnancies (Wroblewska-Seniuk et al., 2009; Vaag et al., 2006). Furthermore, this would seem to indicate that metabolic dysfunction can still occur in spite of normal birth weights.

Glucocorticoids

In utero exposure to glucocorticoids reduces birth weight and causes lasting hypertension, hyperglycemia, and hyperinsulinemia in humans (Seckl, 2001). To investigate this phenomenon, glucocorticoids are administered to the pregnant ewe during late gestation. While glucocorticoid treatment does not alter fetal blood biochemistry or glucose, it increases fetal blood pressure (reviewed in Anthony et al., 2003). Furthermore, administration of glucocorticoids results in 25% reductions in placental weight and a 17-31% reduction in fetal body weight with no changes to fetal oxygenation or glycemic responses (reviewed in Anthony et al., 2003). Uterine and umbilical blood flow and nutrient uptakes have not been assessed. This model most likely directly affects the fetus but does not appear to be a true model of placental insufficiency (reviewed in Anthony et al., 2003). This is supported by the lack of response to early gestational glucocorticoid treatments (reviewed in Anthony et al.,

2003). However, this model still has merit as fetuses in IUGR pregnancies often have elevated cortisol and the lasting impacts of glucocorticoids on postnatal blood pressure make it valuable from a fetal programming standpoint (reviewed in Anthony et al., 2003).

Placental embolization or ligation

Placental embolization is achieved by the infusion of microspheres into the umbilical artery for distribution into the fetoplacental capillary beds (reviewed in Beede et al., 2019). Uterine or umbilical ligation (or occlusion) is achieved surgically through vessel ligation or the placement of an occluder around a single uterine or umbilical artery (reviewed in Anthony et al., 2003; reviewed in Beede et al., 2019). These procedures are applied during late gestation (reviewed in Anthony et al., 2003; reviewed in Beede et al., 2019). This model works by directly reducing either uterine or umbilical blood perfusion of the placenta. Placental embolization results in ~ 30-50% reductions in placental mass which produces a dramatically varying degree of fetal growth restriction, ranging from 15-66% (reviewed in Anthony et al., 2003). Uterine and umbilical blood flow are reduced (as is requisite for this model), and umbilical artery vascular resistance is increased (reviewed in Beede et al., 2019). Additionally, umbilical oxygen and glucose uptakes are reduced (reviewed in Anthony et al., 2003). However, fetal oxygen and glycemic levels vary dramatically (0-50%) as might be anticipated with the wide ranges in fetal weight reductions (reviewed in Anthony et al., 2003). A shortcoming of this model is the direct targeting of uterine or umbilical blood flow. This distinguishes this model from placental insufficiency models like maternal hyperthermia,

as the reduction in blood flow is obstructive rather than a biological progression of placental insufficiency (Anthony et al., 2003). However, this model still provides the opportunity to investigate the direct impacts of blood flow on fetal growth.

Carunclectomies

Carunclectomy procedures involve the surgical removal of the majority of caruncles prior to mating (usually leaving 4), to reduce surface are for nutrient exchange (Alexander, 1964). This results in ~50-60% reductions in placental weight and~30-40% reductions in fetal weight (reviewed in Anthony et al., 2003). Functionally, this causes fetal hypoxia and hypoglycemia by reducing uterine and umbilical blood flow, and the umbilical uptakes of glucose and oxygen (reviewed in Anthony et al., 2003). All of these perturbations are similar to those documented in human IUGR pregnancies. Fetal arterial blood pressure, however, does not increase in this model, unlike maternal glucocorticoid administration (reviewed in Anthony et al., 2003). Interestingly, just like the nutrient restricted ewes, a subset (approximately half) of these carunclectomized pregnancies do not result in growth restriction (Owens et al., 1987). Thus, it could prove quite informative to investigate how these pregnancies are able to adapt to substantial reductions in surface area for nutrient exchange.

Specific gene product deficiency

Until recently, an advantage of using rodent models to investigate pregnancy biology was the ability to genetically manipulate their pregnancies. Rodent models also have disadvantages including the embryonic lethality of many the gene knockouts and

the inability to instrument rodents for steady-state studies, thus limiting their utility as a model for human pregnancy physiology. Fortunately, rodents no longer are the only species that can undergo specific gene product deficiency.

Purcell et al. (2009) utilized lentiviral mediated RNA interference (RNAi) to target proline-rich 15 (PRR15) in the sheep placenta. This methodology involves the infection of the trophectoderm of expanded hatched blastocysts with a lentiviral construct expressing a gene specific shRNA (Purcell et al., 2009; Baker et al., 2016). Using this methodology, Purcell et al. (2009) were successfully able to target PRR15 production by the sheep placenta and detected a phenotype (failure of the conceptus to expand). This methodology has also been utilized to examine the phenotypic consequences of placental hormonal deficiency (Baker et al., 2016; Jeckel et al., 2018) as well as specific placental nutrient transporters (Lynch et al. unpublished). Thus, by combining gene targeting approaches in the sheep placenta along with the ability to instrument those pregnancies, a unique and powerful experimental paradigm emerges for the investigation of placental function and IUGR.

Summary

Intrauterine growth restriction is not only a major contributor to perinatal morbidity and mortality, but also increases the probability of developing chronic diseases such as cardiovascular disease, diabetes, and hypertension. While IUGR can be caused by a variety of factors, the majority of cases are a result of placental insufficiency. While a few factors are known to cause placental insufficiency, the majority of placental insufficiency cases are idiopathic, indicating a substantial knowledge gap. Sheep

models of IUGR provide tremendous utility to interrogate the physiological ramifications of IUGR by closely mirroring perturbations documented in human IUGR pregnancies. Until recently, a shortcoming of using large animal biomedical models was the inability to manipulate gene expression. Recent advances however, addressed this shortcoming using an *in vivo* approach to target specific genes in the sheep placenta with lentiviral-mediated RNA interference. Using this approach, experiments can be designed with the specificity to directly assess gene function within the placenta in tandem with surgical instrumentation to test the physiological ramifications of specific gene product deficiency on placental function and fetal growth.

CHORIONIC SOMATOMAMMOTROPIN

Chorionic somatomammotropin (CSH; aka placental lactogen) is the most abundant placental-derived peptide hormone, belonging to the growth hormone/prolactin family. It is secreted by the placenta into both fetal and maternal circulation and can also be found in the amnion. The first evidence for chorionic somatomammotropin was identified by Erhardt in 1936. Erhardt deduced the substance found in the term placenta was highly lactogenic and immunologically related to pituitary growth hormone (Erhardt, 1936). The substance remained a mystery until Josimovich and MacLaren successfully isolated human CSH from term placentas and from maternal blood perfusing the placenta (Josimovich and MacLaren, 1962). In the 1970's, the existence of CSH in other mammalian species was confirmed including a variety of primate and subprimate species such as the ruminant and rodent (reviewed in Hurley et al., 1977b). It was also during this period that low-birth-weight pregnancies were first

associated with reduced circulating maternal CSH concentrations (Spellacy et al., 1976; Daikoku et al., 1979). Throughout the intervening period, investigators sought to describe the exact biological functions of CSH and understand the significance of its abundance. This review aims to 1) describe the structure and evolution of *CSH* and its regulation, 2) examine the various receptor types for CSH, their binding kinetics, and proposed mechanisms of action, 3) discuss factors impacting CSH secretion and expression, 4) describe studies aimed at understanding CSH function, and 5) conclude with the current proposed function of CSH in regulating fetal and placental physiology.

Evolution & Structure

Most eutherian mammals have placentas capable of producing and secreting products from the growth hormone *(GH)*/prolactin *(PRL)* gene family, including *CSH*. Postnatally, both GH and PRL are necessary for regulating growth, especially through the IGF axis. However, the specific role for GH and PRL during fetal growth remains murky. In some cases, idiopathic deficiencies in GH or GH receptor (GHR) can still lead to fetal growth restriction, but congenital absence of the pituitary gland does not result in growth restriction, thus this relationship is still poorly understood *in utero (*Anthony et al., 1998*)*. It is also difficult to ascertain the exact *in utero* function of PRL as mammary development in the ruminant can proceed without PRL, which may indicate some redundancy in function of the *GH/PRL* gene family (Anthony et al., 1998). As previously discussed on the section in fetal physiology, some of the actions of the IGFs *in utero* are thought to be potentiated through CSH. From an evolutionary standpoint, the primate and subprimate share different evolutionary lineages for *CSH* (reviewed in Anthony et

al., 1995). Primate CSH, especially in the human, has greater amino acid sequence identity with GH (96%) than with PRL (23%) whereas the opposite is true with subprimates such as the ruminant and rodent (reviewed in Anthony et al., 1995).

Primate *CSH* is believed to be a duplication of a common ancestorial gene around ~350 million years ago, causing the segregation of *GH/CSH* and *PRL* onto different loci (reviewed in Anthony et al., 1998). This likely occurred after the separation of the main order of mammals, with the *hCSH* gene segregating ~80 million years ago with the divergence of primates and rodents (Wallis, 1981; Moore et al., 1982). Lastly, two additional duplication events occurred ~10-15 million years ago to separate *GH* from *CSH* (Anthony et al., 1998). From the *pre-GH* gene arose *hGH-N* and hGH-V, and from the *pre-CSH* gene developed *hCSH-1/hCSH-4*, and *hCSH-3* (Anthony et al., 1998). This was followed by the separation of *hCSH-1* and *hCSH-4* through recombination around 5 million years ago, although this is somewhat debated as *hCSH-4* shares greater homology with *hCSH-3* (Hirt et al., 1987; Anthony et al., 1998). *hCSH-3* and *hCSH-4* are the only transcriptionally active forms, with only a single amino acid difference in their signal sequence (cleaved during processing), thus each gene produces identical mature CSH proteins (Barrera-Saldana et al., 1983; Resendez-Perez et al., 1990). Despite the differences in protein structure between species, both the primate and the ruminant CSH appears to have similar function, regulating both fetal growth and mammogenesis (Anthony et al., 1998). While rodents produce two CSH products along with prolactin-related proteins, the placentae of both ruminants and primates produce a single CSH product (Anthony et al., 1998).

Human CSH Gene

Out of all the CSH's investigated, human CSH has been most extensively studied (Ito and Higashi, 1961; Josimovich and MacLaren, 1962). Human CSH is detectable in the cytotrophoblasts early in gestation, by approximately 18 dpc (Beck, 1970) and in maternal serum by 3 weeks post conception (reviewed in Anthony et al., 1998). Maternal concentrations increase exponentially during the first trimester and then steadily rise throughout the rest of gestation (Biwas et al., 1972; Braunstein et al., 1980). hCSH is clearly synthesized and secreted by the syncytiotrophoblast cells in the human placenta as evidenced by immunocytochemistry and *in situ* hybridization, although during the first 6 weeks of gestation, it is the cytotrophoblasts that produce hCSH (Hoshina et al., 1982; Maruo et al., 1992). The content of *CSH* mRNA in the syncytiotrophoblast is fairly constant over pregnancy (reviewed in Anthony et al.,1998). However, as the volume of syncytiotrophoblast cells increase, so do CSH concentrations, nearing ~1.0g/day of CSH production by the placenta by near term (Hoshina et al., 1992; Kaplan et al., 1968). Human *CSH* is encoded by genes within a 55-kb region (5 gene cluster; 2 *hGH* and 3 *hCSH*) on chromosome 17 (reviewed in Anthony et al., 1995). Each gene within the cluster contains 5 exons and 4 introns (reviewed in Anthony et al., 1995) and the two *GH* genes in that cluster are *hGH-N* (normal) and *hGHv* (variant; reviewed in Anthony et al., 1995). The latter is only produced by the placenta and becomes the dominant version of hGH in circulation beginning at 22 weeks of gestation (reviewed in Anthony et al., 1995). hCSH is 191 amino acids long and secreted in a non-glycosylated form by the syncytiotrophoblast cells (reviewed in Anthony et al., 1995). Mature hCSH has two disulfide loops between

Cys^{53} and Cys^{165}, as well as Cys^{182} and Cys^{189} (reviewed in Anthony et al., 1998). Fetal concentrations of CSH are approximately 20-30 ng/mL (Crosignani et al., 1972) near term. Maternal CSH concentration are approximately 5-10 ug/mL near term (Biswas et al., 1972; Braunstein et al., 1980).

Ruminant CSH Gene

CSH has been identified in the placentas of common domestic ruminants including the bovine, ovine and caprine (Murthy et al., 1982; Currie et al., 1990; Martal and Djaine, 1975). In ruminants, CSH has greater homology to PRL (~48-50%) than GH (~24%), which is opposite to the primate (Anthony et al., 1995; Anthony et al., 1998). CSH is produced by the cell type analogous to the syncytiotrophoblast cells called the chorionic binucleate cells (reviewed in Anthony et al., 1995). CSH can be detected in binucleate cells at ~16 dGA in the ovine and ~18 dGA in the bovine (Anthony et al.,1995; Anthony et al., 1998). Both ovine and bovine *CSH* are comprised of five exons, spanning 10-12 Kb (Kessler and Schuler, 1991; Liang et al., 1999; Anthony et al., 1998).

In the bovine, there are multiple isoforms (32,000 and 34,000 M_r) derived from a single *CSH* gene with both O-linked and N-linked glycosylation (reviewed in Antony et al., 1995). In the ovine however, CSH is synthesized and secreted as a single, non-glycosylated protein that has a molecular weight (M_r) of 22,000 with an isoelectric point (PI) of 9.2 (Warren et al., 1990). Caprine CSH has an estimated (M_r) of 22,500 and is believed to lack glycosylation (Currie et al., 1990; Anthony et al., 1998). Ruminant CSH also differs from primate CSH in terms of number of disulfide bridges, containing an

additional disulfide bridge between two additional Cys residues (Anthony et al., 1998). Functionally, CSH is released into maternal circulation by the migration and fusion of BNCs to either the endometrial epithelium or feto-maternal syncytium (Wooding,1992).

In the sheep or goat, approximately one out of five trophectodermal cells are BNCs and approximately 15% of those cells are migrating through the tight junctions to maintain the syncytium at any one time occurring up to parturition (reviewed in Wooding, 1992). In the bovine, the syncytium that forms at implantation is ultimately replaced by uterine unicellular epithelial cells which form transient trinucleate cells (reviewed in Wooding, 1992). This suggests differences in syncytial function and maintenance, supported by functional differences in CSH delivery to maternal circulation. In the bovine, CSH is detectable in maternal circulation by 120 dGA, but remains low (Wallace, 1993; Anthony et al., 1998) in concentrations across gestation (reviewed in Anthony et al., 1995). In the ovine however, CSH is detectable after 40 dGA (Wallace, 1993; Martal and Djiane, 1977; reviewed in Kappes et al., 1992) in maternal circulation. Fetal CSH concentrations continue to rise across gestation until approximately 120-124 dGA, then decline until delivery (Taylor et al., 1980). Maternal CSH concentrations are lower than fetal concentrations until approximately 70-90 dGA where they rapidly increase (reviewed in Kappes et al., 1992) until they peak between 130-139 dGA, and subsequently decrease until term (Taylor et al., 1980). From a transcriptional standpoint, Kappes et al. (1992) documented increases in cotyledonary *oCSH* message until 120 dGA, when concentrations began to decline slightly at 135 dGA. This would support the decreasing fetal oCSH concentrations after 120 dGA.

These increases in CSH production, are thought to originate from increased BNC numbers rather than individual cell production of CSH (Kappes et al., 1992). Fetal uptake of CSH appears to peak by mid-gestation (80-120 dGA) and stabilize or decrease until term in both the bovine and ovine (reviewed in Anthony et al., 1995). However, the mechanism of delivery of CSH into fetal circulation remains unclear (reviewed in Wooding, 1992).

Rodent Csh Gene

In the rodent, all *Csh*, prolactin-like proteins (*Plp*), and *Prl* genes are on the same chromosome (reviewed in Anthony et al., 1995). Two forms of *Csh* (*Csh-I* and *Csh-II*) plus a PL-variant (*Csh-Iv*) have been documented in rats. They are secreted by the trophoblast giant cells, at different timepoints across gestation and each type structurally differs from the other (reviewed in Anthony et al., 1995). The rodent CSH-I is glycosylated and is secreted by the trophoblastic giant cells (TGCs) during mid-gestation (reviewed in Anthony et al., 1995). The second form of CSH (CSH-II) is not glycosylated and is secreted during the latter half of gestation (reviewed in Anthony et al., 1995). The CSH-variant found in the rat is similar structurally to CSH-I but is produced by the spongiotrophoblat cells during the second half of gestation (reviewed in Anthony et al., 1995). Additionally, rodent placentas produce a variety of other related hormones such as prolactin-like proteins, proliferins and proliferin-related proteins, thus highlighting the complications of using the rodent as a model to study CSH (reviewed in Anthony et al.,1995). This brief summary of rodent CSH is necessary due to much of the hypothesized function of CSH being derived from the rodent and to emphasize how

this may be problematic for deriving functional research, due to the unique function and structure of rodent Csh.

Regulation CSH

Transcriptional regulation of *CSH* has been more thoroughly investigated in the human than the ruminant. Human *CSH* transcription can be regulated by cAMP, thyroid hormone, and glucocorticoids (reviewed in Anthony et al., 1998). Using reporter constructs in the rat pituitary GC cells, it was determined that *hGH-N* and *hCSH* respond differently to thyroid hormone, with thyroid hormone stimulating *hCSH-4* promoter activity and inhibiting *hGH-N* (reviewed in Anthony et al., 1998). Additionally, near the transcriptional start sight is a consensus Vitamin D response element and thus, *hCSH* may be responsive to vitamin D (Stephanou et al., 1994). Interleukin-6 (IL-6) has also been shown to enhance *hCSH* gene expression (Stephanou and Handwerger, 1995). A variety of transcription factors can also control *CSH* expression. Their relationships with the expression of *hCSH* is complex and will not be detailed in this review. In summary, placenta-specific expression of *hCSH* is regulated both up and downstream of the structural gene by a complex of positive and negative cis-acting elements (Anthony et al., 1998).

In the bovine, the 5' flanking sequence of *CSH* possesses consensus sequences for a variety of transcription factors as well as a thyroid hormone response element, but this remains to be functionally tested (reviewed in Anthony et al., 1995). Liang et al. (1999) investigated the transcriptional regulation of ovine *CSH;* however, the findings of that investigation are complex and beyond the scope of this literature review. DNAse

footprint analysis of the 5' flanking sequence was conducted on sheep BNC nuclear extracts which revealed that the human and sheep share some transcriptional regulation features (Limesand et al., 1997; Anthony et al., 1998). In terms of oCSH processing, a study by Warren et al. (1990) using solubilized fetal cotyledonary tissue (~100 dGA) found the only intracellular processing of oCSH was the cleaving of an amino-terminal signal sequence.

CSH and its Receptors

One of the factors complicating the understanding of the biological function of CSH is the current failure to characterize a specific CSH receptor and the likely redundancy between growth promoting ligands (GH, PRL, CSH) and receptors. CSH was first purified through heterologous GH or PRL radioreceptor assays (Anthony et al., 1998). Using this approach, it was concluded that CSH (ovine, human and bovine) can bind GH receptor and in some cases, PRL receptor (Lowman et al., 1991; Staten et al., 1993; Fiddes et al., 1992). In the human, both GH-N and CSH have higher affinity for the PRLR receptor, than the GHR (Handwerger and Freemark, 2000). While it has yet to be isolated, there is evidence for a CSH specific receptor including saturable binding kinetics in the fetal sheep liver (Pratt et al., 1995). There is also evidence for a CSH specific receptor in the human (Anthony et al., 1998). This receptor, like its other family members (GH/PRL) is likely capable of JAK/Stat signal transduction (Anthony et al., 1998).

GH Receptor & Potential Actions

Primate

The first potential CSH receptor GHR, is a single-transmembrane domain receptor that does not possess intrinsic tyrosine kinase domains (Anthony et al., 1998). GHR has been immunolocalized to the fetal liver, pancreas, kidney, skin, and cerebral cortex (Hill et al., 1992), and potentiates its actions through dimerization of its receptor by binding one molecule of ligand to two molecules of its receptor (Cunningham et al., 1991). This dimerization activates the GHR signal transduction cascade involving JAK-STAT signaling (Ishizaka-Ikeda et al., 1993; Ilondo et al., 1994). Essentially, when GHR dimerizes through ligand binding, JAK2 and/or JAK1 are activated and autophosphorylate themselves and the GHR (Anthony et al., 1998). This initiates the transcription factors known as STATs (aka signal transducers and activators of transcription) which translocate to the nucleus and stimulate GH-target gene expression (Smit et al., 1996; Gronowski and Rotwein, 1994). Not only does GHR activate the JAK/STAT signaling cascade, but it can also phosphorylate the insulin receptor (Carter-Su et al., 1996) as well as proteins involved in the Ras-MAP Kinase pathway (Anthony et al., 1998). In spite of the obvious role that these pathways play in fetal growth, they can be activated through other receptors as well. Additionally, while primate hGH, hGH-V, and hCSH possess a high degree of structural similarity and thus, have been hypothesized to act through the same hGHR membrane bound receptor, the affinity of hCSH for hGHR is 2300-fold weaker than hGH so this is likely not the case (Anthony et al., 1998).

Ruminants

Unlike the primate, there is limited specific binding of oGH (via microsomes) in ovine fetal liver tissue until near-term (Gluckman et al., 1983; Freemark et al., 1986; Anthony et al., 1998). However, *oGHR* message, can be detected in ovine skeletal muscle and liver by 60 dpc (Adams et al., 1990; Klempt et al., 1993; Pratt and Anthony, 1995). In the bovine, both *bGHR* and *bPRL* message have been detected in bovine endometrium, corpus luteum, and maternal and fetal liver (Scott et al., 1992; Anthony et al., 1998). Bovine CSH binds the GHR in a 1:1 stoichiometry which is not sufficient to initiate JAK/Stat signal transduction through the bGHR, as two GH ligands are necessary to activate bGHR (Ishizaka-Ikeda et al., 1993; Ilondo et al., 1994). While it is possible that experimental techniques may have missed transient dimerization of bGHR with bCSH ligand, cell experiments using bovine cell lines supported the notion that bCSH cannot elicit signal transduction via the bGHR (reviewed in Anthony et al., 1998). Furthermore, based on competition with monoclonal antibodies, bCSH potentially interacts with a different site on bGHR than GH (Staten et al., 1993).

In the ovine, it is likely that this *GHR* message is distinct from adult GHR as it is 300 nucleotides larger with a different exon 1 sequence (Pratt and Anthony, 1995). While this was originally postulated that this variant oGHR might be the CSH specific receptor (Anthony et al., 1998), this does not appear to be the case as the alternative exon 1B isolated from ovine fetal skeletal muscle has structural properties that will likely prevent translational efficiency (Anthony et al., 1998). During around 120 dGA, GHR with exon 1A begins to be expressed, which suggests a developmental switch (Anthony

et al., 1998). Additionally, this may in part explain why *oGHR* message can be detected in the fetal liver prior to specific binding (Pratt et al., 1995; Pratt and Anthony, 1995).

Lastly, it is possible that fetal cortisol concentrations play a role in oGHR expression. This is supported by a study that infused cortisol prior to the normal rise in fetal cortisol concentrations (10-15 days prior to parturition; Challis and Brooks, 1989) and found this increased the expression of *oGHR* message (Anthony et al., 1998). While this study was inconclusive for determining whether the increase in cortisol triggered the developmental switch in *oGHR* splicing/transcription, an *in vitro* experiment treating fetal hepatocyte cultures (100 dGA) with cortisol did induce expression of exon 1A expressing *oGHR* (Anthony et al., 1998).

CSH Receptor & Potential Actions

Primates

While it is not altogether clear that a CSH specific receptor exists in the human, receptor binding studies are certainly suggestive (Hill et al., 1988). Similar to hGH which binds its receptor with greater affinity, hCSH demonstrates a 9-fold greater affinity for hCSH binding sites compared to hGH (reviewed in Anthony et al., 1998). These binding sites are primarily in the fetal liver and fetal skeletal muscle (Hill et al., 1988; Anthony et al., 1998). Evidence suggests that hCSH can stimulate the phosphorylation of both JAK-STAT and uncharacterized intracellular proteins (Gluckman et al., 1983). While it would be tremendously beneficial to identify and characterize CSH's illusory receptor, this research gap does not preclude investigators from studying its actions on fetal growth.

Ruminants

In contrast to the human, more data supports the existence of a CSH specific receptor in ruminants. The first evidence for a CSH specific receptor is essentially no specific binding of ovine GH or oPRL in the sheep fetal liver until near term (Gluckman et al.,1983; Freemark et al., 1986). In spite of this, there is substantial saturable binding of oCSH to fetal liver microsomes during mid and late gestation (Freemark et al., 1986; Pratt et al., 1995; Anthony et al., 1998). Through cross-linking studies, an apparent M_r was determined for a potential oCSH receptor of 39-44,000 (Leung et al., 1987). Due to its small size, it is not a GHR but is a similar size to the extracellular domain of GHR that is proteolytically cleaved (Leung et al., 1987). While attempts have been made to partially purify the oCSH binding site, no amino acid sequence data have been determined (Freemark and Comer, 1989; Anthony et al., 1998).

During late gestation (125-135 dGA), oCSH and oGH were determined to bind to the same or similar receptors through crosslinking immunoprecipitation experiments in the fetal liver (Breier et al., 1994). However, oCSH still has higher specific binding (7.6 vs 1.2%) compared to GH (Breier et al., 1994; Anthony et al., 1998). More supporting evidence for the existence of a CSH-specific receptor in ruminants was determined using Chinese hamster ovary cells or CHO cells (Anthony et al., 1998). This experiment revealed that both oGH and oCSH bound the same number of receptors per cell, but oGH had greater affinity (K_d = 0.30 nM) for oGHR compared to oCSH (vs K_d = 0.76), again suggesting oCSH is not acting through oGHR (Liang et al., 1992)

Receptor Binding Kinetics

Primates

In the human, CSH only weakly binds the growth hormone receptor (hGHR) as compared to GH (Lowman et al., 1991; reviewed in Anthony et al., 1995). However, specific hCSH receptors have been identified in fetal liver and skeletal muscle (Hill et al., 1988; Anthony et al., 1995). More work has been done to characterize CSH – GHR kinetics in the sheep.

Ruminants

The sheep, both maternal and fetal, provide the most insight into the binding of CSH to its various receptors. GHR or a GHR-like protein has been identified in the sheep liver (fetal and maternal) via immunoblot and immunoprecipitation (Freemark et al., 1991; Breier et al. ,1994b). However, their function appears limited until after parturition.

In the fetal lamb, there is limited specific binding of oGH or oPRL in the fetal liver until near term (Freemark et al., 1986; Gluckman et al., 1983). This was corroborated by saturation analysis of fetal liver microsomal membranes and radiolabeled oCSH, oGH, and oPRL, where no specific binding for either oGH or oPRL was detected (Pratt et al., 1995). Due to great variation in the reported K_d values for oCSH-binding sites in the fetal liver (Freemark et al., 1986; Freemark et al., 1987; Freemark and Comer, 1989), the relationship between ligand abundance and available receptor was difficult to determine. By using competitive binding assays, an average K_d of 0.27 nM was reported (Freemark et al., 1986; Freemark et al., 1987; Freemark and Comer, 1989). However, this was clarified by Pratt et al. (1995) using hot saturation analysis of fetal

liver microsomes. They determined more accurately that the true K_d of oCSH-binding sites in the fetal liver was 122.1 pM. This means that based on the average fetal concentrations of 30 ng/mL of CSH, circulating CSH is in 100-fold excess of its receptor. Maternal concentrations of CSH are generally around 10x fetal concentrations (300 ng/mL), thus the excess ligand in respect to receptor K_d is even more disproportional. This relationship is certainly perplexing, especially in regard to receptor saturation. However, in spite of a knowledge gap about how CSH potentiates its actions, this relationship may suggest a binding protein or some other mechanism to alter the bioavailability of CSH in circulation.

Factors Impacting CSH Receptor Expression

In the ruminant, fetal size, length, and plasma glucose concentrations positively correlate with fetal hepatic CSH binding sites, whereas plasma insulin possesses the inverse relationship (Freemark et al., 1992). Fetal CSH concentrations are also robustly increased during extended fasting (72 h), which unsurprisingly decreases the density of CSH receptors in both the fetal and maternal liver (Freemark et al, 1992). By interrupting the fast through the administration of intravenous (IV) glucose to the pregnant ewe, the fetal liver rapidly responds by increasing the number of CSH binding sites (Freemark et al., 1992). This was hypothesized due to increase of fetal plasma and insulin concentrations, which likely prevented the fasting-driven increase in CSH (Freemark et al., 1992). These changes in response to intravenous glucose only occurs in the fetal compartment however, as maternal CSH concentrations and hepatic binding sites did not change with the administration of glucose and subsequent increase in

insulin (Freemark et al., 1992). When the ewe is re-fed, the specific binding of CSH receptors recovered to pre-fasted levels and was positively correlated with glucose, insulin, and IGF-1 concentrations (Freemark et al., 1990). However, free vs. total binding sites were never assessed. Neither fasting, nor re-feeding, nor glucose administration altered the affinity of oPL for its receptor (Freemark et al., 1992).

CSH Secretion

Human

While much work has been done to investigate CSH gene regulation in the human, there is a paucity of work documenting environmental factors that alter its secretion. It appears that hCSH is constitutively expressed after the formation of the syncytiotrophoblasts (Anthony et al., 1998). While a variety of growth factors, metabolic regulators and secretagogues have been suggested to regulate CSH secretion, no clear regulation has been established. There is no direct transfer of hCSH from maternal to fetal circulation (Kaplan et al., 1998), thus, the placenta must distinctly secrete CSH into both circulations.

Ruminant

A multiplicity of factors has been implicated in altering CSH secretions. CSH is believed to be constitutively secreted, lacking circadian rhythms but capable of rapidly and spontaneously fluctuating (Taylor et al., 1980; Butler et al., 1987; Bauer et al., 1995). These maternal CSH fluctuations can occur in a manner unrelated to feeding, fasting, time of day or stage of pregnancy (Butler et al., 1989). While fetal number did

not influence fetal CSH concentrations, increasing fetal number did increase maternal CSH concentrations in the pregnant sheep (Taylor et al., 1980). In addition to litter size, breed composition can also influence the maternal concentrations of oCSH, but this effect is not observed in singleton-bearing ewes (Butler et al., 1981). Interestingly, ewes carrying crossbred lambs had higher oCSH concentrations than purebred fetuses (Butler et al., 1981). However, some factors can somewhat predictably alter CSH concentrations. For instance, in response to surgery, CSH concentrations are elevated in both maternal and fetal circulation for 3-5 days (Taylor et al., 1980). In addition to pregnancy number and genetics, a variety of environmental factors such as nutritional plane and hyperthermia can also influence circulating CSH.

Maternal undernutrition or fasting

Maternal nutritional plane has been demonstrated to impact fetal CSH concentrations. When late gestation (127-134 dGA) ewes were fasted for 72 hours, both fetal glucose and insulin concentrations fell (Freemark et al., 1992). In response to maternal fasting, fetal CSH plasma concentrations but not maternal, increased greatly compared to control fed animals (Freemark et al., 1992). Data from Brinsmead et al. (1981) also agrees with the Freemark et al. (study), also documenting marked increases in fetal CSH concentrations. However, ewes that underwent a 72 hour fast (from their regular diet) but received IV glucose that maintained maternal and fetal euglycemia, did not see changes in fetal CSH concentrations (Freemark et al., 1992). This would likely suggest that secretions of CSH into the fetal compartment are sensitive to glucose, especially fetal hypoglycemia.

However, there are conflicting reports in literature regarding maternal fasting and glycemic status. In contrast to the Freemark et al. (1992) study which documented no maternal changes in CSH concentrations in response to fasting, Brinsmead and colleagues observed increases in maternal CSH concentrations following to a 72 fasting (Brinsmead et al., 1981). However, clamp induced fetal or maternal hyper or hypoglycemia (without fasting) produced no changes in CSH concentrations in either compartment (Brinsmead et al., 1981). This is supported by Butler et al. (1987) that found that no changes to maternal CSH in response to hyperglycemia or insulin-induced hypoglycemia (Butler et al., 1987).

Maternal overnutrition

A study by Lea et al. (2007) found that prolonged maternal overnutrition (7 – 140 dGA), particularly in adolescent ewes pregnant with fetuses from embryo transfer, resulted in reduced peripheral maternal CSH concentrations. These decreases in maternal CSH were documented from mid-gestation onward, however fetal plasma CSH concentrations were not assessed (Lea et al., 2007). Furthermore, this decrease may be due to the decreased placental mass documented by Lea et al. (2007). No significant changes to cotyledonary *CSH* mRNA or protein were observed (Lea et al., 2007).

Nutrient specific stimulation of CSH secretion

Handwerger and colleagues led an effort to document a variety of specific factors that altered maternal CSH concentrations. These specific factors included arginine,

ornithine, high-density lipoproteins (HDL), and arachidonic acid. Arginine infusions into pregnant ewes were shown to suppress maternal CSH concentrations for 1-2 hours, then to increase (79-115% or more) maternal CSH concentrations in a dose dependent manner for the next 5 hours (Handwerger et al., 1978). While arginine can influence NOS3 concentrations, uterine blood flow was not assessed in this study. In the same study, smaller increases (5-58%) in oCSH were documented in response to alanine and glycine (Handwerger et al., 1978). However, this study also demonstrated reductions (20-50%) in CSH in response to hypertonic saline, so it doesn't appear that CSH secretion is simply a factor of infusing oxidative substrates.

Due to the impacts of arginine of maternal CSH, Handwerger and colleagues (1981) investigated the impacts of ornithine and citrulline infusions on CSH concentrations in pregnant ewes. They found that ornithine did stimulate maternal CSH secretions (increasing ~124%) approximately 1 hour after the initiation of the infusion (Handwerger et al., 1981). Citrulline had no consistent effect on maternal CSH or PRL concentrations but did impact plasma GH concentrations (Handwerger et al., 1981). This led them to postulate that arginine-induced oCSH secretions were initiated by the conversion of arginine to ornithine (Handwerger et al., 1981), The effects of ornithine on maternal CSH secretions appears to be contained to the maternal compartment, as fetal ornithine infusions did not alter placental secretions of CSH into fetal circulation (Grandis and Handwerger, 1983).

Like ornithine, arachidonic acid also had no effect on fetal CSH concentrations when infused into fetal circulation (Huyler et al., 1985). However, maternal CSH concentrations rose by 74% in response to arachidonic acid infusions and were not

inhibited by the infusion of cyclooxygenase inhibitors (Huyler et al., 1985). The impacts of HDL infusions on maternal CSH concentrations were also investigated. While in the majority of cases, HDL did increase (274%) maternal CSH concentrations some ewes (~25%) did not experience increases in maternal CSH after infusion and the majority did not respond to lower concentrations of HDL infusions (Grandis et al., 1989). Like the other substances, it took several hours to see an increase in maternal CSH concentrations (Grandis et al., 1989). Unfortunately, neither uterine or umbilical blood flow were assessed in any of the above substrate-specific infusion studies.

Hyperthermia

Placental insufficiency induced through maternal hyperthermia (HT) reduced maternal circulating CSH concentrations (Regnault et al., 1999). Following approximately 20 days in HT conditions, maternal CSH concentrations fell until the conclusion of the study ~93 dGA or 56 days in HT conditions (Regnault et al., 1999). While maternal CSH concentrations were low throughout most of the study, both cotyledonary *CSH* mRNA and protein concentrations was not altered (Regnault et al., 1999). Fetal concentrations of CSH were not assessed in this study. Lea et al. (2017) also documented observed the disconnect between *CSH* mRNA/protein and circulating CSH. This suggests there may at times be a discrepancy between placental *CSH* mRNA or protein concentrations and CSH in circulation, although the reason behind this difference remains to be elucidated. It is clear that a variety of surgical, nutritional, or environmental conditions can alter both fetal and maternal CSH concentrations, although both compartments do not necessarily respond in the same way to the insult.

Thus, much research remains to be conducted to understand the mechanisms involved in compartment specific CSH secretion.

CSH Proposed Function

The central question remaining to be addressed in CSH biology is the exact function of this incredibly abundant pregnancy specific hormone. This section will 1) chronicle the clinical significance of CSH, 2) review the suggestive evidence that CSH may be involved in regulating fetal growth and metabolism, 3) document studies that tried but failed to document the biological function of CSH and 4) conclude with direct evidence that CSH RNAi results in fetal growth restriction. Lastly, this section will conclude with the currently hypothesized role of CSH in regulating fetal growth and metabolism and highlight remaining questions needing to be investigated.

Clinical significance

As previously discussed, fetal weight is correlated with both maternal CSH concentrations and placental mass. The association between circulating maternal CSH concentrations and fetal growth restriction was first noted by Saxena et al. (1969), as early as 28-32 weeks of gestation, but has been more frequently reported during later gestation (after 36 weeks of gestation; Spellacy et al., 1976; Daikoku et al., 1979). Low maternal hCSH concentrations are also associated with fetal death *in utero* (Spellacy et al., 1975). A single maternal blood sample during the last 5 weeks of gestation with CSH concentrations less than 4 ug/mL is associated with an 30% increased risk of neonatal hypoxia and fetal distress (Letchworth and Chard, 1972). Repeated low

maternal CSH samples over the same period increased the likelihood of adverse events by 50% (2 low samples) and 71% (3 low samples) respectively (Letchworth and Chard, 1972). Spellacy et al. (1976) found that 60% of mothers with IUGR babies had reduced circulating concentrations of CSH from 36 weeks to term compared to uncomplicated pregnancies. An investigation by Daikoku et al. (1979) confirmed low hCSH in low-birth-weight pregnancies at both 25-28 as well as 33 weeks of gestation until term. Aberrant hCSH production during pregnancy have also been associated with other pregnancy complications including gestational diabetes mellitus, maternal obesity, preeclampsia, and erythroblastosis (reviewed in Handwerger, 1991; Sibiak et al., 2020).

Interestingly, Daikoku et al. (1979) observed a decrease in circulating hCSH in babies characterized by abnormal growth but not low birth weight. Spellacy et al., (1976) also found that approximately 1-2% of pregnancies with low CSH concentrations resulted in normal birth weight but were still characterized by abnormal growth characteristics. They categorized those newborns as non-LBW IUGR (Spellacy et al., 1976). While the factors ultimately responsible for reduced CSH in maternal circulation are not fully understood, mutations of the *CSH* gene loci have been documented in the human (Rygaard et al., 1998). It is worth noting that while some *CSH* gene loci mutations/deletions can result in fetal growth restriction (Borody and Carlton 1981; Sideri et al. 1983; Trapp et al. 1987; Rygaard et al., 1998), occasionally seemingly normal babies are born with a complete lack of circulating maternal hCSH (Nielsen et al. 1979; Alexander et al. 1982; Barbieri et al. 1986; Simon et al. 1986). This phenomenon is yet another perplexing aspect of CSH biology. However, it does appear that while maternal CSH is correlated with fetal weight, there are two potential phenotypes that

may emerge in response to reduced CSH: fetal growth restricted infants and non-low birth weight yet abnormally grown infants.

The relationship between CSH and fetal weight is also reported in the sheep. In the sheep, Taylor et al. (1980) reported correlations between maternal CSH concentrations and fetal weight. A similar relationship was reported by Schoknecht et al. (1991) to correlate fetal oCSH concentrations and fetal weight. Additionally, they found that combining fetal oCSH concentrations with placental weight could account for 81% of the variation observed in fetal weight differences (Schoknecht et al., 1991). More profoundly, by combining maternal CSH concentrations with cotyledonary *CSH* mRNA concentrations, the same degree of variation (81%) in fetal weight can be explained, further emphasizing the likely relationship between CSH and fetal growth (Kappes et al.,1992).

Metabolism and the Handwerger Hypothesis

One of the main hypothesized functions of CSH is as a partitioning agent to aid in regulating fetal nutrient supply. Handwerger postulated that CSH may acts as the maternal and fetal “growth hormone” (Handwerger et al., 1991). This hypothesis was derived from rodent data that observed hCSH administration increased postnatal weight gain (Arezzuni et al., 1972), DNA synthesis (Kaplan and Grumbach, 1964) as well as epiphyseal growth (Kaplan and Grumbach, 1964). These are the same physiological responses observed with the administration of GH (Handwerger, 1991), although CSH lacks the same potency as GH postnatally. Interestingly, the administration of oCSH in the rat increased postnatal weight gain and epiphyseal growth in equal proportions to

GH (Chan et al., 1976). Furthermore, oCSH administration to rat fibroblasts increased IGF2 secretions *in vitro* (Adams et al., 1983). This does not mean that oCSH functions differently than hCSH. Rather that the affinity for rat plasma membranes for oCSH is greater than hCSH, on par with GH (Handwerger, 1991). As previously discussed, rodent CSH differs in structure and function compared to the human and sheep, thus caution should be applied when extrapolating these results. Handwerger believed CSH was acting on both the maternal and fetal compartments in distinctive ways and thus formulated hypotheses about the actions of CSH in each.

Maternal actions

In the mother, Handwerger (1991) postulated CSH may be responsible for the third trimester rise in maternal IGF1 concentrations along with altering carbohydrate and protein metabolism. He based this hypothesis on several studies including 1) the rise in IGF1 in hypophysectomized rats in response to oCSH infusions (Hurley et al., 1977c), 2) the maintenance of normal IGF1 maternal concentrations in the hypophysectomized pregnant sheep and rat until delivery of the placenta (Handwerger, 1991; Daughaday and Kapadia, 1978), and 3) the concurrent increase in hPL and IGF1 concentrations during pregnancy (Breuer, 1969).

From a nutrient partitioning perspective outlined in work by Grumbach et al, (1968), Handwerger postulated CSH antagonizes insulin to promote glucose intolerance, lipolysis, and proteolysis in the mother to increase the availability of nutrients for transfer to the fetus (Handwerger, 1991). The rationale for this hypothesis was based on several studies. First, the administration of hCSH to pregnant women

enhanced insulin secretion and impaired glucose tolerance (reviewed in Handwerger, 1991). Secondly, in an *ex vivo* experiment, CSH stimulated isolated pancreatic islet insulin synthesis (Martin and Friesen, 1969). Third, CSH administration induced lipolysis in rat adipose tissue (Felber et al., 1972). Limited *in vivo* evidence has supported this hypothesis, and the studies administering hCSH to pregnant women and the impacts on glucose tolerance could not be located for a thoughtful critique. Additionally, due to the excess circulating concentrations of CSH, it is difficult to ascertain whether this effect was truly caused by CSH administration. Furthermore, evidence from maternal oCSH infusions in the sheep suggests limited changes in circulating maternal metabolites in response to CSH. This is also supported by the short duration (~12 h) immunoneutralization of CSH having limited impacts on maternal glucose, lipid, and amino acid concentrations (Waters et al., 1985). To really understand the impacts of CSH on modifying maternal physiology, functional CSH deficient models must be utilized.

Fetal actions

As previously stated, Handwerger proposed that CSH functions as the “fetal growth hormone”, as GH has no effect on fetal growth *in utero* (Browne and Thornburn, 1989; Handwerger, 1991) in spite of concentrations exceeding those in postnatal animals (Hill et al., 1988). While the proportionally lower circulating concentrations of CSH in the fetus initially convinced investigators that the primary action of CSH was on maternal physiology and all fetal effects were indirect, this idea has been discredited. In the sheep oCSH stimulates *in vitro* amino acid uptake (Freemark and Handwerger,

1983; Anthony et al., 1995), glycogen synthesis (Freemark and Handwerger, 1984; Anthony et al., 1995) and ornithine decarboxylase activity (Hurley et al., 1980).

Handwerger postulated that CSH impacts growth via IGFs to produce somatogenic activity in the fetus (Handwerger, 1991). This has been supported through a variety of *in vitro* and *in vivo* experiments. For instance, when human fetal fibroblast cells are treated with hCSH, both IGF1 and IGF2 are released by the cells along with IGFBPs (Hill et al., 1989; Anthony et al., 1995). This effect does not occur when the same cells are treated with hGH (Hill et al., 1989). *In vivo*, this increase in circulating IGF1 and IGF2 occurs when non-lactating dairy cows are treated for 5 days with recombinant bCSH (Byatt et al., 1992). In the late gestation fetal sheep, continuous (14 day) infusion of oCSH (1.2 mg/day) increased circulating concentrations of IGF1 (Schoknecht et al., 1996).

In addition to actions on fetal IGF production, CSH is thought to promote glycogen storage. There are two possible mechanisms for this that have been demonstrated *in vitro*: 1) stimulation of glycogen synthesis and 2) inhibition of glycogenolysis. CSH was shown to stimulate glycogen synthesis in hepatocytes from fetal lambs and rats (Freemark and Handwerger, 1984a; Freemark and Handwerger, 1986b), likely in a synergistic fashion with insulin (Freemark and Handwerger, 1984b). Additionally, using cultured fetal rat hepatocytes, CSH prevented glucagon-mediated glycogenolysis through inhibition of phosphorylase a (Freemark and Handwerger, 1995). Handwerger postulated that one of the functions behind this could be the liberation of hepatic glycogen stores immediately after parturition to assist the neonate in transitioning to postnatal life (Handwerger, 1991). It is interesting that little has been

postulated about the effects of CSH on placental function, especially considering the significant role the placenta plays in facilitating the transfer of nutrients and in producing growth potentiating factors.

Mammogenesis and Lactogenesis

As the name implies, CSH has been postulated to act on the mammary gland in two ways: 1) through the development of the mammary gland (mammogenesis) and/or 2) by altering mammary function for lactation (lactogenesis). The first evidence for the role of CSH is through the inhibition of prolactin. While prolactin has been demonstrated as necessary for induction of mammogenesis in ruminants, the inhibition of prolactin via an antagonist (bromocriptine) during pregnancy does not impede mammogenesis (Schams et al., 1984). Furthermore, bromocriptine administrations do not impact CSH concentrations (Schams et al., 1984). Because mammogenesis can continue during pregnancy in the absence of prolactin, it is plausible that CSH may play a role. Using athymic nude mice, DNA synthesis was stimulated in mammary tissue with bCSH administration (Vega et al., 1989). Similar results were documented when athymic nude mice were implanted with benign tumor tissues from human breasts and administered hCSH (McManus and Welsch, 1984). The administration of bCSH also increased total mammary DNA content and blood concentrations of α-lactalbumin in heifers induced to lactate (Byatt et al., 1997). Functionally, bCSH administration increases milk yield by 22% (in heifers induced to lactate), but was not statistically significant (Byatt et al., 1997). In contrast to PRL, bCSH resulted in more potent stimulation of DNA accretion, but is less effective at increasing α-lactalbumin (Byatt et al., 1994) and thus may be

more critical for mammogenesis vs. lactogenesis. In the human, little is documented about the role of hCSH in mammogenesis and lactogenesis.

Unlike the bovine, oCSH appears to be limited in its contribution to mammary function. The impacts of CSH on mammogenesis have not been adequately assessed. In contrast to the cow, ovine CSH cannot compete for prolactin binding sites in mammary tissue (Emane et al., 1986) and is less efficient at increasing transcription of *β-casein* mRNA in sheep mammary explants (Severly et al., 1983). One study using pseudopregnant ewes documented increased milk production in response to CSH administration on par with GH induced milk production increases (Kann et al., 1999; Gertler and Djiane, 2002). However, in a more biologically relevant model, when early lactating ewes receive exogenous CSH, both mammary weight and milk production are unaltered (Min et al., 1997). In the same study, the administration of GH increased both mammary weight and milk production, and thus, GH is likely more critical for stimulating mammogenesis and lactogenesis in the sheep (Min et al., 1997; Anthony et al., 1998). In conclusion, there is a paucity of research pertaining to the potential mammogenic and lactogenic roles of CSH in both the human and the sheep, and future studies should explore these processes.

Steroidogenesis

In both the rodent and the bovine, it appears that CSH may have luteotropic actions (Galosy and Talamantes, 1995; Lucy et al., 1994). In the cow, injections of bCSH increases the size of the corpora lutea (CL) along with increasing plasma progesterone concentrations (Lucy et al. ,1994). However, bCSH will reduce the size of

the largest follicle (Lucy et al., 1994). While it is possible these effects could be potentiated through GHR as similar effects on the CL were demonstrated with bGH injections (Lucy et al., 1993) little specific binding of bGH to luteal microsomes has been demonstrated (Lucy et al., 1994). Specific binding of bCSH to luteal microsomes has been demonstrated (Lucy et al., 1994), however, it is possible that bCSH is potentiating its actions through the PRLR. This is evidenced by the presence of *PRLR* mRNA in bovine luteal tissue, as well as both bCSH and bPRL having equal affinity towards the PRLR *in vitro* (Scott et al., 1992).

In the human and the sheep, no evidence exists for the role of CSH in regulating steroidogenesis. The administration of hCSH to women fails to extend the luteal phase of the menstrual cycle (Stock et al., 1971) and does not impact progesterone production (Ramasharma and Li, 1987). Additionally, the human CL lacks hCSH binding sites, at least at the end of pregnancy (Goldsmith et al., 1978). However, hCSH administration could have ovarian impacts as it can stimulate IGF2 secretion by granulosa cells (Ramasharma and Li, 1987). In the sheep, ovarian infusion of oCSH does not inhibit luteolysis and fails to increase progesterone production (Schramm et al., 1984). Additionally, short term immunoneutralization by the infusion of anti-CSH antibodies does not alter progesterone concentrations (Thodarson et al., 1987). However, using 45 dGA luteal cells, treatment with oCSH did increase progesterone secretion *in vitro* (Liebovich et al., 2001, Wierzchos et al., 2000; Gerler and Djiane, 2002). One of the reasons for this distinction could be related to the timeline of placental takeover of progesterone production in both the human and the sheep. Thus, continual CL function

across pregnancy is not as necessary for those two species, as it would be for the bovine.

Attempts to Investigate CSH Function in vivo

Infusion of CSH

Attempts to elucidate the *in vivo* function of CSH have focused on 1) the infusion of exogenous CSH into fetal circulation or 2) attempts to alter the bioavailability of CSH through immunoneutralization. Schoknecht et al. (1996) infused 1.2mg daily of purified oCSH into fetal circulation for 14 days during late gestation (122-136 dGA). They found that fetal oCSH infusions increased the circulating IGF1 concentrations and the fetal livers at necropsy had accumulated additional glycogen (Schoknecht et al., 1996). While they did not see changes in fetal growth, they noted increases in aggregate weights of various fetal organs including the brain, liver, lungs, and heart (Schoknecht et al., 1996). It is likely that both dose and duration effect this response. A similar study by Oliver et al. (1995) using only 50% of the daily dose (~600ug) of the Schoknecht study with a distinct infusion strategy (100 ug bolus + 500 ug over 24 hours) during 3 different days opposed to continuous 1.2 mg over 14 days. They found limited changes in either fetal or maternal metabolic factors or hormones (Oliver et al., 1995). Another attempt to assess the biological function of CSH was the infusion of placental extracts containing placental lactogen in the non-pregnant sheep to attempt to directly assess the function of oCSH on maternal physiology in the absence of pregnancy (Thordarson et al., 1987). Using this approach, they documented some increases in plasma NEFAs, glucose, and urea (Thodarson et al., 1987). However, this attempt to assess the impacts of oCSH on

maternal circulation in the absence of pregnancy was interesting. Considering the aims of this experiment, it is baffling they did not use purified oCSH. Rather, the placental extracts they used undoubtedly possessed a conglomeration of factors and hormones capable of modifying maternal physiology, essentially nullifying their attempt to study the effects of oCSH in a non-pregnant physiological state. All of these studies had limited sample size with the Schoknecht et al. (1996) being the highest powered (n=6/treatment) and the Thordarson et al. (1987) being the least powered (n=3 ewes total). If CSH, which is already circulating in fetal circulation at 100x the level to saturate its receptors, it is not surprising that additional exogenous CSH had limited effects. Still, the impacts of CSH on fetal IGF1 concentrations in the Schoknecht et al. (1996) study are suggestive of a potential role of CSH in modulating fetal growth.

CSH neutralization

Another approach used to investigate the potential biological function of CSH is the use of antibody infusion or immunization of sheep against oCSH in order to reduce the bioavailability and thus, action of CSH. Waters and colleagues (1985) first tried this approach with a single day of antibody infusion into 131 dGA minimally catheterized pregnant ewes (n=3/treatment). They found no changes in maternal glucose concentrations but documented a fall in plasma insulin followed by a subsequent increase in circulating maternal insulin (Waters et al., 1985). They saw no changes in either maternal IGF1, IGF2, GH, or PRL concentrations (Waters et al., 1985). Neither did they observe changes in maternal progesterone nor cholesterol concentrations (Waters et al., 1985). In a different study using ewes that had been actively immunized

against oCSH at 5 months of age before breeding at 9 months of age, Leibovich et al. (2000) actually noted increases in birth weight and milk production. While this approach altered the bioactivity of CSH *in vitro*, they actually documented increases of CSH bioactivity of anti-CSH immunized sheep in late gestation (Leibovich et al., 2000). While this increase in fetal weight is perplexing, it does not appear that the actions of CSH were fully neutralized by these approaches and may have even been potentiated in the Leibovich et al. (2000) study. Additionally, while difficult to assess *in vivo*, the bioactivity in vitro of late gestation CSH actually increased in immunized ewes and could in part explain this increase in fetal weights.

The reason these approaches likely failed to elicit the desired changes in available CSH are likely due to profound excess of CSH in circulation in relation to the K_d of its binding site. No known antagonist for CSH is available, and even if there were, it would not be feasible to continuously infuse the antagonist into both compartments across gestation, as simply altering maternal production will not necessarily impact secretion into the fetal compartment. The most ideal way to investigate the function of hormones is by creating a functional gene product deficiency. This can be achieved through gene knockout or by using RNA interference which until recently, could only be accomplished in rodent models. To this end, a methodology has recently been developed utilizing lentiviral-mediated RNA interference to specifically target gene products in the sheep placenta.

CSH RNA interference

The first study utilizing RNA interference to interrogate gene function in the sheep placenta was conducted by Purcell et al. (2009) to test the function of the gene PRR15 on conceptus development. To generate these PRR15 RNAi pregnancies, superovulated sheep were time-mated and expanded hatched blastocysts were collected at 8 dGA and infected with lentiviral (pLL3.7) constructs expressing either a PRR15-specific RNAi, a lentiviral vector with a 3-nucleotide mismatch to PRR15, or an empty lentiviral vector (Purcell et al., 2009). Embryos were cultured in oil drops containing the lentivirus expressing RNAi for 6 hours, then washed and surgically transferred into synchronized recipient ewes (Purcell et al., 2009). Using this methodology, the PRR15 specific RNAi was successful in ablating *PRR15* mRNA concentrations in the trophectoderm.

Using a slight variation of this methodology, Baker et al. (2016) generated the first direct evidence that CSH gene product deficiency actually resulted in both IUGR and placental insufficiency. The slight variations from the Purcell et al. (2009) study included shifting embryo collection, infection, and transfer forward by one day to allow more embryos to hatch from their zona pellucida and not super-ovulating the donor ewes. Baker et al. (2016) also utilized several different vectors (pGIPZ or pLentiLox 3.7) with different promoters (human elongation factor-1 α or human U6) to deliver the constructs (n=4) to the trophectoderm. This was due to the need for continued RNAi production across gestation. They determined the vector/promoter combination that was most efficacious was the hLL3.7 tg6 construct which was delivered by pLentiLox3.7 and had a human U6 promoter (Baker et al., 2016). Using these methodologies followed by

terminal surgeries to collect maternal and fetal blood and tissues at 135 dGA, Baker and colleagues (2016) determined CSH was crucial for fetal growth and likely for placental function. CSH RNAi resulted in 32% reductions in fetal weights and 52% reductions in placental weights (Baker et al., 2016). Fetal liver weights as well as crown-rump lengths were reduced in response to CSH RNAi, as was cotyledonary *CSH* mRNA concentrations (Baker et al., 2016) confirming the model. From an endocrine standpoint, umbilical artery IGF1 was reduced, and umbilical insulin tended to be reduced in CSH RNAi fetuses (Baker et al., 2016). The uterine artery to vein glucose gradient was elevated in CSH RNAi pregnancies which suggested dysfunction in placental glucose transport (Baker et al., 2016). Not only were fetal livers smaller in mass, but the mRNA concentrations of IGF factors such as *IGF1* and *IGF2*, as well as *IGFBP2* and *IGFBP3* were reduced (Baker et al., 2016). Placental *IGF1* and *IGF2* mRNA concentrations were also reduced in these CSH RNAi pregnancies (Jeckel et al., 2018). Nutrient transporter mRNA concentrations were also impacted by CSH RNAi as concentrations of both *SLC2A1* and *SLC2A3* were reduced and *SLC2A8* was increased (Jeckel et al., 2018). However, only SLC2A3 protein was reduced in CSH RNAi pregnancies but mirrored reductions (32%) in fetal weight (Jeckel et al., 2018). This study also examined maternal IGF1 and insulin concentrations, which were not impacted by CSH RNAi, contradicting the hypothesized function of CSH for maternal physiology.

Clearly, deficiency of the CSH gene product throughout gestation and assessed near term results in IUGR. Investigators however, wanted to interrogate the progression of CSH-RNAi induced IUGR, thus, they used the same methodologies to create CSH RNAi pregnancies in the sheep but took them only to the end of the first-third (50 dGA)

of pregnancy. By 50 dGA of pregnancy, fetal growth restriction was detected in response to CSH RNAi, as fetuses were 20.5% smaller than CON RNAi and placental weights were reduced by 16.5% (Jeckel et al., 2018). Fetal liver weights were reduced by 20.9%, but fetal liver *IGF1* and *IGF2* mRNA concentrations were not different at this point in gestation (Jeckel et al., 2018). However, placental mRNA concentrations of *IGF1* were reduced, as was placental concentrations of *SLC2A1* (Jeckel et al., 2018).

Not every CSH RNAi pregnancy however, results in fetal growth restriction. In the Baker et al. (2016) study, approximately 50% of the CSH RNAi pregnancies resulted in fetal growth restriction (± 2 SD from mean of CON RNAi), and 50% resulted in pregnancies with similar fetal and placental weights compared to controls (Ali et al., 2020). This is very similar to the dual phenotypes observed in the human (Borody and Carlton 1981; Sideri et al. 1983; Trapp et al. 1987; Rygaard et al., 1998, Nielsen et al. 1979; Alexander et al. 1982; Barbieri et al. 1986; Simon et al. 1986). Just as suggested in human pregnancies characterized by low maternal CSH concentrations, a non-LBW IUGR phenotype is observed with metabolic perturbations similar to CSH RNAi IUGR pregnancies. For instance, both CSH RNAi phenotypes had elevated uterine artery-vein glucose gradients, indicating similar dysfunction in placental glucose transfer (Ali et al., 2020). Thus Ali et al. (2020) set out to document the shared metabolic perturbations between CSH RNAi IUGR and normal weight CSH RNAi pregnancies.

Fetuses from both CSH RNAi phenotypes had similar reductions in *CSH* mRNA concentrations, suggesting it wasn't a failure of the lentiviral infection to impact *CSH* transcription that resulted in this dual phenotype (Ali et al., 2020). Next, they noted similar reductions in umbilical artery IGF1 concentrations between both CSH RNAi

phenotypes, along with similar reductions in fetal liver *IGF1, IGF2, IGFBP1, IGFBP2*, and *IGFBP3* mRNA (Ali et al., 2020). Unlike the CSH RNAi IUGR pregnancies, those with normal weights had reductions in *IGFBP1* which could potentially hint at increased bioavailability of IGF in spite of its suppression in fetuses with normal weights (Ali et al., 2020). Both CSH RNAi phenotypes also had documented increases in fetal liver IRβ concentrations, which is biologically consistent with reductions in umbilical insulin concentration although not quite statistical ($P = 0.15$) in the normal weight CSH RNAi group (Ali et al., 2020).

Paradoxically to the postulated role of CSH in glycogenesis, fetal glycogen concentrations were elevated in both CSH RNAi phenotypes (Ali et al., 2020). This could in part, be explained by increased fetal liver glycogen synthase 1 concentrations in both phenotypes, as well as reductions in the inactive form of glycogen synthase, p-glycogen synthase (Ali et al., 2020). While this increase in fetal liver glycogen concentrations appears to conflict with previous reports about the role of CSH in increasing glycogen storage, the pregnant ewes on this study were fasted for ~16 h prior to sample collection and underwent anesthesia, both of which have been documented to increase circulating CSH especially in the fetal compartment. However, this emphasizes the weaknesses of performing maternal and fetal blood sampling under non-steady state conditions. Thus, there is great need to use this model to investigate the physiological ramifications of CSH RNAi under non-stressed, non-anesthetized conditions using the transplacental diffusion technique to directly assess placental and fetal metabolism.

Current Hypothesis for CSH function

Based on the literature reviewed in this section, the current hypothesis for the biological function of CSH is illustrated by **Figure 3**. CSH is postulated to act in endocrine fashion on both the fetal and maternal compartments as supported by extensive investigations described in this review, and most likely, acts directly on the placenta as evidenced by the severe placental growth restriction observed in Baker et al. (2016). CSH has been suggested to act directly on the uterus, increasing endometrial gland density (Spencer et al., 1999). Perhaps, CSH continues to act directly on the uterus to promote growth throughout pregnancy. CSH is also hypothesized to influence nutrient transfer to the fetus as evidenced by elevated transplacental glucose gradients and reduced SLC2A3 concentrations. This would be further supported in future studies through assessing nutrient uptake by the uterus and the fetus in CSH RNAi animals with the transplacental diffusion technique. Additionally, CSH is hypothesized to alter placental utilization of oxidative substrates which can also be directly assessed by transplacental diffusion technique and if true, would result in less nutrients available for uptake by the fetus. While there has been no direct evidence yet for the role of CSH on placental metabolism, the dramatically smaller placentas of CSH RNAi pregnancies are suggestive of this hypothesis. Lastly, CSH is postulated to act in an endocrine fashion on the fetal liver, due to the presence of CSH-specific binding sites, and reductions in fetal liver mass and IGF1 production in CSH RNAi pregnancies. Additionally, it is possible that CSH acts on the pancreas to alter insulin production and signaling as suggested by reduced fetal insulin concentrations and increases in fetal liver IR β. In

conclusion, the hypothesized function of CSH is to regulate nutrient transfer to and uptake by the fetus, supporting appropriate fetal growth and placental function.

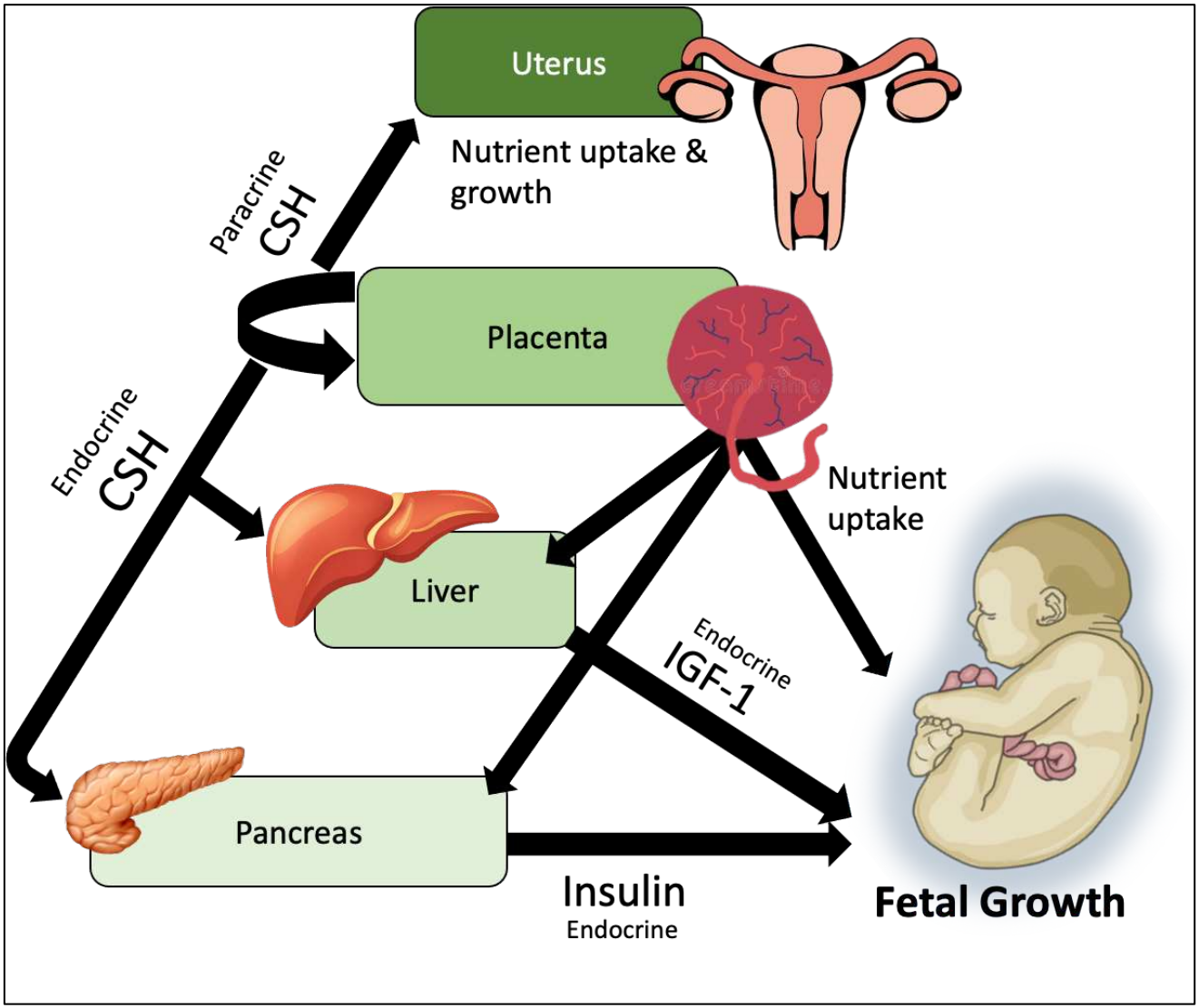


Figure 1.3: Current hypothesis for the role of CSH on the uterus, placenta, and fetus.

Summary

Despite its discovery ~60 years ago, the specific biological function of the placental hormone CSH remains to be elucidated. However, it does appear that CSH plays a similar role in regulating fetal growth and placental function in both the human and the sheep. The necessity of CSH for adequate fetal and placental growth was clearly demonstrated in recent experiments using a model of CSH specific RNAi in the sheep placenta. CSH RNAi reduces circulating fetal IGF1 as well as placental and fetal liver mRNA concentrations of *IGF1* and *IGF2*. However, not all CSH RNAi result in fetal growth restriction. It appears that CSH RNAi results in similar metabolic perturbations, independent of fetal weight reductions, including elevated uterine artery to vein glucose gradients, reduced umbilical IGF1, and elevated fetal liver IR- β concentrations. This supports the crucial role for CSH in regulating fetal and likely placental metabolism. Finally, by using the mathematical and methodological approaches described in previous sections (Fick principle, transplacental diffusion technique, etc) to investigate placental and fetal physiology, it is possible to directly assess the ramifications of CSH RNAi on placental physiology and how CSH RNAi leads to fetal growth restriction. By investigating both the IUGR and normal weight phenotypes, it may be possible to parse out the direct and indirect effects of CSH and finally begin to address the biological function of CSH on modulating fetal growth.

CHAPTER II: IMPACT OF CHORIONIC SOMATOMAMMOTROPIN RNA INTERFERENCE ON UTERINE BLOOD FLOW AND PLACENTAL GLUCOSE UPTAKE IN THE ABSENCE OF INTRAUTERINE GROWTH RESTRICTION

INTRODUCTION

The placenta-specific hormone chorionic somatomammotropin (CSH) is one of the most abundantly produced placental hormones and belongs to the growth hormone/prolactin gene family. Syncytiotrophoblast cells in the human placenta are responsible for the production and secretion of CSH whereas chorionic binucleate cells in the sheep serve the same purpose (Gootwine, 2004). Recently, we demonstrated through lentiviral mediated RNA interference (RNAi) of CSH, that CSH deficiency resulted in the development of intrauterine growth restriction in sheep, both at near-term (135 dGA; Baker et al., 2016) and at end of the first one-third of pregnancy (50 dGA; Jeckel et al., 2018). These recent studies confirm an established association between reduced CSH in maternal circulation and IUGR in both human (Daikoku et al., 1979; Spellacy et al., 1976) and sheep pregnancies (Lea et al., 2007), thus emphasizing the importance of CSH in maintaining healthy pregnancies. In spite of being discovered over 60 years ago, the exact biological role of CSH remains to be elucidated.

As reported previously (Baker et al., 2016), CSH RNAi does not always result in IUGR. Two phenotypes emerged in response to CSH RNAi, 1) pregnancies with placental and fetal growth restriction and 2) pregnancies with fetal and placental weights similar to control RNAi pregnancies (Ali et al., 2020). While perplexing, this phenomenon is not unique to sheep. In the human, pregnancies have been observed to

possess gene mutations or deletions that either resulted in IUGR (Borody and Carlton, 1981; Rygaard et al., 1998; Sideri et al., 1983) or non-IUGR phenotypes (Alexander et al., 1982; Barbieri et al., 1986; Simon et al., 1986). Recently (Ali et al., 2020) we expanded the comparison of CSH RNAi pregnancies (Baker et al., 2016) to include both phenotypes. With both near-term phenotypes, CSH RNAi resulted in increased uterine artery to uterine vein glucose gradients, reduced umbilical artery IGF1 concentrations and increased fetal liver insulin receptor concentrations, amongst other commonalities between the two CSH RNAi phenotypes. While these near-term pregnancies (Baker et al., 2016) were harvested under fasted and anesthetized conditions, some of the commonalities (Ali et al., 2020) between the two phenotypes (e.g., uterine artery to uterine vein glucose gradients) led us to hypothesize that CSH RNAi may be impacting uterine blood flow and uteroplacental glucose uptake, regardless of fetal growth restriction. As little is known about basic *in vivo* CSH physiology, the objective of our current research was to determine the *in vivo* impacts of CSH RNAi on uteroplacental blood flow and nutrient transport in the absence of IUGR.

MATERIALS AND METHODS

All animal procedures were approved by the Colorado State University Institutional Animal Care and Use Committee (Protocol # 18-7866A), the Institutional Biosafety Committee (18-029B) and the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee (Protocol #00714).

Lentiviral generation

Lentiviral generation of hLL3.7 tg6 (target 6; CSH RNAi) and hLL3.7 NTS (scramble control/non-targeting sequence; control RNAi) was described previously (Baker et al., 2016; Jeckel et al., 2018). Briefly, both the NTS and tg6 sequences (Baker et al., 2016; Jeckel et al., 2018) were cloned into the LL3.7 vector in accordance with the procedures described extensively (Baker et al., 2016). The shRNA sequences for the hLL3.7 NTS and hLL3.7 tg6 constructs are summarized in **Table 1.** All virus generation and titering were completed in accordance with our previously described procedures (Baker et al., 2016).

Table 2.1. Control and CSH-targeting shRNA sequences.

Oligonucleotide	Sequence (5′-3′)
Control (NTS) shRNA sense	GAGTTAAAGGTTCGGCACGAATTCAAGAGATTCGTGCCGAACCTTTAACTC
CSH-targeting (tg6) shRNA sense	AAGGCCAAAGTACTTGTAGACTTCAAGAGAGTCTACAAGTACTTTGGCCTT

Generation of CSH RNAi pregnancies

Sheep were group housed in pens at the Colorado State University Animal Reproduction and Biotechnology Laboratory and provided access to hay, trace mineral, and water in order to meet or slightly exceed their National Research Council (2007) requirements. Animal management, estrus synchronization and embryo transfers were done as previously described extensively (Baker et al., 2016). In summary, after synchronization and subsequent breeding (Columbia-Rambouillet rams), fully expanded and hatched blastocysts were collected by flushing the uteri at 9 days post conception. Each blastocyst was infected with 100,000 transducing units of either control RNAi or CSH RNAi lentivirus. After infection for approximately 5 hours, each blastocyst was washed thoroughly in HCDM-2 media and a single blastocyst was transferred surgically (Baker et al., 2016; Jeckel et al., 2018) into a synchronized Dorper ewe. Dorper ewes were used as recipients in order to meet size requirements for the metabolic studies. Each recipient ewe was monitored daily for return to standing estrus and confirmed pregnant at 50 days of gestational age (dGA) by ultrasound (ALKOA SSD-500V, Wallingford, CT).

Surgical instrumentation of fetus and ewe

At approximately 115 dGA, pregnant recipient ewes were transported to the University of Colorado Anschutz Medical Campus, Perinatal Research Center (Aurora, CO). Animals had access to *ad libitum* alfalfa pellets (Standlee Hay, Kimberly, ID) and water. Six control RNAi (4 males and 2 females) and six CSH RNAi (2 males and 4 females) ewes underwent surgical placement of fetal and maternal catheters at 126

dGA, to determine blood flow and nutrient flux as previously described (Bonds et al., 1980; Brown et al., 2015; Hay et al., 1981; Jones et al., 2019; Regnault et al., 2003). Briefly, the uterus was exteriorized via midventral laparotomy and the fetus was exposed through a 6 cm incision in the uterine wall (Hay et al., 1981; Regnault et al., 2003). Fetuses were instrumented with indwelling catheters in their descending aorta (representing umbilical artery blood), femoral vein, and umbilical vein (Battaglia and Meschia, 1986; Brown et al., 2015; Hay et al., 1981; Regnault et al., 2003). Maternal femoral artery (representing uterine artery blood), femoral vein, and uterine vein were also catheterized (Battaglia and Meschia, 1986; Hay et al., 1981; Regnault et al., 2003). All catheters were tunneled subcutaneously to the paralumbar fossa, where they were exteriorized into a pouch and maintained with 5% heparinized saline flushes while the ewe was given a minimum of 5 days for recovery from surgery (Hay et al., 1981; Regnault et al., 2003).

Blood flow calculations and tissue collection

At 132 dGA, uterine and umbilical blood flows were determined by the steady state $^{3}H_2O$ transplacental diffusion technique (Meschia et al., 1966; Van Veen et al., 1984). Baseline samples (draw 0) were collected from maternal femoral artery (A), uterine vein (V), umbilical vein (γ) and fetal descending aorta (α) simultaneously. Then, a 3 ml bolus of $^{3}H_2O$ was infused into the fetal femoral vein, and isotopic steady state was reached by continuous infusion at 3 ml/h (15μCi/ml) for 90-minute (Jones et al., 2019). Four samples (draws 1-4) were then collected from the four catheters

simultaneously at 20-minute intervals for analysis of blood gas, nutrient content, $^{3}H_2O$ and CSH concentrations (Jones et al., 2019; Regnaul et al., 2003).

Uterine and umbilical blood flows were calculated by the steady state diffusion technique described previously (Meschia et al., 1966). Plasma flows for either the uterine or umbilical artery were calculated by using the corresponding vessel blood flows respectively multiplied by 1 minus the fractional maternal or fetal hematocrits (Regnault et al., 2003). The diffusion rate of $^{3}H_2O$ was calculated as a ratio by dividing the transplacental $^{3}H_2O$ diffusion rate by the $^{3}H_2O$ concentrations in umbilical artery to uterine artery (Regnault et al., 2003). Oxygen content was determined in accordance with calculations previously described (Regnault et al., 2003). Uterine and umbilical uptakes of oxygen, glucose, lactate and amino acids were calculated by the Fick Principal (Meschia et al., 1980) and reported as an average of draws one through four. Uteroplacental utilization of the aforementioned nutrients were calculated as the difference between uterine and umbilical uptakes (Battaglia and Meschia, 1986). The fraction of uterine glucose uptake taken up by the placenta is calculated as uteroplacental glucose uptake divided by uterine glucose uptake. The fraction of uterine glucose uptake being taken up by the fetus is calculated by the umbilical glucose uptakes divided by the uterine glucose uptakes. Maternal glucose to oxygen quotients are defined as the maternal artery to uterine vein (A-V) glucose gradient x 6 carbons/glucose, divided by the A-V oxygen gradient (Morris et al., 1973). Maternal lactate to oxygen quotients are defined as the A-V lactate gradient x 3 carbons/lactate, divided by the A-V oxygen gradient. Maternal amino acid to oxygen quotients are defined as the A-V gradient of an individual amino acid x number of carbons that can be

oxidized for that amino acid divided by the A-V oxygen gradient. Fetal umbilical nutrient to oxygen quotients are calculated the same way as maternal, except the umbilical vein to fetal artery (γ-α) difference replaced the (A-V) difference. To calculate the overall amino acid: oxygen quotient, each individual amino acid quotient is added together. Uteroplacental lactate production is defined as the addition of uterine lactate uptake and umbilical lactate uptake.

Ewes and fetuses were euthanized at 132 dGA by a lethal dose of sodium pentobarbital (390mg/mL, Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI), and the gravid uterus (uteroplacenta) was removed and weighed (Regnault et al., 2003). Placentomes were trimmed from the endometrium and after recording a total placentome weight and number, 10 placentomes were selected from each placenta and separated into cotyledonary (fetal) and caruncular (maternal) components then snap frozen in liquid N and stored at -80ºC (Regnault et al., 2003). Excess fluid was removed from fetal membranes and weighed, then the uterus was weighed after removal of all placentomes, fluids, and fetal membranes. Fetal crown rump length (CRL) was recorded (Mellor and Matheson, 1979). Fetal weight and dissected organ weights were recorded, and tissues were snap frozen in liquid nitrogen. Ponderal index was calculated as fetal weight (g) x 100 / CRL $(cm)^3$ and fetal brain (g) /liver weight (g) ratios were calculated as indicators of asymmetric fetal growth (Bell et al., 1987; Regnault et al., 2003).

Biochemical analysis of blood samples

Whole blood O_2 content, hemoglobin O_2 saturation (SO_2) partial pressure of oxygen (PO_2), partial pressure of carbon dioxide (PCO_2), pH and hematocrit measurements were analyzed by an ABL 800 Blood Gas Analyzer (radiometer; Brown et al., 2015; Jones et al., 2019; Regnault et al., 2003). Plasma glucose and lactate were measured by Yellow Springs Instrument 2900 (YSI Incorporated, Yellow Springs, OH) as described previously (Baker et al., 2016; Brown et al., 2015; Limesand et al., 2007). Plasma amino acids were measured by HPLC (Maliszewski et al., 2012; Wai et al., 2018). CSH concentrations in maternal and fetal plasma were analyzed by radioimmunoassay as previously described (Baker et al., 2016; Jeckel et al., 2018; Kappes et al., 1992; Lea et al., 2007; Regnault et al., 1999).

Western blot analysis

Protein isolation and analysis was done in accordance with methods described in (Baker et al., 2016; Jeckel et al., 2018; Kappes et al., 1992; Regnault et al., 1999). Cotyledonary or caruncular tissue (100 mg) was lysed in 500 µL of lysis buffer (0.48 M Tris, pH 7.4; 10 mM EGTA, pH 8.6; 10mM EDTA, pH 8; 0.1 mM PMSF; 0.1 mM AEBSF; 0.0015 mM pepstatin A; 0.0014 mME-64; 0.004 mM bestatin; 0.002 mM leupeptin; and 0.00008 mM aprotinin) and sonicated on ice. For cotyledonary CSH analysis, 5 µg of protein from each sample was electrophoresed through a 4-15% Tris-Glycine Stain-Free gel (BioRad, Hercules, California) and transferred to a 0.45-µm pore nitrocellulose membrane. The protein in each lane was visualized after transfer to the nitrocellulose and protein loading was imaged using the ChemiDoc XRS+ chemiluminescence system

(BioRad) to use for normalization. To visualize CSH, the blot was incubated in a 1:25,000 dilution (in 5% Non-Fat Dry Milk / 1X Tris-Bis Solution+ 1% Tween) of rabbit α-oPL-S4 (Kappes et al., 1992) for 24 h at 4ºC. After washing the membrane, the membrane was transferred into a 1:100,000 dilution (in 5% Non-Fat Dry Milk / 1X Tris-Bis Solution+ 1% Tween) of mouse α-rabbit IgG conjugated to horse radish peroxidase (sc-2357; Santa Cruz Biotechnology Inc., Dallas, TX). Nitrocellulose membranes were developed using an ECL Western Blotting Detection Reagent chemiluminescent kit (Amersham, Pittsburgh, PA) and imaged using the ChemiDoc XRS+ chemiluminescence system. Densitometry calculations were performed using Image Lab Software (BioRad). To account for technical error between membranes, a common sample was included in each Western immunoblot and densitometry measurements were adjusted based on the average densitometry measurement of the common sample.

For analysis of caruncular and cotyledonary concentrations of NOS3, 20 μg of each sample was electrophoresed through 4-15% Tris- Glycine Stain-Free gels (BioRad), transferred and analyzed as described for CSH. NOS3 was detected using a 1:2000 dilution of mouse α-NOS3 (BD 610297; BD Biosciences, San Jose, CA) and a 1:5,000 dilution of goat α-mouse IgG conjugated to horse radish peroxidase (sc-2005; Santa Cruz Biotechnology Inc). For analysis of caruncular (1 μg/sample) and cotyledonary (2.5 μg/sample) concentrations of SLC2A1 (GLUT1), samples were electrophoresed, transferred and analyzed as described above. SLC2A1 was detected using a 1:2000 dilution of rabbit α-SLC2A1 (07-1401; EMD Millipore) and a 1:5000 dilution of goat α-rabbitt IgG conjugated to horse radish peroxidase (ab205718; Abcam).

As described above, densitometry of SLC2A1 was normalized on total protein/lane, using Image Lab Software (BioRad).

To analyze the concentration of SLC2A3 (GLUT3), we generated a rabbit α-sheep SLC2A3 antiserum, following the protocol of Erhardt and Bell (1997). The COOH-terminal sequence of sheep SLC2A3 (SIQPTKDTNA) was synthesized and conjugated to keyhole limpet hemocyanin, and used to immunize rabbits as described (Ehrhardt and Bell, 1997; Kappes et al., 1992). The resulting antiserum (CSU-α-SCL2A3-22) was titered and validated by detection of sheep SLC2A3 expressed in immortalized wild-type sheep OTR cells (Ali et al., 2020), and cells transduced to overexpress sheep SLC2A3. With both, a single immunoreactive band was detected at an apparent M_r of 50,000. In addition, CSU-α-SCL2A3-22 detected SLC2A3 in human placental extracts, equivalent to an α-human SLC2A3 antisera that we had previously used (Jeckel et al., 2018). Subsequently, 10 μg samples of caruncular or cotyledonary tissue were electrophoresed through NuPAGE 4-12% Bis-Tris Gels (Life Technologies), transferred to nitrocellulose, and the resulting blots stained with Ponceau S to assess total/lane using the ChemiDoc XRS+ (BioRad). Subsequent procedures as are described above, using a 1:1000 dilution of CSU-α-SLC2A3-22, and a 1:5000 dilution of a goat α-rabbit IgG conjugated to horse radish peroxidase (ab97051; Abcam). As described above, densitometry of SLC2A3 was normalized on total protein/lane, using Image Lab Software (BioRad).

Statistical analysis

Data were analyzed by 2-way analysis of variance using GraphPad Prism (8.3.1) to analyze the main effects of treatment and fetal sex as well as the treatment x sex interaction. There were no treatment by fetal sex interactions, potentially due to the limited n/group, therefore the data are presented as the main effect of treatment only. Statistical significance was set at $P \leq 0.05$ and a statistical tendency at $P \leq 0.10$. Data are reported as the mean ± standard error of the mean (SEM). The data figures are presented as scatter plots, with the horizontal line representing the mean, and the capped vertical lines representing the SEM.

RESULTS

Fetal and placental assessments

A summary of all fetal measurements at necropsy (132 dGA) are provided in **Table 2**. CSH RNAi fetal weights, organ weights and measurements of fetal growth did not differ from control RNAi. While measures of fetal growth were unchanged, placental and fetal membrane weights from CSH RNAi pregnancies tended ($P \leq 0.10$) to be lighter compared to control RNAi. Furthermore, uteroplacental weight also tended ($P \leq 0.10$) to be reduced in CSH RNAi pregnancies, while uterine weight was unchanged. Placental efficiency tended ($P \leq 0.10$) to be increased in CSH RNAi pregnancies. Placentome number was not affected by treatment. Reductions in uterine vein CSH concentrations (ng/ml) did not reach statistical significance, but were reduced by 24%, mirroring the reduction (26%) in cotyledonary CSH concentration ($P \leq 0.10$; **Figure 1**).

Table 2.2. Fetal and maternal measurements collected at necropsy (132 dGA).

	Control RNAi (n = 6)	CSH RNAi (n = 6)	% change	P-Value
Fetal body weight, kg	3.74 ± 0.14	3.82 ± 0.19	2.14	0.87
Maternal weight, kg	58.97 ± 1.94	62.75 ± 2.97	6.41	0.31
Uterine vein oCSH, ng/ml	538.87 ± 129.39	406.66 ± 51.75	24.53	0.31
Umbilical vein oCSH, ng/ml	41.90 ± 5.05	40.08 ± 3.58	4.34	0.46
Uteroplacental weight, kg	2.60 ± 0.29	1.99 ± 0.12	23.37	0.10
Total uterus weight, kg	0.64 ± 0.03	0.60 ± 0.04	5.11	0.46
Placental weight, kg	0.43 ± 0.02	0.38 ± 0.01	11.63	0.06
Placental efficiency	8.75 ± 0.50	9.92 ± 0.35	13.37	0.09
Total membrane weight, kg	0.69 ± 0.07	0.49 ± 0.04	29.07	0.06
Total placentomes	69 ± 5	66 ± 7	5.06	0.89
Crown-rump length, cm	50.8 ± 0.59	52.23 ± 0.68	2.81	0.22
Ponderal index*	2.86 ± 0.12	2.67 ± 0.05	6.64	0.17
Lower leg length (BCB), cm	38.7 ± 1.34	35.63 ± 0.63	7.93	0.11
Brain weight:Liver weight	0.45 ± 0.04	0.51 ± 0.05	13.88	0.34

Data are shown as mean values ± SEM for all ewes in each treatment group.
*Ponderal Index = Fetal body weight (g) x 100 / crown-rump length (cm^3).

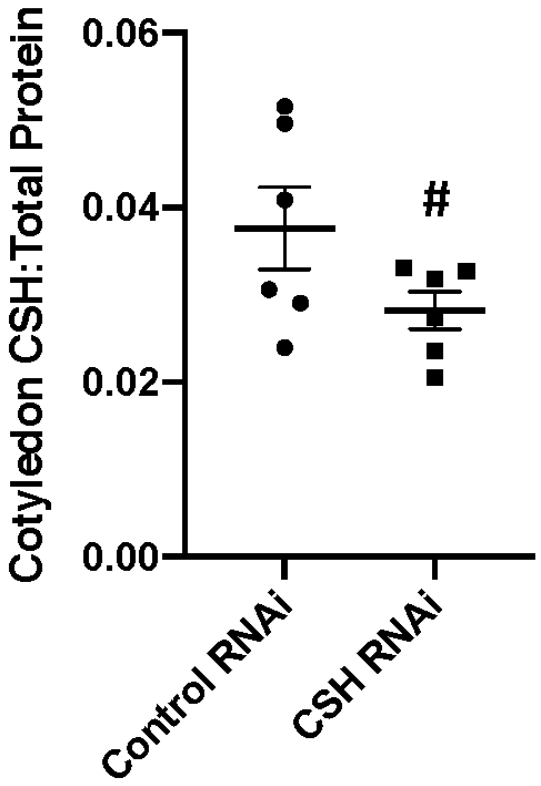


Figure 2.1. Cotyledonary CSH concentration, relative to total protein transferred, in control RNAi (n = 6) vs CSH RNAi (n = 6) pregnancies. Individual data points are presented, with the horizontal line representing the mean, and the capped vertical lines representing the SEM. $^{\#}P \leq 0.10$ for CSH RNAi pregnancies compared with control RNAi pregnancies.

Uterine and umbilical blood flows

At 132 dGA, uterine and umbilical blood flows were determined by the transplacental diffusion technique. Uterine (ml/min) blood flow (**Figure 2**) and uterine blood flow relative to fetal weight (ml/min/kg fetus) were reduced ($P \leq 0.05$) in CSH RNAi pregnancies by 22% and 24% respectively. Uterine blood flow relative to placental weight (435.70 ± 28.28 vs 377.79 ± 24.72 ml/min/100 g placenta; control RNAi vs CSH RNAi, respectively) did not statistically differ ($P > 0.10$) between treatments but was decreased by 13%. A summary of uterine and umbilical plasma flows is presented in **Table 3**. Uterine plasma flow (ml/min) and uterine plasma flow relative to fetal weight (ml/min/kg fetus) were also reduced ($P \leq 0.05$) in CSH RNAi pregnancies, however uterine plasma flow relative to placental weight (ml/min/100 g placenta) did not differ. In contrast, umbilical (ml/min) blood flow (**Figure 3**), umbilical blood flow relative to fetal weight (ml/min/kg fetus), and umbilical blood flow relative to placental weight (187.94 ± 20.77 vs. 204.88 ± 8.44 ml/min/100 g placenta; control RNAi vs CSH RNAi, respectively) were not altered by CSH RNAi. Neither were umbilical plasma flow (**Table 3**) or relative umbilical plasma flow (ml/min/kg fetus) or plasma flow relative to 100 g placental weight (ml/min/100 g placenta). However, the uterine to umbilical flow ratio, tended to be reduced ($P \leq 0.10$) in CSH RNAi pregnancies. Neither uterine nor umbilical hematocrits differed by treatment. To assess potential mediators of altered blood flow, NOS3 was assessed in the maternal caruncles and fetal cotyledons. As evidenced in **Figure 4**, in response to CSH RNAi, caruncular expression of NOS3 was reduced ($P \leq 0.05$) by 24%. In contrast, cotyledonary NOS3 (**Figure 4**) was increased 35% in response to CSH RNAi, but this change did not reach statistical significance ($P \approx 0.14$).

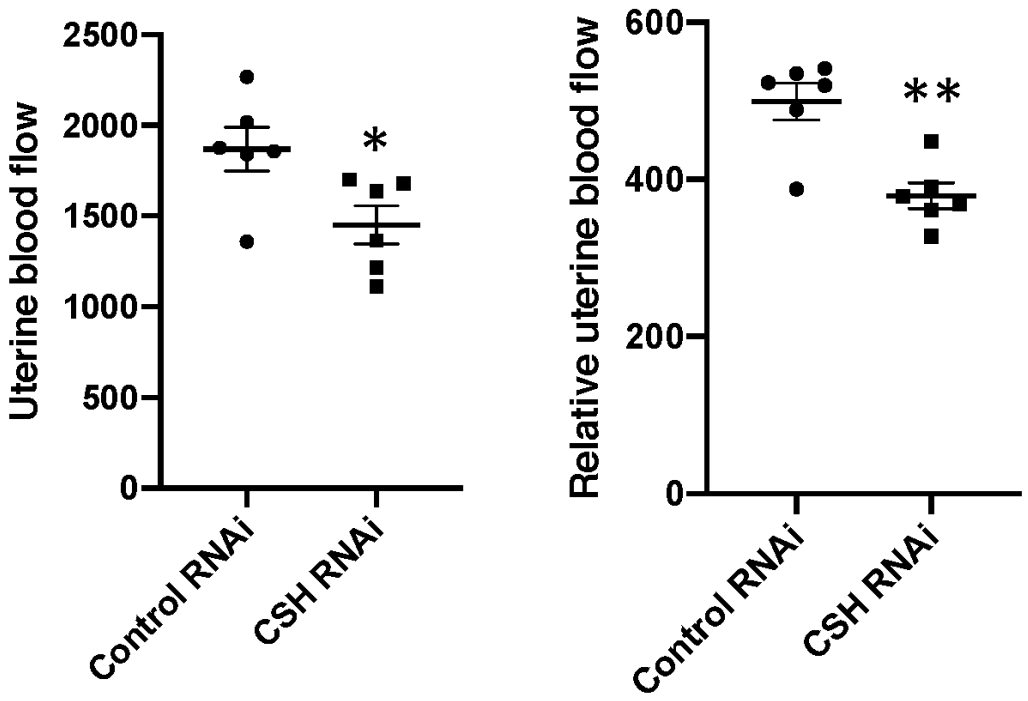


Figure 2.2. Uterine blood flow (mL/min) and relative uterine blood flow (ml/min/kg fetus) in control RNAi (n = 6) vs CSH RNAi (n = 6) pregnancies. Individual data points are presented, with the horizontal line representing the mean, and the capped vertical lines representing the SEM. *$P \leq 0.05$ and **$P \leq 0.01$ when CSH RNAi pregnancies are compared with control RNAi pregnancies.

Table 2.3. Uterine and umbilical plasma flow based upon transplacental diffusion technique.

	Control RNAi (n = 6)	CSH RNAi (n = 6)	% change	*P*-value
Uterine				
Uterine plasma flow (ml/min)	1231.20 ± 79.40	940.59 ± 67.00	23.60	0.02
Uterine plasma flow (ml/min) /kg fetus	328.61 ± 15.18	245.63 ± 10.12	24.02	0.006
Uterine plasma flow (ml/min)/ 100 g placenta	286.95 ± 18.81	244.74 ± 15.90	14.71	0.12
Average uterine arterial hematocrit	0.34 ± 0.00	0.35 ± 0.01	3.07	0.39
Umbilical				
Umbilical plasma flow (ml/min)	533.6 ± 58.98	529.1 ± 37.32	0.80	0.88
Umbilical plasma flow (ml/min)/kg fetus	141.08 ± 10.63	138.97 ± 7.82	1.49	0.80
Umbilical plasma flow (ml/min)/100g placenta	125.48 ± 15.97	137.01 ± 5.97	9.19	0.51
Average umbilical arterial hematocrit	0.34 ± 0.02	0.33 ± 0.01	1.39	0.51
Uterine: Umbilical Flow	2.40 ± 0.19	1.87 ± 0.17	22.21	0.09

Data are shown as mean values ± SEM for all ewes in each treatment group.

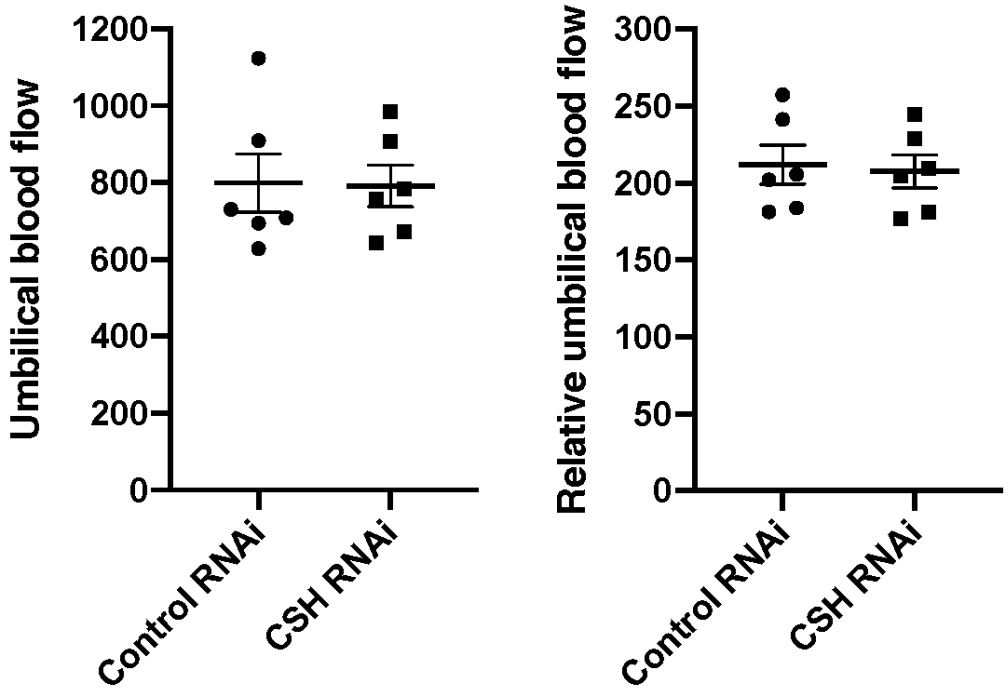


Figure 2.3. Umbilical blood flow (ml/min) and relative umbilical blood flow (ml/min/kg fetus) in control RNAi (n = 6) vs CSH RNAi (n = 6) pregnancies. Individual data points are presented, with the horizontal line representing the mean, and the capped vertical lines representing the SEM.

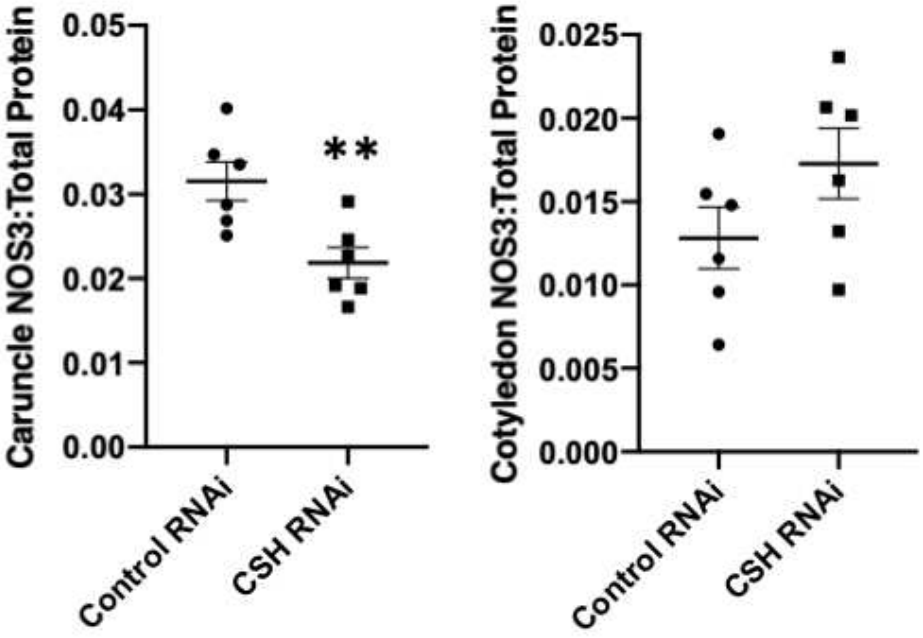


Figure 2.4. Densiometric analysis of NOS3, relative to total protein transferred, in maternal caruncles and fetal cotyledons from control RNAi and CSH RNAi pregnancies (n=6/treatment). Individual data points are presented, with the horizontal line representing the mean, and the capped vertical lines representing the SEM. $^{**}P \leq 0.01$ when CSH RNAi pregnancies are compared with control RNAi pregnancies.

Blood gas and oxygen uptakes

A summary of uterine, umbilical and uteroplacental oxygen uptakes and concentrations can be found in **Table 4**. The uterine artery to vein O_2 concentration gradient (mmol/min) was elevated ($P \leq 0.05$) in response to CSH RNAi. However, due to the reduction in uterine blood flow in CSH RNAi, uterine O_2 uptake was not different. Limited differences were observed in uterine and umbilical blood gas measurements, which are provided in **Table 5 & 6**. Uterine artery methemoglobin levels were elevated ($P \leq 0.05$) in CSH RNAi pregnancies and uterine venous pO_2 was reduced ($P \leq 0.01$**)**. No other uterine or umbilical blood gas measurements were different. The lack of response in oxygen uptake continued on the fetal side, as umbilical uptakes of oxygen did not differ from control RNAi. Uteroplacental O_2 uptakes (mmol/min), oxygen utilization and fraction of oxygen being transferred to the fetus did not change as a result of CSH RNAi.

Table 2.4. *In vivo* measurements of oxygen transfer and uptake based upon the Fick Principal.

	Control RNAi (n = 6)	CSH RNAi (n = 6)	% change	*P*-value
Uterine				
Uterine artery oxygen content (mmol/l)	6.22 ± 0.11	6.58 ± 0.15	5.90	0.14
Uterine vein oxygen content (mmol/l)	4.80 ± 0.09	4.85 ± 0.16	1.04	0.95
Uterine artery to vein oxygen gradient (mmol/l)	1.42 ± 0.08	1.73 ± 0.08	22.35	0.02
Uterine oxygen uptake (mmol/min)	2.63 ± 0.18	2.48 ± 0.12	5.65	0.51
Umbilical				
Umbilical artery oxygen content (mmol/l)	3.11 ± 0.23	3.10 ± 0.11	0.27	0.77
Umbilical vein oxygen content (mmol/l)	4.99 ± 0.21	4.89 ± 0.09	2.09	0.84
Umbilical vein to artery oxygen gradient (mmol/l)	1.88 ± 0.12	1.79 ± 0.09	5.09	0.59
Umbilical oxygen uptake (mmol/min)	1.47 ± 0.06	1.40 ± 0.08	4.43	0.76
Fetal oxygen uptake (mmol/min/kg fetus)	0.39 ± 0.01	0.37 ± 0.02	5.96	0.51
Uteroplacental				
Uteroplacental oxygen uptake (mmol/min)	1.16 ± 0.17	1.08 ± 0.11	7.18	0.61
Uteroplacental oxygen utilization (mmol/min/kg placenta)	2.67 ± 0.33	2.82 ± 0.30	5.65	0.71
Relative placental oxygen transfer (mmol/min/kg)	3.43 ± 0.21	3.65 ± 0.19	6.42	0.20
Uterine oxygen uptake utilized by the placenta	0.43 ± 0.03	0.43 ± 0.03	0.28	0.89
Uterine oxygen uptake transferred to the fetus	0.57 ± 0.03	0.57 ± 0.03	0.21	0.89

Data are shown as mean values ± SEM for all ewes in each treatment group.

Table 2.5. *In vivo* measurements of uterine blood gasses.

	Control RNAi (n = 6)	CSH RNAi (n = 6)	% change	*P*-value
Maternal Artery (A)				
pH $^{37.9C}$	7.46 ± 0.01	7.47 ± 0.01	0.18	0.47
pCO_2 $^{37.9C}$ mmHg	39.17 ± 2.44	33.81 ± 0.79	13.68	0.06
pO_2 $^{37.9C}$ mmHg	86.88 ± 1.67	89.70 ± 1.98	3.24	0.30
tCO_2 (B) mmol/L	22.11 ± 0.97	21.45 ± 0.55	2.98	0.57
tCO_2 (P) mmol/L	25.53 ± 1.14	24.95 ± 0.59	2.30	0.66
HCO^3-	24.54 ± 1.13	24.00 ± 0.58	2.19	0.68
tHb mmol/L	6.84 ± 0.05	7.09 ± 0.15	3.59	0.15
O_2 Hb %	89.74 ± 0.86	91.41 ± 0.82	1.86	0.19
SO_2 %	94.30 ± 0.97	96.38 ± 1.00	2.21	0.17
CO Hb %	3.78 ± 0.12	3.89 ± 0.08	2.98	0.46
Met Hb %	1.070 ± 0.04	1.28 ± 0.08	19.07	0.05
O_2 ct mmol/L	6.22 ± 0.11	6.58 ± 0.15	5.90	0.08
Na^+ meq/L	146.42 ± 0.31	145.96 ± 0.47	0.31	0.43
K^+ meq/L	4.18 ± 0.07	4.10 ± 0.02	1.79	0.35
Ca^{2+} meq/L	2.67 ± 0.09	2.59 ± 0.03	2.70	0.46
Cl^- meq/L	110.88 ± 1.25	111.88 ± 0.51	0.90	0.48
Uterine Vein (V)				
pH $^{37.9C}$	7.43 ± 0.01	7.43 ± 0.01	0.03	0.88
pCO_2 $^{37.9C}$ mmHg	39.08 ± 1.55	40.51 ± 0.69	3.65	0.42
pO_2 $^{37.9C}$ mmHg	57.88 ± .98	53.86 ± 0.73	6.93	0.01
tCO_2 (B) mmol/L	23.65 ± 1.00	22.43 ± 0.84	5.17	0.37
tCO_2 (P) mmol/L	27.10 ± 1.16	26.81 ± 0.55	1.09	0.82
HCO^3-	25.96 ± 1.15	25.68 ± 0.56	1.06	0.83
tHb mmol/L	6.88 ± 0.06	7.09 ± 0.15	3.09	0.21
O_2 Hb %	69.25 ± 0.86	67.12 ± 1.53	3.07	0.26
SO_2 %	71.95 ± 0.92	69.80 ± 1.70	2.99	0.29
CO Hb %	2.74 ± 0.10	2.64 ± 0.08	3.65	0.46
Met Hb %	1.01 ± 0.05	1.16 ± 0.07	14.40	0.11
O_2 ct mmol/L	4.80 ± 0.09	4.85 ± 0.16	1.04	0.79
Na^+ meq/L	146.88 ± 0.39	146.79 ± 0.57	0.06	0.91
K^+ meq/L	4.18 ± 0.07	4.10 ± 0.02	1.70	0.38
Ca^{2+} meq/L	2.62 ± 0.09	2.54 ± 0.03	3.13	0.38
Cl^- meq/L	110.92 ± 1.23	111.25 ± 0.52	0.30	0.81

Data are shown as mean values ± SEM for all ewes in each treatment group.

Table 2.6. *In vivo* measurements of umbilical blood gasses.

	Control RNAi (n = 6)	CSH RNAi (n = 6)	% change	*P*-value
Umbilical artery (α)				
pH $^{37.9C}$	7.36 ± 0.01	7.36 ± 0.00	0.00	1.00
pCO_2 $^{37.9C}$ mmHg	52.13 ± 1.17	50.98 ± 0.35	2.21	0.37
pO_2 $^{37.9C}$ mmHg	20.90 ± 1.45	19.43 ± 0.60	7.04	0.37
tCO_2 (B) mmol/L	26.18 ± 0.97	25.63 ± 0.39	2.10	0.61
tCO_2 (P) mmol/L	29.61 ± 1.32	28.89 ± 0.37	2.42	0.61
HCO^3-	28.17 ± 1.29	27.48 ± 0.36	2.47	0.61
tHb mmol/L	6.77 ± 0.43	6.66 ± 0.19	1.60	0.82
O_2 Hb %	46.50 ± 4.54	62.67 ± 17.56	34.76	0.39
SO_2 %	47.63 ± 4.67	46.88 ± 1.28	1.58	0.88
CO Hb %	2.07 ± 0.22	2.16 ± 0.06	4.44	0.70
Met Hb %	0.28 ± 0.08	0.28 ± 0.03	2.94	0.92
O_2 ct mmol/L	3.11 ± 0.23	3.10 ± 0.11	0.27	0.97
Na^+ meq/L	141.54 ± 0.67	141.58 ± 0.30	0.03	0.96
K^+ meq/L	4.24 ± 0.18	3.78 ± 0.22	10.72	0.14
Ca^{2+} meq/L	2.84 ± 0.05	2.88 ± 0.06	1.26	0.68
Cl^- meq/L	105.58 ± 1.64	106.04 ± 0.92	0.43	0.81
Umbilical vein (γ)				
pH $^{37.9C}$	7.40 ± 0.01	7.40 ± 0.00	0.02	0.88
pCO_2 $^{37.9C}$ mmHg	44.80 ± 1.26	44.13 ± 0.34	1.49	0.62
pO_2 $^{37.9C}$ mmHg	33.33 ± 3.08	28.98 ± 0.92	13.05	0.21
tCO_2 (B) mmol/L	24.46 ± 0.93	24.00 ± 0.43	1.89	0.66
tCO_2 (P) mmol/L	27.74 ± 1.31	27.30 ± 0.41	1.58	0.76
HCO^3-	26.91 ± 1.45	26.04 ± 0.40	3.24	0.58
tHb mmol/L	6.73 ± 0.43	6.62 ± 0.19	1.61	0.82
O_2 Hb %	75.48 ± 5.63	73.42 ± 1.40	2.73	0.73
SO_2 %	78.07 ± 5.78	76.05 ± 1.45	2.59	0.74
CO Hb %	3.38 ± 0.15	3.47 ± 0.07	2.84	0.57
Met Hb %	- 0.05 ± 0.09	- 0.02 ± 0.04	57.14	0.79
O_2 ct mmol/L	4.99 ± 0.21	4.89 ± 0.09	2.09	0.66
Na^+ meq/L	140.58 ± 0.45	141.67 ± 0.58	0.77	0.17
K^+ meq/L	4.28 ± 0.22	3.77 ± 0.23	11.88	0.14
Ca^{2+} meq/L	2.93 ± 0.05	2.94 ± 0.06	0.33	0.91
Cl^- meq/L	106.33 ± 1.36	107.38 ± 1.30	0.98	0.59

Data are shown as mean values ± SEM for all ewes in each treatment group.

Glucose uptakes

Uterine, umbilical and uteroplacental glucose gradients and concentrations are summarized in **Table 7**. Uterine arterial plasma glucose tended to be elevated ($P \leq 0.10$) in CSH RNAi pregnancies, however uterine venous glucose was unchanged. The uterine artery to vein glucose gradient (mmol/L) was elevated ($P \leq 0.01$) in CSH RNAi pregnancies, yet the uterine uptake of glucose was not altered (**Table 7**; **Figure 5**). CSH RNAi had no effect on umbilical plasma arterial or venous glucose concentrations as well as the umbilical vein to artery glucose gradient. Due to the lack of response to CSH RNAi on umbilical blood flow and the umbilical glucose gradient, the umbilical uptake of glucose (**Figure 5**) was not impacted by CSH RNAi. In contrast to the uterine and umbilical measures, CSH RNAi resulted in a 27% increase in ($P \leq 0.05$) uteroplacental glucose uptake relative to placental weight (μmol/min/kg; **Figure 5**) and a tendency ($P \leq 0.10$) for uteroplacental glucose uptake (μmol/min) to be increased (**Table 7**). This observation is supported by the increased fraction of uterine glucose uptake being utilized by the placenta ($P \leq 0.05$; **Figure 6**) in CSH RNAi pregnancies. Since more glucose was actually being utilized by the placenta, the fraction of uterine glucose uptake being transferred to the fetus was decreased ($P \leq 0.05$; **Figure 6**). To further assess the changes in glucose uptake and transport, SLC2A1 and SLC2A3 concentrations in maternal caruncles and fetal cotyledons were determined. As evidenced in **Figure 7**, caruncular SLC2A1 concentration was not impacted by CSH RNAi, but cotyledonary SLC2A1 tended ($P \leq 0.10$) to be reduced (25%). By contrast, there appeared to be no significant impact of CSH RNAi on SLC2A3 concentration (**Figure 8**) in either the maternal caruncles or fetal cotyledons.

Table 2.7. *In vivo* measurements of glucose transfer and uptake based upon the Fick Principal.

	Control RNAi (n = 6)	CSH RNAi (n = 6)	% change	*P*-value
Uterine				
Uterine artery plasma glucose concentration (mmol/l)	3.40 ± 0.08	3.68 ± 0.10	8.24	0.10
Uterine vein plasma glucose concentration (mmol/l)	3.15 ± 0.09	3.31 ± 0.08	5.17	0.30
Uterine artery to vein plasma glucose concentration (mmol/l)	0.25 ± 0.02	0.37 ± 0.03	48.00	0.006
Uterine glucose uptake (µmol/min)	431.77 ± 44.27	479.08 ± 34.16	10.96	0.42
Umbilical				
Umbilical artery plasma glucose concentration (mmol/l)	0.85 ± 0.05	0.92 ± 0.07	8.24	0.35
Umbilical vein plasma glucose concentration (mmol/l)	1.06 ± 0.06	1.11 ± 0.07	4.72	0.55
Umbilical vein to artery plasma concentration (mmol/l)	0.22 ± 0.02	0.19 ± 0.01	13.64	0.22
Umbilical glucose uptake (µmol/min)	155.70 ± 12.37	139.29 ± 10.47	10.54	0.34
Uterine glucose uptake transferred to the fetus	0.37 ± 0.04	0.29 ± 0.02	21.54	0.05
Uteroplacental				
Uterine artery-umbilical vein glucose gradient (mmol/l)	2.53 ± 0.11	2.78 ± 0.07	9.88	0.14
Uteroplacental glucose uptake (µmol/min)	276.07 ± 40.40	339.79 ± 28.57	23.08	0.06
Uterine glucose uptake utilized by the placenta	0.63 ± 0.04	0.71 ± 0.02	12.92	0.05

Data are shown as mean values ± SEM for all ewes in each treatment group.

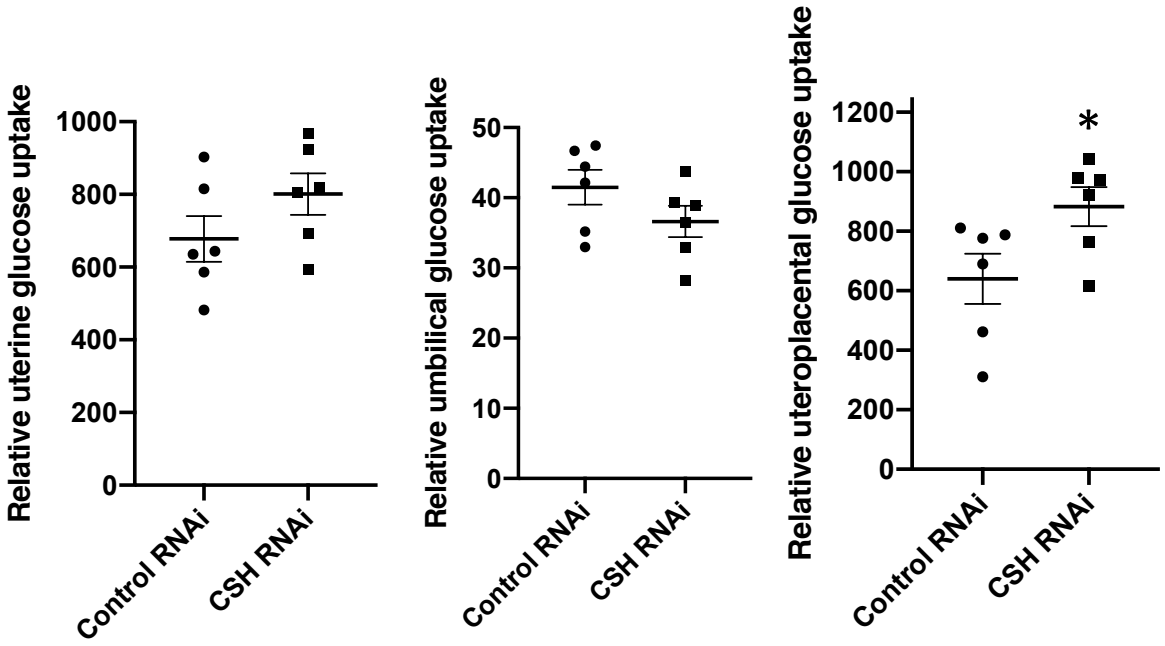


Figure 2.5. Relative uterine (µmol/min/kg uterus), umbilical (µmol/min/kg fetus) and uteroplacental (µmol/min/kg placenta) uptakes of glucose in control RNAi (n = 6) vs CSH RNAi (n = 6) pregnancies. Individual data points are presented, with the horizontal line representing the mean, and the capped vertical lines representing the SEM. *$P \leq 0.05$ when CSH RNAi pregnancies are compared with control RNAi pregnancies.

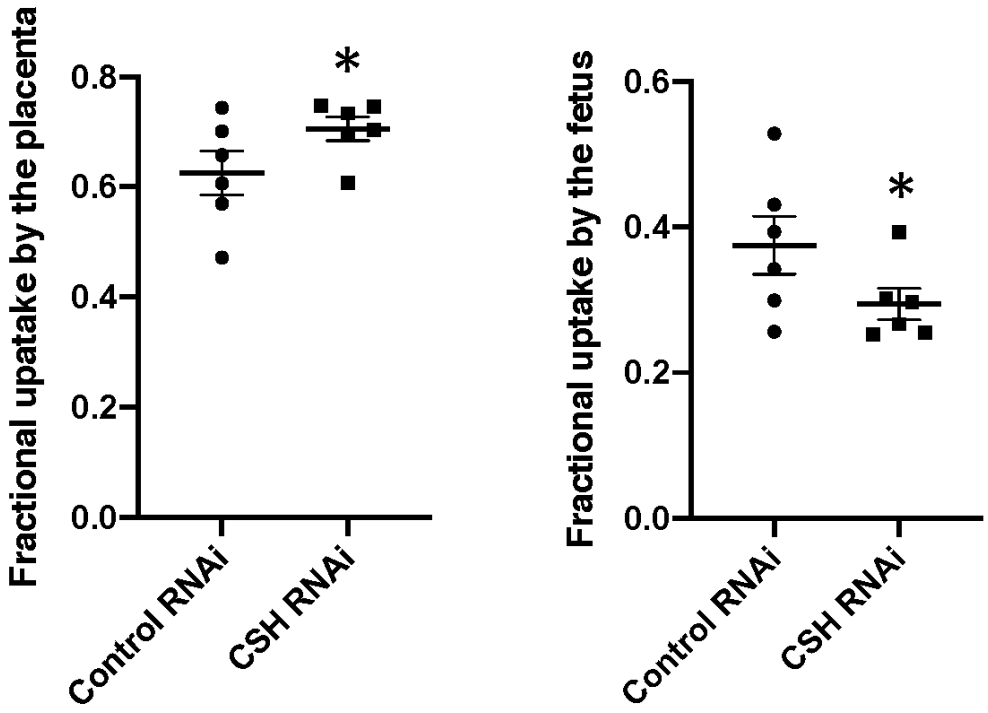


Figure 2.6. Fraction of uterine uptake of glucose being utilized by the placenta vs. transferred to the fetus in control RNAi (n = 6) vs CSH RNAi (n = 6) pregnancies.. Individual data points are presented, with the horizontal line representing the mean, and the capped vertical lines representing the SEM. *$P \leq 0.05$ when CSH RNAi pregnancies are compared with control RNAi pregnancies.

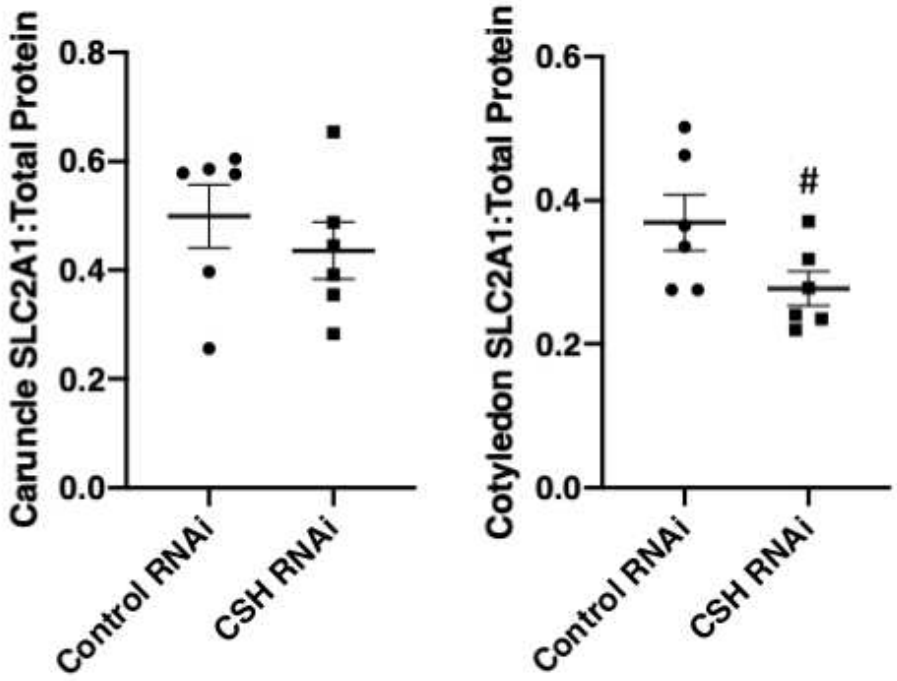


Figure 2.7. Densiometric analysis of SLC2A1, relative to total protein transferred, in maternal caruncles and fetal cotyledons from control RNAi and CSH RNAi pregnancies (n=6/treatment). Individual data points are presented, with the horizontal line representing the mean, and the capped vertical lines representing the SEM. $^{\#}P \leq 0.10$ when CSH RNAi pregnancies are compared with control RNAi pregnancies.

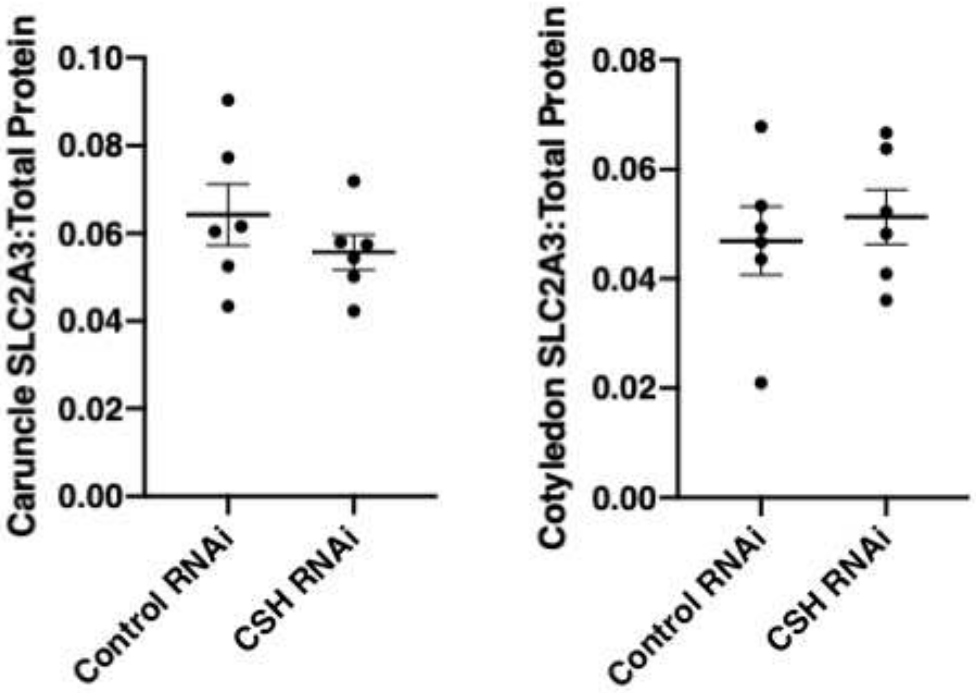


Figure 2.8. Densiometric analysis of SLC2A3, relative to total protein transferred, in maternal caruncles and fetal cotyledons from control RNAi and CSH RNAi pregnancies (n=6/treatment). Individual data points are presented, with the horizontal line representing the mean, and the capped vertical lines representing the SEM.

Uptake of other nutrients

Uterine, umbilical, and uteroplacental lactate uptakes and concentrations are provided in **Table 8**. CSH RNAi pregnancies tended ($P \leq 0.10$) to have decreased total placental lactate production as well as umbilical lactate uptake relative to placental mass (μmol/kg/min). However, no other differences were observed in lactate uptakes or concentrations. Individual amino acid uptakes by the uterine and umbilical vasculature are provided in **Tables 9, 10 & 11** Relative uterine (μmol/min/kg uterine weight) uptake of taurine and glycine was reduced ($P \leq 0.05$) in CSH RNAi pregnancies, without significant changes in the uterine uptake of other amino acids. Only relative uteroplacental (μmol/min/kg placenta) glycine uptake was reduced ($P \leq 0.05$), with no changes in the uteroplacental utilization of other amino acids. CSH RNAi did not significantly impact relative umbilical uptake (μmol/min/kg fetus) of any amino acids.

Table 2.8. *In vivo* measurements of lactate transfer and uptake based upon the Fick Principal.

	Control RNAi (n = 6)	CSH RNAi (n = 6)	% change	*P*-value
Uterine				
Uterine artery lactate content (mmol/l)	0.79 ± 0.07	0.80 ± 0.04	1.20	0.82
Uterine vein lactate content (mmol/l)	0.84 ± 0.07	0.85 ± 0.04	1.76	0.80
Uterine vein to artery lactate gradient (mmol/l)	0.05 ± 0.01	0.06 ± 0.01	10.37	0.87
Uterine lactate uptake (µmol/min)	91.46 ± 12.17	79.86 ± 16.45	12.68	0.47
Umbilical				
Umbilical artery lactate content (mmol/l)	2.23 ± 0.24	2.15 ± 0.17	3.64	0.38
Umbilical vein lactate content (mmol/l)	2.40 ± 0.25	2.29 ± 0.14	4.47	0.33
Umbilical vein to artery lactate gradient (mmol/l)	0.17 ± 0.02	0.15 ± 0.01	15.04	0.22
Umbilical lactate uptake (µmol/min)	135.84 ± 10.10	116.28 ± 9.84	14.40	0.20
Umbilical lactate uptake from placenta (µmol/kg/min)	36.31 ± 2.22	30.44 ± 2.30	16.18	0.07
Umbilical lactate: oxygen quotient	0.28 ± 0.02	0.25 ± 0.02	10.99	0.11
Uteroplacental				
Lactate production per kg of placenta (µmol/min/kg)	530.97 ± 36.07	510.01 ± 32.44	3.95	0.61
Lactate transferred per kg placenta (µmol/kg/min)	315.29 ± 18.86	302.41 ± 25.75	4.09	0.72
Total placental lactate production (µmol/min)	227.3 ± 14.48	196.13 ± 14.70	13.71	0.10
Uteroplacental lactate production transferred to uterine vein	0.40 ± 0.04	0.39 ± 0.06	0.63	0.93
Uteroplacental lactate production transferred to the fetus	0.60 ± 0.04	0.61 ± 0.06	0.41	0.93

Data are shown as mean values ± SEM for all ewes in each treatment group.

Table 2.9. Uterine amino acid uptake relative to uterine weight (μmol/min/kg uterus).

	Control RNAi (n = 6)	CSH RNAi (n = 6)	% change	*P*-value
TAU Amount	1.90 ± 1.24	-1.95 ± 1.04	203.01	0.04
ASP Amount	-0.88 ± 0.39	-1.17 ± 0.25	32.83	0.55
M.S. Amount	0.87 ± 0.82	0.25 ± 0.71	71.36	0.58
THR Amount	24.89 ± 2.66	23.31 ± 2.91	6.35	0.70
SER Amount	15.27 ± 2.63	19.49 ± 3.67	27.61	0.37
ASPG Amount	7.75 ± 1.35	6.39 ± 1.92	17.54	0.58
GLU Amount	1.00 ± 1.43	-0.51 ± 1.43	150.88	0.47
GLN Amount	48.34 ± 6.66	35.87 ± 3.71	25.80	0.13
PRO Amount	18.22 ± 4.59	8.00 ± 5.80	56.09	0.20
GLY Amount	9.43 ± 5.14	-12.14 ± 6.42	228.83	0.03
ALA Amount	24.60 ± 4.91	22.92 ± 2.88	6.83	0.77
CIT Amount	29.43 ± 5.46	18.32 ± 2.59	37.76	0.10
a-ABA Amount	0.99 ± 0.68	0.67 ± 0.48	31.73	0.72
VAL Amount	44.62 ± 8.50	41.25 ± 7.02	7.55	0.77
CYS Amount	1.93 ± 1.21	0.60 ± 1.29	68.91	0.47
MET Amount	6.30 ± 0.91	5.92 ± 1.46	6.02	0.83
ILEU Amount	23.72 ± 3.92	25.00 ± 3.78	5.39	0.82
LEU Amount	32.26 ± 4.86	32.12 ± 6.72	0.45	0.99
TYR Amount	7.76 ± 1.47	8.47 ± 3.04	9.19	0.84
PHE Amount	8.43 ± 2.25	5.82 ± 2.23	30.97	0.43
TRP Amount	5.04 ± 2.71	4.25 ± 1.30	15.59	0.80
ORNI Amount	20.10 ± 3.19	15.36 ± 3.64	23.58	0.35
LYS Amount	20.38 ± 3.87	16.09 ± 2.80	21.06	0.39
n-M-LYS Amount	-0.93 ± 5.43	0.88 ± 1.84	194.18	0.76
HIS Amount	8.26 ± 1.32	7.69 ± 1.11	6.93	0.75
3-MeHIS Amount	2.06 ± 1.54	-0.30 ± 0.33	114.36	0.16
ARG Amount	22.33 ± 4.86	16.51 ± 4.11	26.09	0.38

Data are shown as mean values ± SEM for all ewes in each treatment group.

Table 2.10. Umbilical amino acid uptake relative to fetal weight (μmol/min/kg fetus).

	Control RNAi (n = 6)	CSH RNAi (n = 6)	% change	*P*-value
TAU Amount	0.39 ± 0.39	0.53 ± 0.37	33.71	0.81
ASP Amount	-0.04 ± 0.05	-0.07 ± 0.06	84.46	0.70
M.S. Amount	-0.07 ± 0.29	0.00 ± 0.20	100.50	0.85
THR Amount	3.15 ± 0.70	3.09 ± 0.48	1.94	0.94
SER Amount	-1.45 ± 0.98	0.02 ± 1.19	101.44	0.36
ASPG Amount	1.56 ± 0.11	1.69 ± 0.13	8.25	0.46
GLU Amount	-5.68 ± 0.73	-4.40 ± 0.72	22.45	0.24
GLN Amount	10.08 ± 0.93	10.00 ± 0.96	0.72	0.96
PRO Amount	2.36 ± 0.34	1.79 ± 0.36	24.45	0.27
GLY Amount	3.74 ± 0.52	3.85 ± 0.55	2.88	0.89
ALA Amount	3.66 ± 0.65	4.12 ± 0.49	12.60	0.58
CIT Amount	0.27 ± 0.33	0.28 ± 0.32	1.90	0.99
a-ABA Amount	0.04 ± 0.37	0.26 ± 0.09	590.51	0.57
VAL Amount	4.06 ± 0.49	4.93 ± 0.61	21.49	0.29
CYS Amount	0.31 ± 0.10	0.41 ± 0.07	28.77	0.46
MET Amount	1.26 ± 0.31	1.45 ± 0.21	15.06	0.62
ILEU Amount	2.62 ± 0.24	2.72 ± 0.25	3.86	0.77
LEU Amount	4.12 ± 0.22	4.20 ± 0.35	2.08	0.84
TYR Amount	1.34 ± 0.26	1.90 ± 0.29	41.75	0.18
PHE Amount	1.39 ± 0.14	1.59 ± 0.16	14.12	0.38
TRP Amount	0.47 ± 0.08	0.51 ± 0.14	9.76	0.78
ORNI Amount	0.54 ± 0.39	0.36 ± 0.22	33.48	0.69
LYS Amount	3.74 ± 0.63	4.00 ± 0.49	7.00	0.75
n-M-LYS Amount	0.09 ± 0.38	0.48 ± 0.19	427.21	0.38
HIS Amount	0.79 ± 0.08	1.05 ± 0.15	33.18	0.17
3-MeHIS Amount	-0.03 ± 0.10	0.18 ± 0.16	802.57	0.30
ARG Amount	3.23 ± 0.29	3.36 ± 0.28	4.00	0.76

Data are shown as mean values ± SEM for all ewes in each treatment group.

Table 2.11. Uteroplacental amino acid utilization relative to placental weight (μmol/min/kg placenta).

	Control RNAi (n = 6)	CSH RNAi (n = 6)	% change	*P*-value
TAU Amount	-1.20 ± 3.99	-8.84 ± 5.00	638.94	0.26
ASP Amount	-1.2 ± 0.87	-1.22 ± 0.78	1.59	0.99
M.S. Amount	1.14 ± 3.38	0.50 ± 2.11	56.26	0.88
THR Amount	7.96 ± 6.36	5.84 ± 6.20	26.71	0.82
SER Amount	33.48 ± 10.29	29.58 ± 15.89	11.66	0.84
ASPG Amount	-2.47 ± 2.10	-6.79 ± 4.18	174.31	0.38
GLU Amount	51.82 ± 7.39	42.29 ± 8.11	18.39	0.41
GLN Amount	-18.63 ± 13.56	-41.64 ± 13.38	123.49	0.25
PRO Amount	6.35 ± 7.49	-5.48 ± 7.89	186.37	0.30
GLY Amount	-19.66 ± 8.73	-56.78 ± 11.66	188.83	0.03
ALA Amount	3.17 ± 8.32	-5.79 ± 5.38	282.61	0.39
CIT Amount	41.02 ± 8.57	25.49 ± 5.03	37.86	0.15
a-ABA Amount	0.96 ± 3.39	-1.64 ± 1.15	270.52	0.48
VAL Amount	29.08 ± 10.62	15.03 ± 14.16	48.32	0.45
CYS Amount	-0.14 ± 2.08	-3.12 ± 2.17	2066.66	0.35
MET Amount	-1.80 ± 3.16	-5.22 ± 2.47	189.87	0.41
ILEU Amount	11.40 ± 4.69	12.40 ± 6.11	8.75	0.90
LEU Amount	10.51 ± 6.13	8.50 ± 9.28	19.07	0.86
TYR Amount	-0.43 ± 2.68	-5.99 ± 4.72	1289.85	0.33
PHE Amount	-0.17 ± 2.85	-6.80 ± 3.83	3972.69	0.19
TRP Amount	3.21 ± 3.95	1.37 ± 2.68	57.47	0.71
ORNI Amount	24.24 ± 4.09	20.24 ± 7.71	16.50	0.66
LYS Amount	-4.20 ± 8.77	-13.52 ± 6.99	222.29	0.42
n-M-LYS Amount	-4.42 ± 6.61	-3.80 ± 1.90	13.98	0.93
HIS Amount	4.88 ± 1.80	1.75 ± 2.11	64.26	0.28
3-MeHIS Amount	3.35 ± 2.55	-2.30 ± 1.94	168.56	0.11
ARG Amount	5.61 ± 8.72	-7.07 ± 7.57	226.05	0.30

Data are shown as mean values ± SEM for all ewes in each treatment group.

Total nutrient uptakes

Uterine and umbilical carbon and nitrogen uptakes as well as nutrient to oxygen quotients are provided in **Table 12**. Overall, uterine carbon and nitrogen uptakes did not differ between CSH RNAi and control RNAi pregnancies. In CSH RNAi pregnancies, umbilical carbon and nitrogen uptake from amino acids tended ($P \leq 0.10$) to be increased but those effects were canceled out as carbon uptake from lactate tended ($P \leq 0.10$) to be decreased. Umbilical carbon uptake from glucose was not altered by treatment. While minor changes in the proportion of carbon sources being taken up by the umbilicus between different oxidative sources might exist, the total carbon uptake and the nutrient quotients available for oxidative metabolism did not differ between treatments

Table 2.12. Umbilical and uterine carbon, nitrogen and nutrient uptakes.

	Control RNAi (n = 6)	CSH RNAi (n = 6)	% change	*P*-value
Uterine				
Uterine glucose carbon uptake	2590.60 ± 265.60	2874.49 ± 204.96	10.96	0.81
Uterine lactate carbon uptake	647.04 ± 91.64	622.80 ± 125.53	3.75	0.52
Uterine amino acid carbon uptake	326.56 ± 48.09	248.81 ± 42.38	23.81	0.17
Uterine carbon uptake	3564.21 ± 199.94	3746.11 ± 326.32	5.10	0.52
Uterine nitrogen uptake	105.09 ± 15.37	75.71 ± 11.86	27.95	0.26
Umbilical				
Umbilical amino acid carbon uptake	211.91 ± 23.58	237.61 ± 28.13	12.13	0.07
Umbilical glucose carbon uptake	248.89 ± 14.86	219.68 ± 13.32	11.73	0.31
Umbilical lactate carbon uptake	108.93 ± 6.67	91.31 ± 6.91	16.18	0.07
Total fetal carbon uptake	569.73 ± 30.45	548.6 ± 30.51	3.71	0.71
Umbilical nitrogen uptake	70.11 ± 7.84	76.66 ± 8.79	9.34	0.10
Umbilical glucose: oxygen quotient	0.63 ± 0.03	0.62 ± 0.04	2.26	0.41
Umbilical lactate: oxygen quotient	0.28 ± 0.02	0.25 ± 0.02	10.99	0.11
Umbilical amino acid: oxygen quotient	0.57 ± 0.06	0.68 ± 0.06	18.48	0.45
Total umbilical nutrient quotient	1.48 ± 0.07	1.54 ± 0.09	4.13	0.25

Data are shown as mean values ± SEM for all ewes in each treatment group.

DISCUSSION

Baker et al. (2016) previously reported two distinct phenotypes associated with lentiviral-mediated CSH RNAi in near term pregnancies (135 dGA). The first phenotype had placental weight values 2 SDs below the mean of control pregnancies. In those pregnancies, significant reductions in fetal body weight (32%) and placental weight (52%) occurred. The second phenotype of “nonresponder pregnancies” did not exhibit the same degree of reductions in fetal and placental weight. These dual phenotypes agree with observations in human pregnancies. Daikoku et al. (1979) reported suppressed maternal CSH concentrations in IUGR pregnancies at 25-28 weeks of gestational age and again from 33-40 weeks. Pregnancies with reduced CSH yielded babies ≤3% of birth weight which tracks with the CSH RNAi IUGR phenotype described in sheep (Baker et al., 2016). Interestingly, Daikoku et al. (1979) also observed that some pregnancies had suppressed maternal CSH from 33-40 weeks of gestational age but gave birth to non-low birth weight pregnancies. This second phenotype mimics what was observed in the non-IUGR CSH-deficient phenotype in the Baker et al. (2016) study, as well as in the present study. In fact, numerous case studies report CSH loci deletions or mutations that either result in IUGR pregnancies (Borody and Carlton, 1981; Rygaard et al., 1998; Sideri et al., 1983) or non-IUGR phenotypes (Alexander et al., 1982; Barbieri et al., 1986; Simon et al., 1986). One study reported up to 40% of pregnancies that had low maternal levels of CSH had no obvious obstetric abnormality (related to birth weight; Lindberg and Nilsson, 1973). More studies are needed to investigate the true prevalence of human IUGR and non-IUGR outcomes associated with CSH deficiencies, with emphasis placed on whether or not pregnancies and/or

offspring are truly physiologically normal. This need is highlighted by our recent report (Ali et al., 2020) that the CSH RNAi pregnancies from the Baker et al. (2016) study exhibited significant changes in umbilical concentrations of IGF1 and elevated fetal liver concentrations of the insulin receptor. These recent data (Ali et al., 2020) imply that the progression of fetal development was altered by CSH RNAi, even in the absence of placental and fetal growth restriction.

While it is clear that two phenotypes can emerge in spite of a deficiency in CSH, no studies have yet to examine the physiological ramifications of CSH deficiency on uteroplacental function. This is the rationale for creating sheep pregnancies with CSH deficiency based on RNAi. Larger uterine artery-vein glucose gradients in both the IUGR and non-IUGR CSH RNAi pregnancies compared to control pregnancies (Ali et al., 2020; Baker et al., 2016) suggest the potential for metabolic perturbations in response to CSH deficiency regardless of fetal weight outcome. Therefore, the main objective of this study was to examine the impacts of CSH RNAi in the absence of IUGR on placental and fetal physiology. Our results support the idea that CSH RNAi impairs uteroplacental function even in the absence of IUGR.

In the present study, fetal weights did not differ between treatments, yet uteroplacental, placental and fetal membrane weights tended to be reduced as a result of CSH RNAi. Experimentally, one factor that might explain the milder fetal growth phenotype in this study compared to the previous report (Baker et al., 2016) is a lower efficiency in CSH RNAi in this cohort. While uterine vein CSH and cotyledonary CSH concentrations were reduced by approximately 25%, in the Baker et al. (2016) study, the CSH RNAi pregnancies with IUGR had a 38% reduction in cotyledonary CSH

concentrations. Several factors can change RNAi efficacy including integration rate of RNAi vector, length of time between initiation of RNAi and sample harvest, and effective competition for RISC complexes (Huppi et al., 2005; Kim and Rossi, 2008; Koller et al., 2006). Additionally, if CSH RNAi was more robustly active during placental development, it is possible that a more severe phenotype would be produced like the ones we observed previously (Baker et al., 2016; Jeckel et al., 2018). Unfortunately, no feasible methods exist to measure RNAi expression *in vivo* across pregnancy without terminating those pregnancies prior to their experimental endpoints. Another difficulty in determining CSH concentrations is the well documented variability in CSH secretion (Bauer et al., 1995; Butler et al., 1987; Taylor et al., 1980). In the current cohort, uterine and umbilical vein samples were only collected at time of metabolic study (132 dGA). Since CSH in circulation can fluctuate rapidly and lacks circadian rhythms for secretion (Bauer et al., 1995; Butler et al., 1987; Taylor et al., 1980), increased sampling frequency and duration maybe warranted. It is also worth noting that in two different models of IUGR in sheep (Lea et al., 2007; Regnault et al., 1999), maternal CSH was significantly depressed in the absence of any change in cotyledonary CSH mRNA or protein concentrations. This infers that there is not always a direct correlation between CSH mRNA and protein concentrations, and what is secreted, suggesting that there is still a deficit in our knowledge of CSH regulation and secretion.

Even in the absence of IUGR, as a result of CSH RNAi, physiological changes were observed in this study. Previous studies have attempted to associate CSH concentrations and blood flow. In pregnant women, Gitsch et al. (1980) and Qaio et al. (1995) observed concomitant decreases in CSH in maternal circulation and decreases

in placental perfusion or uteroplacental blood flow in cases of pregnancy related hypertension or placental insufficiency. However, in goats (Hayden et al.,1983), uterine blood flow was measured simultaneously with blood sampling to examine CSH concentrations, and no direct relationship in short-term uterine blood flow was observed. In the present study, we found that even in the absence of growth restriction, profound reductions in uterine blood and plasma flow occur as a result of CSH RNAi suggesting a potential role for CSH in modulating uterine blood flow. To our knowledge, this is the first study to examine the direct impact of CSH deficiency on uterine and umbilical blood flow and supports the concept that even in the absence of IUGR, CSH-deficient pregnancies have altered uteroplacental function. We also determined that CSH RNAi resulted in significant reductions in maternal caruncle NOS3 concentration, which coincides with the reduction in uterine blood flow, as the relationship between NOS3 and uterine blood flow has been demonstrated previously (Kulandavelu et al., 2012; Rosenfeld et al., 1996). *NOS3* null mice exhibit major reductions in uterine blood flow during mid-to late-gestation (Kulandavelu et al., 2012). This relationship was also observed in sheep using a NOS-specific antagonist (L-NAME) to investigate estrogen-mediated effects on NOS and subsequent changes in uterine blood flow (Rosenfeld et al., 1996). It is possible that the impact of CSH RNAi on uterine blood flow and NOS3 is indirect, and may be mediated by placental steroids. In contrast to uterine blood flow, there were no significant changes in umbilical blood flow. Perhaps, in more severe cases of CSH deficiency, where IUGR is apparent (Baker et al., 2016; Jeckel et al., 2018), umbilical blood flows might be decreased as well.

Glucose is a key oxidative substrate for the fetus and many IUGR pregnancies are characterized by a reduction in placental glucose transport (Hay, 2006). Even in the absence of fetal growth restriction, CSH RNAi pregnancies exhibited altered uteroplacental glucose uptake. The maternal artery-uterine vein gradient was greater in CSH RNAi pregnancies which agrees with previous findings (Ali et al., 2020; Baker et al., 2016). However, the uterine uptake of glucose was not statistically different, likely due to the decrease in uterine blood flow in CSH RNAi pregnancies. Maternal caruncle SLC2A1 concentrations were not significantly impacted by CSH RNAi, but fetal cotyledon concentrations of SLC2A1 was reduced 25%, similar to what we observed with CSH RNAi pregnancies exhibiting fetal growth restriction (Jeckel et al., 2018). Neither caruncular or cotyledonary SLC2A3 concentrations were significantly altered in these CSH RNAi pregnancies, which is in contrast to the significant reduction of cotyledonary SLC2A3 observed in growth restricted CSH RNAi pregnancies (Jeckel et al., 2018). This might infer a compensatory maintenance of SLC2A3 in response to CSH RNAi in our non-growth restricted pregnancies, not dissimilar to what was observed in late-onset IUGR human pregnancies (Janzen et al., 2013) The placenta is a highly metabolic organ and can consume upwards of 65% of the glucose it transports for its own oxidative needs (Battaglia and Meschia, 1986; Hay et al., 2003; Morris et al., 1973). In this study, relative uteroplacental utilization of glucose was increased by 27% in CSH RNAi pregnancies but the umbilical uptake of glucose was not altered. This may suggest that CSH deficiency, at least in the absence of IUGR, is actually increasing the glucose demands of the placenta. This is supported by the increased fraction of uterine glucose uptake being utilized by the placenta and reductions in the fraction of uterine

glucose being transferred to the fetus. We have previously reported (Jeckel et al., 2018) altered placental gene expression in response to CSH RNAi. Perhaps in more severe cases of IUGR as a result of CSH deficiency, placental glucose consumption increases to the point where it can no longer support normal fetal growth. However, in order to address this question, IUGR phenotypes in response to CSH RNAi need to be studied in the same fashion.

In this cohort of CSH RNAi pregnancies, in the absence of IUGR, uteroplacental glucose uptake was significantly increased, suggesting metabolic perturbations. Adult onset disease risk is increased in offspring due to *in utero* exposure to adverse events, leading to long-term irreversible changes in physiology and metabolism (Barker and Osmond, 1988; Barker et al., 1990; Barker, 1990; Hales and Barker, 2001). In sheep, a variety of models exist to examine the impacts of placental insufficiency and/or altered fetal growth ranging from elevated core body temperatures, maternal inflammation, uteroplacental embolization, uterine artery ligation or occlusion, carunclectomies, and nutritional manipulation (Anthony et al., 2003). Sheep models of nutritional manipulation have been followed postnatally to study fetal programming consequences in adulthood (Ford and Long, 2011). When ewes were either overfed (150% NRC from -60 dpc to term) or underfed (50% NRC from 28-78 dGA then realimented to 100% NRC from 78 dGA to term), their phenotypes diverged at mid-gestation (78 dGA) with overfed having fetuses larger than controls and underfed smaller than controls (Ford and Long, 2011). However, both phenotypes then converged by late gestation and had similar lamb weights at birth (Ford and Long, 2011). In adulthood, offspring from both over and underfed dams displayed compensatory growth, increased insulin resistance, and

increased adiposity (Ford and Long, 2011). These effects proved to be multigenerational as F2 offspring from overfed ewes also had increased adiposity, glucose and insulin at birth (Shasa et al., 2015). In the current study, changes in uteroplacental glucose utilization and uterine blood flow, as well as changes in uterine A-V glucose gradients show evidence of metabolic perturbations. Taken together with our previous studies (Ali et al., 2020; Baker et al., 2016; Jeckel et al., 2018), it appears that CSH deficiency could predispose lambs to fetal programming outcomes regardless of birth weight. Furthermore, it potentially raises a concern about the adult outcomes of human pregnancies in the normal birthweight range, following a CSH-deficient pregnancy.

CONCLUSIONS

While CSH RNAi did not result in significant reductions in near-term fetal weight in this cohort, this was the first study to examine the direct impacts of *in vivo* CSH RNA interference on *in vivo* uteroplacental nutrient transport and hemodynamics. We established the relationship between CSH RNAi and reduced uterine blood flow and increased placental glucose utilization. This study highlights that significant physiological changes may occur during pregnancy without noticeable impacts on birthweight, likely predisposing the offspring to altered health and well-being. By examining *in vivo* placental physiology and fetal development in both IUGR and non-IUGR pregnancies in the future, we may potentially define the cause and effect relationship between CSH and normal pregnancy physiology.

CHAPTER III: CSH RNA INTERFERENCE REDUCES GLOBAL NUTRIENT UPTAKE AND UMBILICAL BLOOD FLOW RESULTING IN INTRAUTERINE GROWTH RESTRICTION

INTRODUCTION

Intrauterine growth restriction (IUGR), the second-leading cause of perinatal mortality, results from a failure of the fetus to reach its growth potential *in utero*, and impacts up to 6% of pregnancies worldwide (Gagnon, 2003). This pathology has also been linked epidemiologically to the development of adult-onset diseases such as cardiovascular disease, diabetes, and hypertension (Barker, 1988; Barker et al., 1990; Barker, 1990; Hales and Barker, 2011). While many causes of IUGR remain unknown, deficiency of the placental hormone chorionic somatomammotropin (CSH) has been associated with fetal growth restriction in both humans (Daikoku et al., 1979; Spellacy et al., 1976) and sheep (Lea et al., 2007). This relationship was directly demonstrated by our laboratory, utilizing lentiviral mediated RNA interference (RNAi) in the sheep placenta (Baker et al., 2016). Using this model, CSH RNAi resulted in fetal and placental growth restriction in near-term (135 dGA) pregnancies (Baker et al., 2016), and by the end of the first third (50 dGA) of pregnancy (Jeckel et al., 2018). However, CSH deficiency does not always result in IUGR, a phenomenon which is also documented in human pregnancies (Daikoku et al., 1979; Rygaard et al., 1998). We have previously described this normal weight phenotype in response to CSH RNAi, which is characterized by decreased umbilical IGF1 (Ali et al., 2020), reduced uterine blood flow and increased placental glucose utilization (Tanner et al., 2021a). These

perturbations, in spite of normal fetal and placental weights, support the necessity of CSH for maintaining adequate uterine blood flow and regulating placental glucose consumption. Additionally, these CSH dependent effects hint at potential mechanisms that could be responsible for the development of IUGR in more severe CSH RNAi phenotypes. Thus, to better understand the actions of CSH in modulating fetal growth, our objective was to assess the physiological ramifications of CSH RNAi induced IUGR, to ascertain how CSH deficiency leads to the progression of fetal and placental growth restriction. We hypothesized that CSH RNAi induced IUGR results from altered uterine blood flow and nutrient uptake. It was determined that CSH RNAi resulted in decreased uterine and umbilical blood flows, reduced global nutrient transport and altered fetal IGF1 concentrations, driving the development of IUGR.

METHODS & MATERIALS

All animal procedures were approved by the Colorado State University Institutional Animal Care and Use Committee (Protocol # 18-7866A), the Institutional Biosafety Committee (18-029B) and the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee (Protocol #00714).

Lentiviral generation

Lentiviral generations of hLL3.7 tg6 (target 6; CSH RNAi) and hLL3.7 NTS (scramble control/non-targeting sequence; control RNAi) are summarized in **Table 1** as described previously (Baker et al., 2016; Jeckel et al., 2018; Tanner et al., 2021a). Briefly, both the NTS and tg6 sequences (Baker et al., 2016; Jeckel et al., 2018; Tanner

et al., 2021a) were cloned into the LL3.7 vector, and all subsequent virus generation and titration were completed in accordance with our previously described procedures (Baker et al., 2016).

Table 3.1. Scrambled control and CSH-targeting shRNA sequences.

Oligonucleotide	Sequence (5′-3′)
NTS shRNA sense	GAGTTAAAGGTTCGGCACGAATTCAAGAGATTCGTGCCGAACCTTTAACTC
tg6 shRNA sense	AAGGCCAAAGTACTTGTAGACTTCAAGAGAGTCTACAAGTACTTTGGCCTT

Generation of CSH RNAi pregnancies

All ewes (Dorper breed composition) were group housed in pens at the Colorado State University Animal Reproduction and Biotechnology Laboratory, and were provided access to hay, trace mineral, and water in order to meet or slightly exceed their National Research Council (2007) requirements. All animal procedures were conducted as previously described (Baker et al., 2016; Jeckel et al., 2018; Tanner et al., 2021a). In summary, after synchronization and subsequent breeding, fully expanded and hatched blastocysts were collected by flushing the uteri at 9 days post conception. Each blastocyst was infected with 100,000 transducing units of either NTS/control RNAi or CSH RNAi virus. After infection for approximately 5 hours, each blastocyst was washed and a single blastocyst was transferred surgically (Baker et al., 2016; Jeckel et al., 2018; Tanner et al., 2021a) into a synchronized recipient ewe. Each recipient ewe was monitored daily for return to standing estrus, and confirmed pregnant at 50 days of gestational age (dGA) by ultra-sound (Mindray Medical Equipment, Mahwah, NJ). Using these methods, 6 Control RNAi and 6 CSH RNAi pregnancies were generated.

Doppler velocimetry

At 90 dGA, all pregnancies (n = 12) underwent Doppler velocimetry using a Mindray M7 Premium (Mindray) with a convex C5-2s probe (2.1-5.1 MHz) abdominal transducer by a single technician. All ewes were examined supine in a recumbent position. After confirming fetal viability, fetal binocular distance (cm), biparietal circumference (cm), abdominal circumference (cm), femur (cm) and tibia length (cm) were recorded and averaged across 3 independent measurements. Pulse-waved

Doppler velocimetry measurements of the umbilical artery were performed with color flow Doppler with an angle of insonation of 30 degrees (Galan et al., 1998; Galan et al., 2005). Doppler indices of perfusion including pulsatility indices (PI; Table 2) and resistance indices (RI; Table 2), as well as a systolic:diastolic ratio (S/D), were recorded on 3 independent sets of 3 cardiac cycles (minimum of 9 cardiac waveforms) and averaged (Galan et al., 1998; Galan et al., 2005). Fetal heart rate was also recorded as a 3 cardiac cycle average. Umbilical blood flow (mL/min) was calculated by time-averaged mean velocity (cm/s) x π/4 x vessel cross-sectional area (cm^2) x 60. One control RNAi ewe had umbilical hemodynamic measurements excluded due to failure to achieve a 30-degree angle of insonation, but all fetal measurements from that animal were included.

Surgical instrumentation of fetus and ewe

At approximately 115 dGA, pregnant recipient ewes were transported to the University of Colorado Anschutz Medical Campus, Perinatal Research Center (Aurora, CO). Animals had access to ad libitum alfalfa pellets (Standlee Hay, Kimberly, ID) and water. All animals (6 = control RNAi and 6 = CSH RNAi pregnancies) underwent surgical placement of fetal and maternal catheters at 126 dGA, to determine blood flow and nutrient flux as previously described (Bonds et al., 1980; Brown et al., 2015; Hay et al., 1981; Jones et al., 2019; Regnault et al., 2003; Tanner et al., 2021a). Briefly, the following catheters were placed: fetal descending aorta (representing umbilical artery blood), fetal femoral vein and umbilical vein, maternal femoral artery (representing uterine artery blood), maternal femoral vein, and uterine vein. Due to two fetal demises

(one CSH RNAi and one control RNAi) and catheters failing to draw on the study day, a total of four control RNAi and four CSH RNAi animals completed the full study and were included in the final analysis. Fetal sex was balanced between both treatments, with 3 males and 1 female in each treatment group.

Blood flow calculations and tissue collection

At 130 dGA, uterine and umbilical blood flows were determined by the steady state $^{3}H_2O$ transplacental diffusion technique, as summarized previously (Tanner et al., 2021a). Briefly, samples were simultaneously collected from the maternal femoral artery (A), uterine vein (V), umbilical vein (γ) and fetal descending aorta (α) every 20 minutes and averaged across 4 draws for analysis of blood biochemistry, nutrient content, $^{3}H_2O$ and hormone concentrations (Tanner et al., 2021a).

As described extensively in **Table 2**, uterine and umbilical blood flows were calculated by the transplacental diffusion technique described previously (Tanner et al., 2021a; Meschia et al., 1966; Cilvik et al., 2021). Uterine, umbilical, and uteroplacental utilization of oxygen, glucose, lactate and amino acids were calculated by the transplacental diffusion technique (Meschia et al., 1980) and reported as an average of draws one through four. All other calculations were described extensively in Tanner et al. (2021a).

Ewes and fetuses were euthanized, and tissues were harvested at 130 dGA as previously described by Tanner et al. (2021a). Briefly, after trimming, placentomes were selected from each placenta and separated into cotyledonary (fetal) and caruncular (maternal) components, then snap frozen in liquid N2 and stored at -80ºC. Fetal weight

and dissected organ weights were recorded, and tissues were snap frozen in liquid nitrogen. Ponderal index and fetal brain:liver weight ratios were calculated, as previously described (Tanner et al., 2021a).

Table 3.2. Calculations[1] for blood flow, nutrient uptake, nutrient utilization, and quotients.

Blood flow (Doppler ultrasound)	
Pulsatility index	(PSV – EDV)/ Timed-average mean velocity
Resistance index	(PSV-EDV)/PSV
Umbilical Blood Flow (mL/min)	TAMV * (π/4) * Umb A cross-sectional area * 60

Blood Flow (^3H_2O tracer)	
R_{inf} ^3H_2O (dpm/min)	Pump rate * [infusate]
$R_{acc(f)}$ (dpm/min)	α_{pl} slope * (0.8 * fetal weight)
$R_{acc(m)}$ (dpm/min)	$R_{acc(f)}$ + [α_{pl} slope * 0.8(uterine weight)]
Umbilical Blood Flow (UBF; mL/min)	$(R_{inf} – R_{acc(f)}) / ([^3H_2O]_{\alpha(WB)} - [^3H_2O]_{\gamma(WB)})$
Umbilical Plasma Flow (UPF; mL/min)	UBF * [1 – $Hct_{f(avg)}$]
Uterine Blood Flow (UtBF; mL/min)	$(R_{inf} – R_{acc(m)}) / ([^3H_2O]_{V(WB)} - [^3H_2O]_{A(WB)})$
Uterine Plasma Flow (UtPF; mL/min)	UtBF * [1 – $Hct_{m(avg)}$]

Nutrient Uptake and Utilization Rates	
Umbilical Oxygen Uptake (fetal oxygen utilization; UOU; mmol/min)	UBF * $([O_2]_{\gamma(WB)} – [O_2]_{\alpha(WB)})$
Uterine Oxygen Uptake (UtOU; mmol/min)	UtBF * $([O_2]_{A(WB)} – [O_2]_{V(WB)})$
Uteroplacental Oxygen Utilization (mmol/min)	UtOU – UOU
Plasma to WB Glucose Conversion	$[G]_{pl}$ * [1 – (0.24 * Hct)] – (3.3 * Hct)
Umbilical Glucose Uptake (UGU; µmol/min)	UBF * $([G]_{\gamma(WB)} – [G]_{\alpha(WB)})$
Uterine Glucose Uptake (UtGU; µmol/min)	UtBF * $([G]_{A(WB)} – [G]_{V(WB)})$
Uteroplacental Glucose Utilization (µmol/min)	UtGU – UGU
Umbilical Lactate Uptake (µmol/min; ULU)	UBF * $([L]_{\gamma(pl)} – [L]_{\alpha(pl)})$
Uterine Lactate Secretion (µmol/min; UtLS)	UtBF * $([L]_{V(pl)} – [L]_{A(pl)})$
Uteroplacental Lactate Production (µmol/min)	ULU + UtLS
Umbilical AA Uptake (µmol/min; UAAU)	UPF * $([AA]_{\gamma(pl)} – [AA]_{\alpha(pl)})$
Uterine AA Uptake (µmol/min; UtAAU)	UtPF * $([AA]_{A(pl)} – [AA]_{V(pl)})$
Umbilical AA Carbon Uptake (µmol/min; UCU)	(#AA carbons) * UAAU
Uterine AA Carbon Uptake (µmol/min; UtCU)	(#AA carbons) * UtAAU
Umbilical AA Nitrogen Uptake (µmol/min; UNU)	(#AA nitrogens) * UAAU
Uterine AA Nitrogen Uptake (µmol/min; (UtNU)	(#AA nitrogens) * UtAAU

Fetal Nutrient:Oxygen Quotients	
Glucose:Oxygen (G:O) quotient	6 * $([G]_{\gamma(WB)} – [G]_{\alpha(WB)}) / ([O_2]_{\gamma(WB)} – [O_2]_{\alpha(WB)})$
Lactate:Oxygen (L:O) quotient	3 * $([L]_{\gamma(pl)} – [L]_{\alpha(pl)}) / ([O_2]_{\gamma(WB)} – [O_2]_{\alpha(WB)})$
Amino Acid:Oxygen (AA:O) quotient	Q * $([AA]_{\gamma(pl)} – [AA]_{\alpha(pl)}) / ([O_2]_{\gamma(WB)} – [O_2]_{\alpha(WB)})$
Total Nutrient:Oxygen quotient	G:O quotient + L:O quotient + Total AA:O quotient

[1]Calculations are derived from Cilvik et al., 2021.

Biochemical analysis of blood samples

Whole blood O_2 content, hemoglobin O_2 saturation (SO_2) partial pressure of oxygen (PO_2), partial pressure of carbon dioxide (PCO_2), pH and hematocrit measurements were analyzed by an ABL 800 Blood Gas analyzer, as previously described (Tanner et al., 2021a). Plasma glucose and lactate were measured by Yellow Springs Instrument 2900 (YSI Incorporated, Yellow Springs, OH), as described previously (Tanner et al., 2021a). Plasma amino acids were measured by HPLC (Tanner et al., 2021a). Maternal (uterine) and fetal (umbilical) concentrations of insulin, IGF1, and cortisol were assessed by enzyme-linked immunosorbent assay (ALPCO Immunoassays 80-INSOV-E01, 22-IGFHU-E01, and 11-CORHU-E01-SLV, respectively), as described previously (Andrews et al., 2015; Benjamin et al., 2017; Cilvik et al., 2021). Estradiol was analyzed by radioimmunoassay, as described by Gonzalez-Padilla et al. (1975).

Western blot analysis

Protein isolation and analysis was conducted in accordance with methods described previously (Tanner et al., 2021a). Cotyledonary or caruncular tissue (100 mg) was lysed in 500 µL of lysis buffer and sonicated on ice. For analysis of cotyledonary CSH, 5 µg of protein was electrophoresed through a 4%–15% Tris-Glycine Stain-Free gel (BioRad, Hercules, Cali-fornia) and transferred via a Trans-Blot Turbo semi-dry transfer system (Bio-Rad) to a 0.20-µm pore nitrocellulose membrane. Total protein per lane was visualized using the ChemiDoc XRS+ chemiluminescence system (BioRad) to use for normalization. To visualize CSH, the blot was incubated in a 1:25,000 dilution

(in 5% Non-Fat Dry Milk / 1X Tris-Bis Solution+ 1% Tween) of rabbit α- oPL-S4 (Kappes et al., 1992) for 24 h at 4ºC. After washing the membrane, the membrane was transferred into a 1:5,000 dilution (in 5% Non-Fat Dry Milk / 1X Tris-Bis Solution+ 1% Tween) of mouse α-rabbit IgG conjugated to horse radish peroxidase (ab-97051; Abcam, Cambridge, MA). Nitrocellulose membranes were developed using an ECL Western Blotting Detection Reagent chemiluminescent kit (Amersham, Pittsburgh, PA) and imaged using the ChemiDoc XRS+ chemiluminescence system. Densitometry calculations were performed using Image Lab Software (BioRad).

For analysis of caruncular and cotyledonary concentrations of NOS3, 20 μg of each sample was electrophoresed through 4%–15% Tris-glycine stain-free gels (Bio-Rad), transferred and analyzed as described for CSH. NOS3 was detected using a 1:2,000 di-lution of mouse α-NOS3 (BD 610297; BD Biosciences, San Jose, CA) and a 1:5,000 dilution of goat α-mouse IgG conjugated to horseradish peroxidase (sc-2005; Santa Cruz Bio-technology Inc., Dallas, TX). For analysis of caruncular and cotyledonary (5 μg/sample) concentrations of SLC2A1 (GLUT1), samples were electrophoresed, transferred, and analyzed as described above. SLC2A1 was detected using a 1:40,000 dilution of rabbit α-SLC2A1 (07–1401; EMD Millipore) and a 1:80,000 dilution of goat α-rabbit IgG conjugated to horseradish peroxidase (ab205718; Abcam). As described above, densitometry of SLC2A1 was normalized on total protein/lane. Analysis of SLC2A3 (GLUT3) was conducted as previously detailed in Tanner et al. (2021a). Briefly, 10 μg samples of caruncular or cotyledonary tissue were electrophoresed through NuPAGE 4%–12% Bis-Tris Gels (Life Technologies), transferred to nitrocellulose, and the resulting blots were stained with Ponceau S to

assess total protein per lane using the ChemiDoc XRS+ (Bio-Rad). Subsequent procedures were as described above, using a 1:1,000 dilution of CSU-α-SLC2A3-22, and a 1:5,000 dilution of a goat α-rabbit IgG conjugated to horseradish peroxidase (ab97051; Abcam). All densitometry was conducted in accordance with the description above.

Statistical analysis

Data were analyzed by Student's t-test in GraphPad Prism (8.3.1). Due to the limited number of females in each treatment group (n = 1), fetal sex x treatment interactions were not examined. Statistical significance was set at $P \leq 0.05$ and a statistical tendency at $P \leq 0.10$. Data are reported as the mean ± standard error of the mean (SEM).

RESULTS

All equations used to calculate blood flow, nutrient uptake, nutrient utilization, and nutrient quotients are summarized in **Table 2**.

90 dGA Doppler velocimetry

As assessed by Doppler ultrasound and velocimetry, fetal binocular distance (cm), biparietal circumference (cm), abdominal circumference (cm), femur length (cm), and tibia length (cm) did not differ ($P \geq 0.10$; **Table 3**) between treatments. Umbilical blood flow (mL/min) was reduced ($P = 0.05$; **Figure 1**) by 36% in CSH RNAi pregnancies. Umbilical artery cross sectional area (CSA, cm^2) and cross-sectional

diameter (CSD, cm) both tended (P ≤ 0.10; **Table 3**) to be reduced in CSH RNAi pregnancies. Resistance indices (RI), pulsatility indices (PI), systolic/diastolic ratios (S/D ratios), and fetal heart rate (bpm) did not differ between treatments.

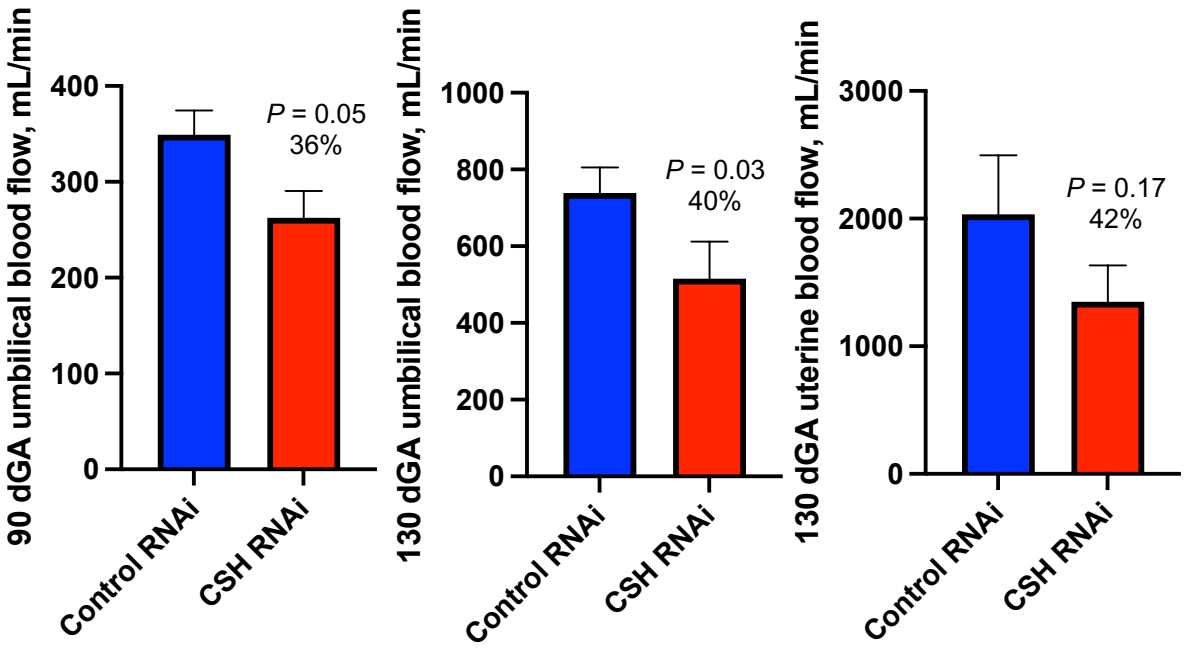


Figure 3.1. Umbilical and uterine blood flows (mL/min) as assessed by Doppler ultrasound at 90 dGA and $^{3}H_{2}0$ transplacental diffusion at 130 dGA. Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference.

Table 3.3. Measures of blood flow and fetal growth as assessed by 90 dGA Doppler velocimetry and 130 dGA $^3H_20$ transplacental diffusion.

	CON RNAi	CSH RNAi	% change	P-value
90 dGA Doppler ultrasound measurements	**(n = 6)**	**(n = 6)**		
Binocular distance, cm	4.91 ± 0.25	4.63 ± 0.19	5.70	0.48
Biparietal circumference, cm	16.56 ± 0.63	15.11 ± 0.54	8.72	0.19
Abdominal circumference, cm	22.03 ± 0.95	20.30 ± 1.27	7.82	0.40
Femur length, cm	4.24 ± 0.08	4.17 ± 0.09	1.81	0.62
Tibia length, cm	3.05 ± 0.11	3.03 ± 0.04	0.75	0.87
Pulsatility Index	1.98 ± 0.16	2.04 ± 0.14	2.88	0.84
Resistance Index	0.69 ± 0.04	0.70 ± 0.02	1.12	0.89
Systolic:Diastolic	3.63 ± 0.34	3.59 ± 0.28	0.97	0.95
Fetal heart rate, bpm	190.48 ± 3.38	199.81 ± 8.91	4.90	0.48
Umbilical artery cross-sectional area, cm^2	0.24 ± 0.02	0.18 ± 0.02	25.23	0.09
Umbilical artery cross-sectional diameter, cm	0.55 ± 0.02	0.47 ± 0.03	15.31	0.08
130 dGA transplacental diffusion blood flow measurements	**(n = 4)**	**(n = 4)**		
Uterine plasma flow (mL/min)	1417.74 ± 328.48	807.99 ± 208.40	43.01	0.17
Relative uterine blood flow (mL/min/kg fetus)	500.07 ± 86.44	400.98 ± 59.35	19.81	0.38
Relative uterine plasma flow (mL/min/kg fetus)	348.20 ± 61.93	274.78 ± 41.11	21.09	0.36
Uterine blood flow/100 g placenta	429.05 ± 54.03	320.80 ± 59.32	25.23	0.23
Umbilical plasma flow (mL/min)	490.84 ± 52.03	293.18 ± 63.87	40.27	0.05
Relative umbilical blood flow (mL/min/kg fetus)	185.35 ± 8.81	155.61 ± 11.26	16.04	0.08
Relative umbilical plasma flow (mL/min/kg fetus)	123.17 ± 8.27	101.08 ± 12.74	17.93	0.20
Umbilical blood flow/100 g placenta	163.27 ± 14.51	123.84 ± 13.63	24.15	0.09
Uterine: umbilical blood flow	2.68 ± 0.37	2.58 ± 0.37	3.74	0.85
Average umbilical arterial hematocrit	0.34 ± 0.02	0.36 ± 0.04	6.25	0.65
Average uterine arterial hematocrit	0.30 ± 0.01	0.31 ± 0.02	2.94	0.64

Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference.

130 dGA Uterine and umbilical blood flows

As calculated by the transplacental diffusion technique, uterine blood (**Figure 1**) and plasma flows (mL/min; **Table 3**) in CSH RNAi pregnancies were not statistically different between treatments, nor were relative uterine blood or plasma flow (mL/min/kg fetus or mL/min/100 g placenta) different between treatments (**Table 3**). While caruncular endothelial nitric oxide synthase (NOS3) was numerically reduced by 38% in CSH RNAi pregnancies, this difference was not statistically significant (P = 0.38; **Figure 2**).

Total umbilical (**Figure 1**) and plasma blood flows (mL/min) were reduced (P ≤ 0.05; **Table 3**) by 40% in CSH RNAi pregnancies. Umbilical blood flow relative to fetal or placental weight (mL/min/kg fetus and mL/min/100 g placenta) both tended to be reduced (P ≤ 0.10) in CSH RNAi pregnancies, but umbilical plasma flow relative to fetal weight was not different. Cotyledonary NOS3 was numerically elevated by 59% (P = 0.11; **Figure 2**). Neither the uterine to umbilical blood flow ratio nor uterine and umbilical hematocrits were significantly altered by treatment.

Fetal and uteroplacental characteristics near-term (130 dGA)

Fetal weight was reduced (P = 0.04; Figure 3) by 30% by CSH RNAi, but the fetal measurements (**Table 4**) of crown-rump length (cm) and ponderal index did not differ between treatments. Fetal hindlimb leg length (cm) however, was reduced (P = 0.02) in CSH RNAi fetuses. Fetal liver weights tended (P = 0.10; **Table 4**) to be reduced in CSH RNAi pregnancies, with right liver lobe mass tending to be lighter (P = 0.06) by 37%. Fetal heart weight was also numerically smaller by 23% (P = 0.11) in CSH RNAi

fetuses, with left ventricular weights significantly reduced (P = 0.05) by 26%. Fetal brain weight did not differ (P = 0.68) between treatments, but brain weight relative to fetal weight was in-creased (P = 0.03) by 43% in CSH RNAi fetuses, an indicator of asymmetric fetal growth. Fetal muscle mass including the biceps femoris, soleus, flexor digitorum superficialis (FDS), tibialis anterior (TA), and extensor digitorum longus (EDL) were all reduced (P ≤ 0.05) in CSH RNAi fetuses.

Placental weight of CSH RNAi pregnancies was not different from controls (**Figure 3**), however uterine weights were 43% smaller (P = 0.03; **Figure 3**) compared with control pregnancies. Uteroplacental weights also tended (P = 0.09; **Table 4**) to be reduced by 27% in CSH RNAi pregnancies. Fetal membrane weight and placentome number were not significantly altered by CSH RNAi. Cotyledonary CSH was numerically reduced by 36% in CSH RNAi placentae, but did not reach statistical significance (P = 0.17; **Figure 4**).

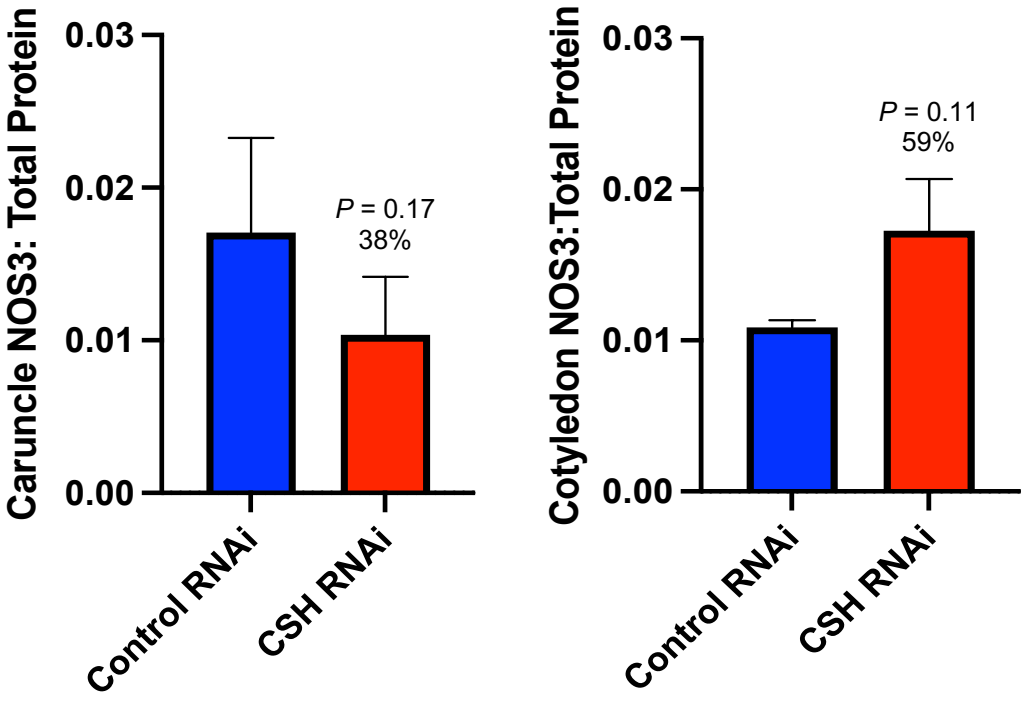


Figure 3.2. Caruncular and cotyledonary NOS3 protein concentrations relative to total protein. Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference.

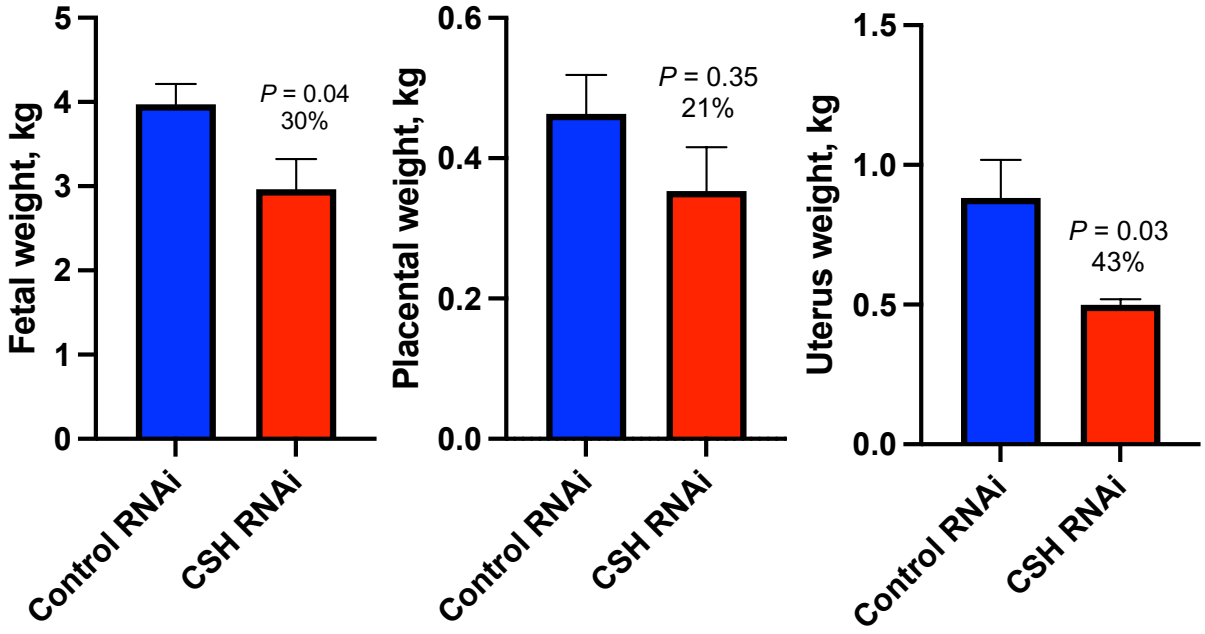


Figure 3.3. Fetal and uteroplacental measurements. Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference.

Table 3.4. Fetal and maternal measurements collected at necropsy (130 dGA).

	CON RNAi	CSH RNAi	% change	P-value
Fetal weights, growth parameters	**(n = 4)**	**(n = 4)**		
Crown-rump length, cm	49.98 ± 1.67	45.73 ± 2.10	8.50	0.16
Ponderal index	3.19 ± 0.15	2.86 ± 0.24	10.48	0.28
Lower leg length, cm	37.25 ± 1.16	32.38 ± 1.15	13.09	0.02
Brain, g	47.71 ± 1.21	46.05 ± 3.72	3.48	0.69
Brain: fetal weight	0.0121 ± 0.000	0.0172 ± 0.002	42.63	0.03
Liver, g	100.33 ± 9.13	65.50 ± 15.80	34.71	0.10
Brain: liver	0.45 ± 0.03	0.79 ± 0.21	73.65	0.17
Liver: fetal weight	0.0267 ± 0.001	0.0247 ± 0.004	7.64	0.62
Left liver lobe, g	27.19 ± 4.53	17.74 ± 4.07	34.76	0.17
Right liver lobe, g	75.46 ± 3.24	47.88 ± 11.71	36.54	0.06
Heart, g	22.48 ± 1.79	17.28 ± 2.14	23.15	0.11
Heart: fetal weight	0.0056 ± 0.000	0.0063 ± 0.001	12.29	0.23
Left ventricle, g	9.02 ± 0.81	6.68 ± 0.48	25.91	0.05
Right ventricle, g	4.99 ± 0.32	3.81 ± 0.70	23.56	0.18
Lungs, g	123.18 ± 8.76	101.61 ± 15.44	17.51	0.27
Lungs: fetal weight	0.031 ± 0.001	0.0364 ± 0.001	17.31	0.01
Pancreas, g	3.25 **±** 0.33	2.40 ± 0.42	26.21	0.16
Kidneys, g	20.05 ± 1.21	15.61 ± 2.33	22.17	0.14
Perirenal adipose tissue (PRAT), g	13.36 ± 1.96	10.58 ± 1.23	20.80	0.27
Spleen, g	6.48 ± 0.58	5.24 ± 1.24	19.24	0.40
Adrenal glands, g	0.32 ± 0.02	0.34 ± 0.06	4.38	0.86
Biceps femoris (BF), g	28.69 ± 1.22	17.97 ± 2.19	37.35	0.01
Soleus, g	0.35 ± 0.06	0.14 ± 0.04	60.28	0.02
Flexor digitorum superficialis (FDS), g	3.08 ± 0.27	1.93 ± 0.31	37.45	0.03
Tibialis anterior (TA), g	3.8 ± 0.35	2.37 ± 0.32	37.59	0.02
Extensor digitorum longus (EDL), g	1.01 ± 0.10	0.59 ± 0.10	42.22	0.03
Uteroplacental weight, g	1829.40 ± 136.67	1333.65 ± 207.93	27.10	0.09
Membrane weight, g	483.55 ± 32.18	470.28 ± 112.73	2.75	0.91
Total placentome #	67.25 ± 4.09	71.75 ± 10.09	6.69	0.69

Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference.

Blood gas and oxygen uptakes

As calculated by the $^{3}H_2O$ transplacental diffusion technique, uterine oxygen uptake (mmol/min) was reduced (P = 0.05; **Figure 5**) by 43% in CSH RNAi pregnancies, but not on a relative basis (mmol/min/kg uterus; **Table 5**). Umbilical oxygen uptake (mmol/min) was reduced (P = 0.02; **Figure 5**) by 37% in CSH RNAi pregnancies, with relative (mmol/min/kg fetus) oxygen uptakes also tending (P = 0.07) to be suppressed. Uteroplacental oxygen utilization, both absolute (mmol/min; **Figure 5**) and relative (mmol/min/kg placenta; **Table 5**) were numerically lower (P = 0.12) in CSH RNAi pregnancies, but did not reach statistical significance. Uterine artery and vein blood gas and biochemistry measurements were not altered by CSH RNAi, (**Table 6**), but both umbilical artery and vein oxygen content (O_2 ct; mmol/L) tended to be reduced (P ≤ 0.10; **Table 7**).

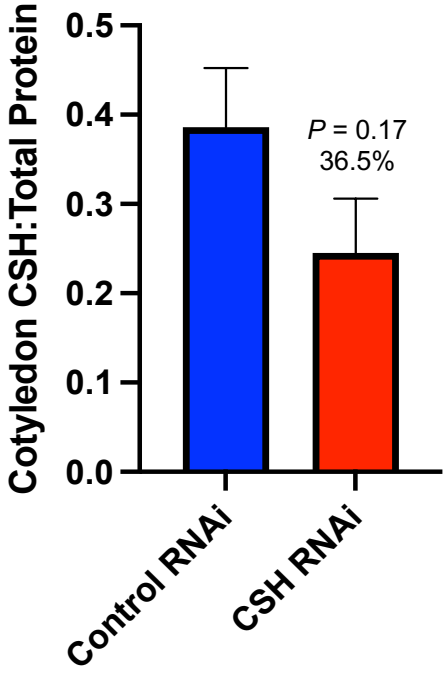


Figure 3.4. Densiometric analysis of CSH, relative to total protein transferred, in fetal cotyledons from control RNA interference (RNAi) and chorionic somatomammotropin (CSH) RNAi pregnancies (n = 4/treatment). Data are shown as means ± SEM for all pregnancies in each treatment group.

Table 3.5. *In vivo* measurements of nutrient transfer and uptake.

	CON RNAi	CSH RNAi	% change	P-value
130 dGA nutrient uptake	**(n = 4)**	**(n = 4)**		
Relative umbilical oxygen uptake (mmol/min/kg fetus)	0.33 ± 0.01	0.29 ± 0.02	12.25	0.07
Relative uterine oxygen uptake (mmol/min/kg uterus)	3.18 ± 0.54	2.96 ± 0.45	6.93	0.76
Relative uteroplacental oxygen utilization (mmol/min/kg placenta)	2.78 ± 0.42	1.90 ± 0.25	31.61	0.12
Relative umbilical glucose uptake (μmol/min/kg fetus)	34.07 ± 2.98	24.75 ± 2.70	27.36	0.06
Glucose transferred per placental weight (μmol/kg/min)	301.82 ± 41.64	199.24 ± 32.59	33.99	0.10
Relative uterine glucose uptake (μmol /min/kg uterus)	515.33 ± 85.74	461.76 ± 70.98	10.39	0.65
Relative uteroplacental glucose utilization (μmol/min/kg placenta)	634.00 ± 59.81	472.72 ± 76.49	25.44	0.15
Umbilical lactate uptake (μmol/min)	123.16 ± 5.97	75.52 ± 16.11	38.68	0.03
Relative umbilical lactate uptake (μmol/min/kg fetus)	31.23 ± 1.78	26.26 ± 2.37	15.91	0.14
Uterine lactate secretion (μmol/min)	134.17 ± 1.78	-80.41 ± 154.00	159.93	0.22
Relative uterine lactate secretion (μmol/min/kg uterus)	301.02 ± 49.60	-518.48 ± 692.82	272.24	0.28
Uteroplacental lactate production (μmol/min)	257.33 ± 16.19	-4.89 ± 168.14	101.90	0.17
Relative uteroplacental lactate production (μmol/min/kg placenta)	576.83 ± 68.83	-311.58 ± 705.10	154.02	0.26

Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference.

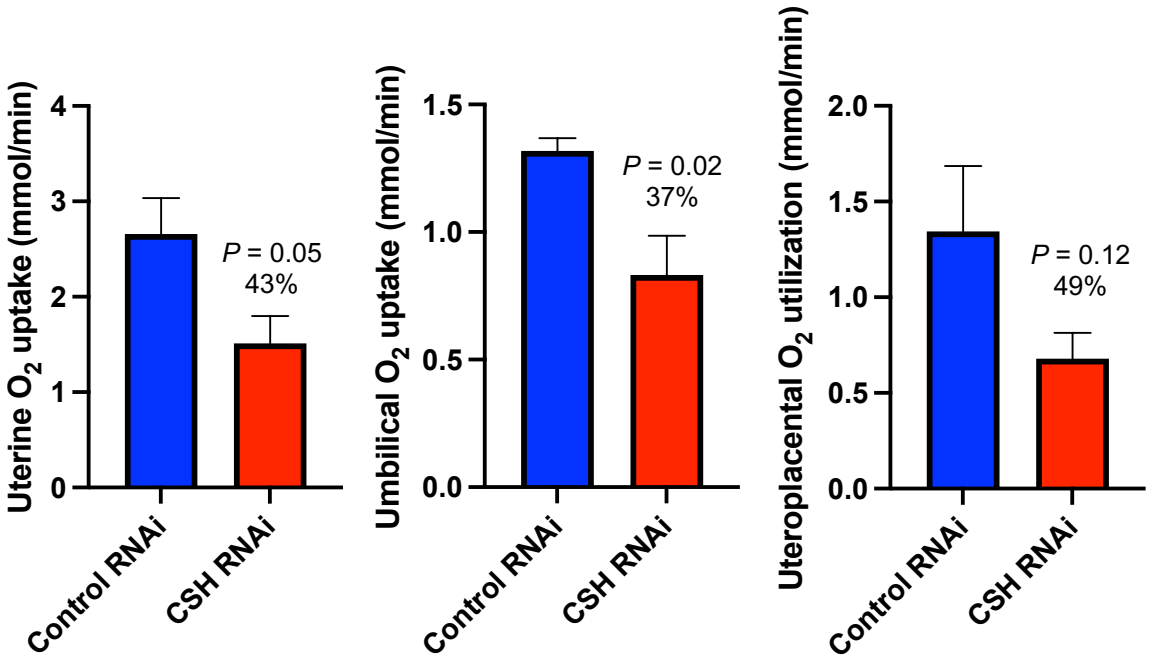


Figure 3.5. Uterine, umbilical, and uteroplacental oxygen uptakes as assessed by $^{3}H_20$ transplacental diffusion at 130 dGA. Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference.

Table 3.6. Maternal (uterine) blood gas measurements.

	CON RNAi	CSH RNAi	% change	P-value
Uterine Artery (A)	**(n = 4)**	**(n = 4)**		
pH $^{37.9C}$	7.458 ± 0.012	7.457 ± 0.009	0.01	0.96
pCO_2 $^{37.9C}$ mmHg	36.456 ± 1.877	35.375 ± 1.243	2.97	0.65
pO_2 $^{37.9C}$ mmHg	88.875 ± 0.704	90.219 ± 1.633	1.51	0.48
tCO_2 (B) mmol/L	22.669 ± 0.686	21.950 ± 0.725	3.17	0.50
tCO_2 (P) mmol/L	25.744 ± 0.814	24.981 ± 0.644	2.96	0.49
HCO^3-	24.744 ± 0.776	24.006 ± 0.621	2.98	0.49
Hct %	30.556 ± 0.593	31.088 ± 1.689	1.74	0.78
tHb mmol/L	6.131 ± 0.123	6.244 ± 0.353	1.83	0.77
O_2 Hb %	90.863 ± 0.929	91.988 ± 1.112	1.24	0.47
SO_2 %	96.363 ± 0.425	96.900 ± 1.322	0.56	0.71
CO Hb %	3.831 ± 0.092	4.106 ± 0.154	7.18	0.18
Met Hb %	1.244 ± 0.158	0.956 ± 0.066	23.12	0.14
O_2 ct mmol/L	5.700 ± 0.099	5.838 ± 0.332	2.41	0.71
Na^+ meq/L	146.438 ± 2.401	147.750 ± 0.595	0.90	0.61
K^+ meq/L	4.131 ± 0.123	4.419 ± 0.101	6.96	0.12
Ca^{2+} meq/L	2.734 ± 0.033	2.776 ± 0.029	1.53	0.38
Uterine Vein (V)	**(n = 4)**	**(n = 4)**		
pH $^{37.9C}$	7.446 ± 0.015	7.424 ± 0.008	0.30	0.24
pCO_2 $^{37.9C}$ mmHg	40.025 ± 1.280	40.275 ± 1.227	0.62	0.89
pO_2 $^{37.9C}$ mmHg	54.675 ± 1.422	54.281 ± 1.342	0.72	0.85
tCO_2 (B) mmol/L	23.731 ± 0.635	23.300 ± 0.689	1.82	0.66
tCO_2 (P) mmol/L	26.769 ± 0.763	26.325 ± 0.579	1.66	0.66
HCO^3-	25.681 ± 0.731	25.200 ± 0.559	1.87	0.62
Hct %	30.756 ± 0.621	31.213 ± 1.746	1.48	0.81
tHb mmol/L	6.169 ± 0.123	6.256 ± 0.362	1.42	0.83
O_2 Hb %	69.319 ± 2.116	69.775 ± 3.772	0.66	0.92
SO_2 %	72.131 ± 2.239	72.631 ± 4.144	0.69	0.92
CO Hb %	2.706 ± 0.112	2.994 ± 0.304	10.62	0.41
Met Hb %	1.163 ± 0.126	0.906 ± 0.077	22.04	0.13
O_2 ct mmol/L	4.325 ± 0.096	4.456 ± 0.419	3.03	0.77
Na^+ meq/L	147.500 ± 2.588	149.000 ± 1.177	1.02	0.62
K^+ meq/L	4.131 ± 0.103	4.344 ± 0.138	5.14	0.26
Ca^{2+} meq/L	2.704 ± 0.030	2.718 ± 0.016	0.53	0.68

Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference.

Table 3.7. Fetal (umbilical) blood gas measurements.

	CON RNAi	CSH RNAi	% change	P-value
Umbilical artery (α)	**(n = 4)**	**(n = 4)**		
pH $^{37.9C}$	7.346 ± 0.004	7.352 ± 0.006	0.08	0.46
pCO_2 $^{37.9C}$ mmHg	51.313 ± 1.758	52.106 ± 1.470	1.55	0.74
pO_2 $^{37.9C}$ mmHg	21.238 ± 0.241	18.031 ± 2.066	15.10	0.17
tCO_2 (B) mmol/L	24.850 ± 0.979	25.369 ± 0.556	2.09	0.66
tCO_2 (P) mmol/L	27.988 ± 0.994	28.819 ± 0.864	2.97	0.55
HCO^3-	26.575 ± 0.940	27.350 ± 0.807	2.92	0.55
Hct %	33.144 ± 1.807	35.713 ± 4.117	7.75	0.59
tHb mmol/L	6.800 ± 0.308	7.181 ± 0.855	5.61	0.69
O_2 Hb %	49.525 ± 1.660	40.200 ± 7.859	18.83	0.29
SO_2 %	51.856 ± 1.367	41.288 ± 7.758	20.38	0.23
CO Hb %	1.931 ± 0.108	1.500 ± 7.758	22.33	0.19
Met Hb %	0.250 ± 0.071	0.338 ± 0.161	35.00	0.64
O_2 ct mmol/L	3.538 ± 0.145	2.719 ± 0.345	23.14	0.07
Na^+ meq/L	141.813 ± 2.796	143.688 ± 1.028	1.32	0.55
K^+ meq/L	3.463 ± 0.155	3.913 ± 0.189	13.00	0.12
Ca^{2+} meq/L	2.977 ± 0.050	2.899 ± 0.104	2.62	0.52
Umbilical vein (γ)	**(n = 4)**	**(n = 4)**		
pH $^{37.9C}$	7.388 ± 0.004	7.392 ± 0.003	0.06	0.46
pCO_2 $^{37.9C}$ mmHg	43.588 ± 1.503	44.750 ± 1.656	2.67	0.62
pO_2 $^{37.9C}$ mmHg	32.881 ± 0.484	27.406 ± 3.772	16.65	0.20
tCO_2 (B) mmol/L	23.369 ± 1.204	23.688 ± 0.602	1.36	0.82
tCO_2 (P) mmol/L	26.225 ± 0.961	27.175 ± 0.890	3.62	0.50
HCO^3-	25.013 ± 0.925	25.938 ± 0.854	3.70	0.49
Hct %	33.494 ± 1.471	35.419 ± 4.151	5.75	0.68
tHb mmol/L	6.744 ± 0.310	7.125 ± 0.857	5.65	0.69
O_2 Hb %	79.244 ± 0.961	68.063 ± 9.995	14.11	0.31
SO_2 %	82.281 ± 1.159	69.963 ± 10.365	14.97	0.28
CO Hb %	3.500 ± 0.448	2.656 ± 0.353	24.11	0.19
Met Hb %	0.106 ± 0.232	0.031 ± 0.165	70.59	0.80
O_2 ct mmol/L	5.350 ± 0.187	4.613 ± 0.308	13.79	0.09
Na^+ meq/L	142.375 ± 2.260	144.063 ± 1.625	1.19	0.57
K^+ meq/L	3.463 ± 0.155	3.925 ± 0.193	13.36	0.11
Ca^{2+} meq/L	3.050 ± 0.057	2.984 ± 0.107	2.15	0.61

Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference.

Glucose and lactate uptakes

Uterine glucose uptake (μmol/min) was reduced (P = 0.04; **Figure 6**) by 45% in CSH RNAi pregnancies but relative uterine glucose uptake (μmol/min/kg uterus; **Table 5**) was not changed by treatment. Umbilical glucose uptake (μmol/min; **Figure 6**) was suppressed (P = 0.02) by 47% in CSH RNAi fetuses, and by 27% (P = 0.06) relative to fetal weight (μmol/min/kg fetus). Uteroplacental glucose utilization (μmol/min) was 44% lower (P = 0.05; **Figure 6**) in CSH RNAi pregnancies whereas relative uteroplacental glucose utilization (μmol/min/kg placenta) was not statistically impacted (P = 0.15). On a placental weight basis, the quantity of glucose transferred to the fetus (μmol/kg placenta/min) tended (P = 0.10) to be reduced by 34% in CSH RNAi pregnancies. Due to the decrease in glucose uptake and utilization, we assessed the concentrations of the key placental glucose transporters, SLC2A1 and SLC2A3. The placental concentrations of SLC2A1 and SLC2A3 were not altered by treatment (**Figures 7 and 8**).

Uterine absolute (μmol/min; P = 0.22) and relative (μmol/min/kg uterus; P = 0.28) lactate secretions were not significantly impacted (**Table 5**) in CSH RNAi pregnancies. In contrast to control pregnancies which had positive uterine lactate secretion (**Table 5**), CSH RNAi pregnancies had negative uterine lactate secretion which indicates a net uptake of lactate by the uterine tissues. This was also reflected by negative uteroplacental utilization of lactate in CSH RNAi pregnancies (**Table 5**). In spite of limited uteroplacental lactate utilization, umbilical lactate uptake (μmol/min) was reduced (P = 0.03) by 39% in CSH RNAi fetuses.

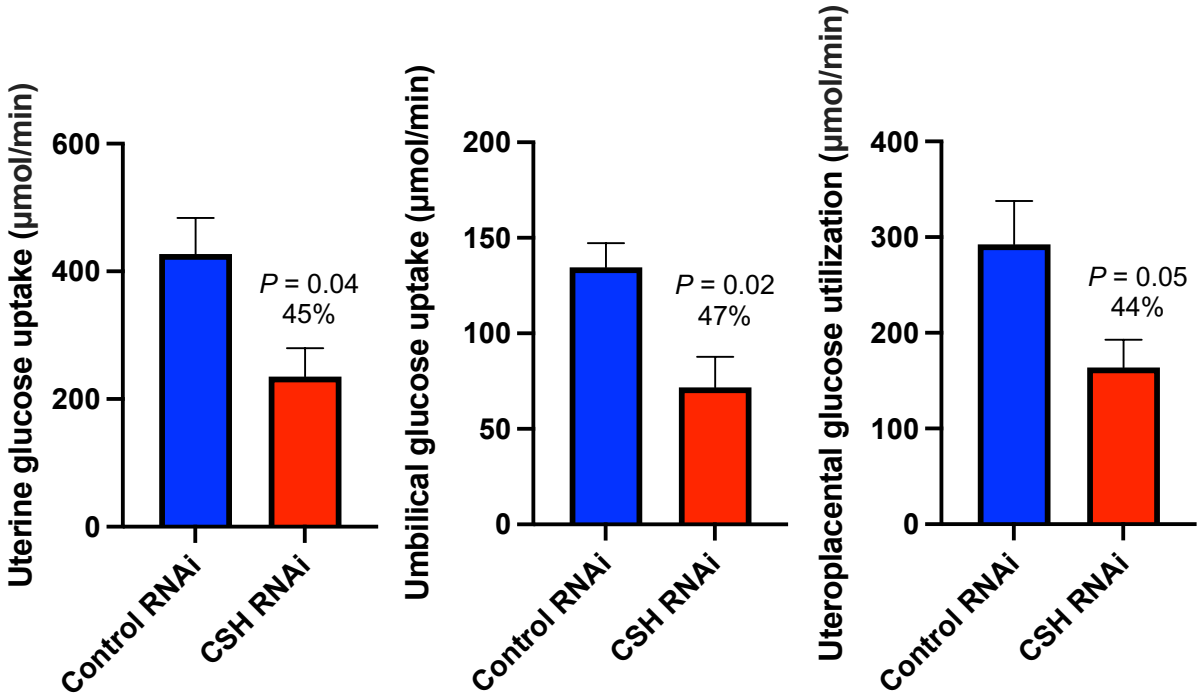


Figure 3.6. Uterine, umbilical, and uteroplacental glucose uptakes as assessed by $^{3}H_{2}0$ transplacental diffusion at 130 dGA. Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference.

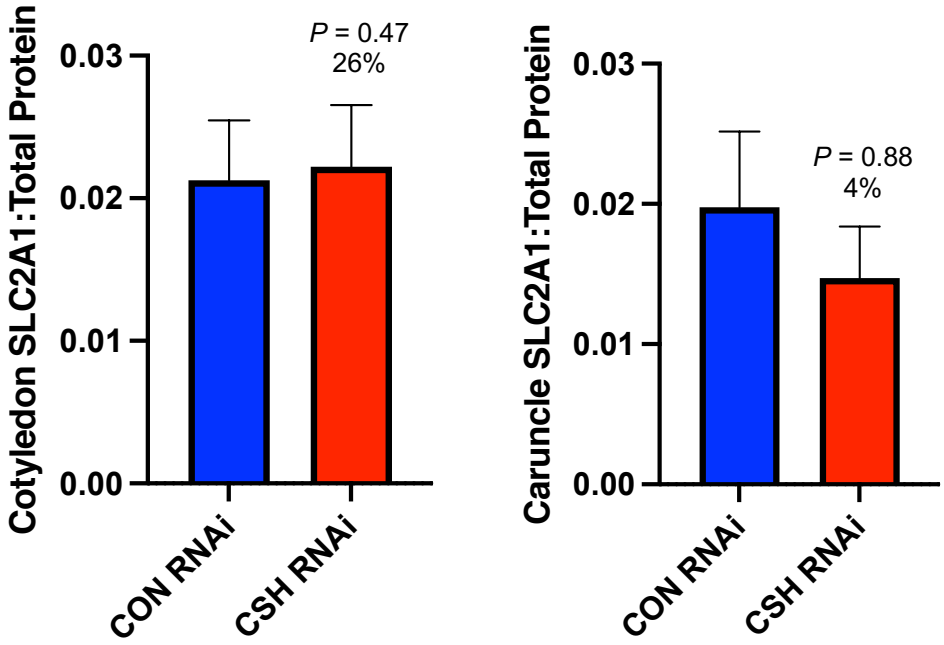


Figure 3.7. Densiometric analysis of SLC2A1 relative to total protein transferred, in fetal cotyledons from control RNA interference (RNAi) and chorionic somatomammotropin (CSH) RNAi pregnancies (n = 4/treatment). Data are shown as means ± SEM for all pregnancies in each treatment group.

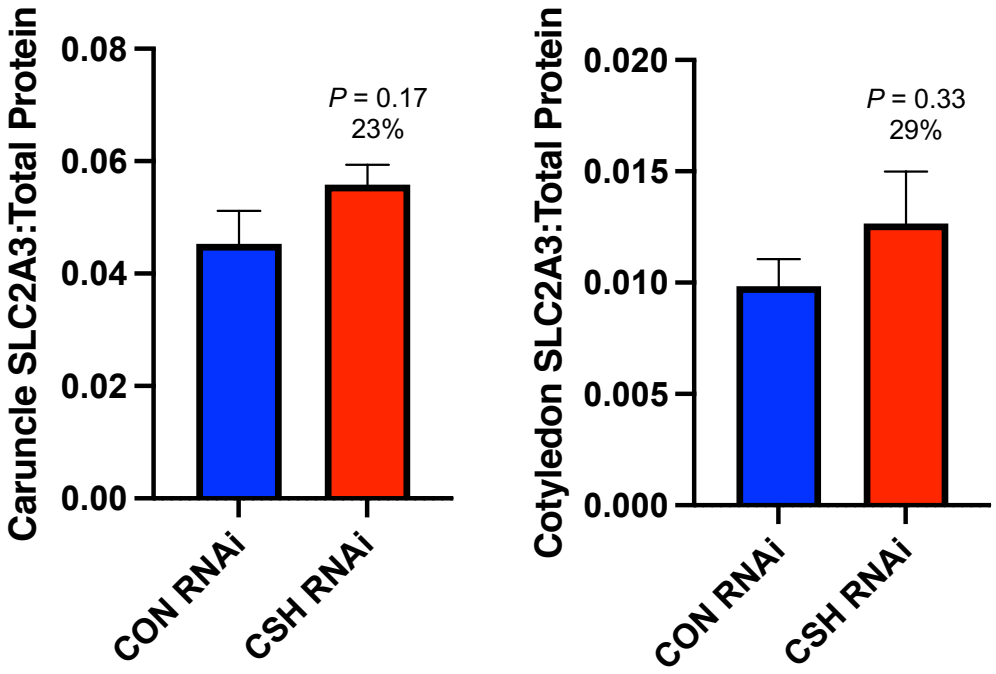


Figure 3.8: Densiometric analysis of SLC2A3 relative to total protein transferred, in fetal cotyledons from control RNA interference (RNAi) and chorionic somatomammotropin (CSH) RNAi pregnancies (n = 4/treatment). Data are shown as means ± SEM for all pregnancies in each treatment group.

Amino acid uptakes

The uterine uptakes (μmol/min) of alanine, arginine, asparagine, glutamine, histidine, isoleucine, leucine, lysine, ornithine, phenylalanine, serine, threonine, tyrosine, and valine were all reduced ($P \leq 0.05$; **Figure 9**) in CSH RNAi pregnancies, with the uptakes of both citrulline and methionine also tending ($P \leq 0.10$; **Figure 9**) to be reduced. The relative uterine uptakes of alanine and lysine (μmol/min/kg uterus) were also reduced ($P \leq 0.05$; **Table 8**) in CSH RNAi pregnancies, while the relative uterine uptake of phenylalanine tended ($P = 0.07$) to be reduced.

Not only were the uterine uptakes of amino acids impaired in CSH RNAi pregnancies, but the umbilical uptakes (μmol/min) of asparagine, leucine, and tyrosine were also reduced, whereas the fetal production of glutamate was decreased ($P \leq 0.05$; **Figure 10**). Additionally, the umbilical uptakes of glutamine, alanine, and isoleucine also tended ($P \leq 0.10$; **Figure 10**) to be reduced in CSH RNAi fetuses. The relative fetal production of taurine (μmol/min/kg fetus) tended ($P = 0.08$; **Table 9**) to be increased in CSH RNAi pregnancies whereas the relative umbilical uptake of asparagine tended ($P = 0.09$) to be reduced.

The placenta was also impacted by perturbed amino acid utilization in CSH RNAi pregnancies. The uteroplacental utilization (μmol/min) of glutamate and ornithine was reduced ($P \leq 0.05$; **Figure 11**) in CSH RNAi pregnancies, whereas the uteroplacental utilization of isoleucine and lysine was negative ($P \leq 0.10$). This response may indicate a lack of utilization to facilitate transfer to the fetus. On a relative basis (μmol/min/kg placenta), the uteroplacental utilization of lysine was negative ($P = 0.03$; Table 10), again indicating a lack of utilization to facilitate transfer to the fetus. Additionally, the

uteroplacental utilization of glutamate and isoleucine tended ($P \leq 0.10$) to be reduced in CSH RNAi pregnancies.

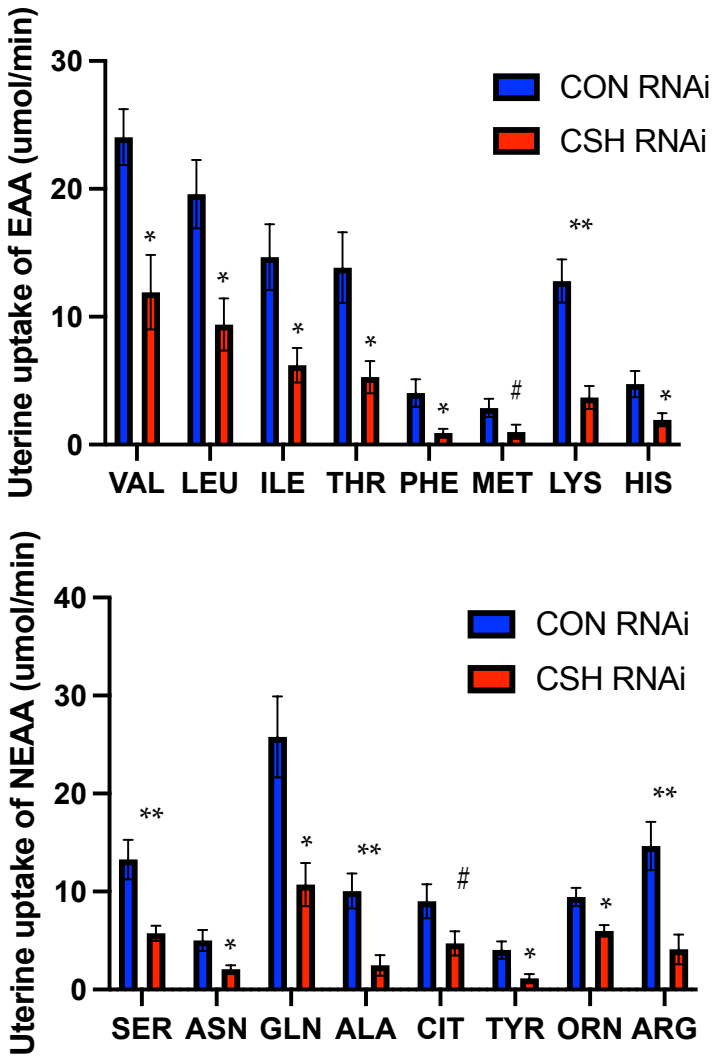
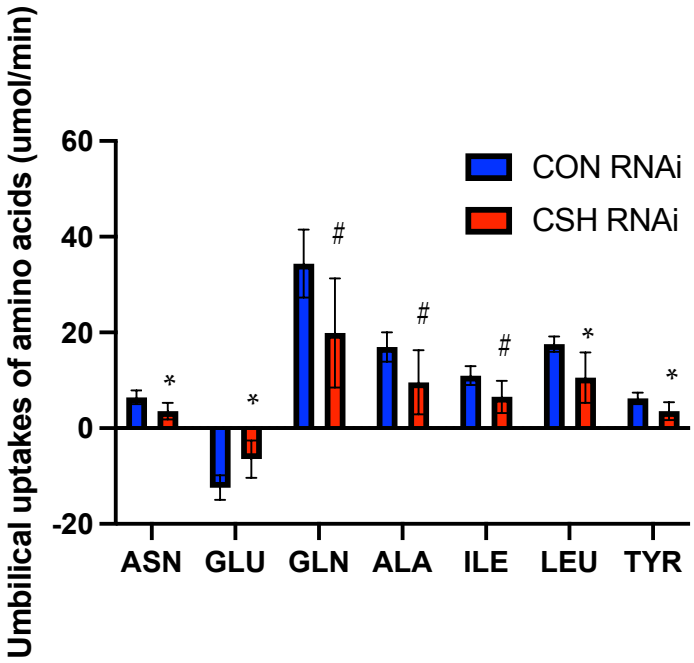


Figure 3.9. Uterine uptakes of essential (EEA) and nonessential (NEAA) amino acids significantly altered by treatment as assessed by $^{3}H_{2}0$ transplacental diffusion at 130 dGA. Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference. Ala, alanine; Arg, arginine; Asn, asparagine; Cit, citrulline; Gln, glutamine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Orn, ornithine; Phe, phenylalanine; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine. **P≤0.01, *P≤0.05, #P≤0.10, when CSH RNAi pregnancies are compared with control RNAi pregnancies.

Table 3.8. Relative uterine uptake of individual amino acids (μmol/min/kg uterus).

	CON RNAi	CSH RNAi	% change	P-value
Amino acid	**(n = 4)**	**(n = 4)**		
Tau	0.07 ± 0.66	-2.42 ± 1.19	3560.98	0.12
Asp	-1.52 ± 0.38	-0.99 ± 0.77	34.63	0.56
Thr	16.29 ± 3.36	10.36 ± 2.19	36.44	0.19
Ser	15.98 ± 2.82	11.37 ± 1.04	28.87	0.18
Asn	5.97 ± 1.52	4.07 ± 0.64	31.80	0.29
Glu	-1.53 ± 0.37	-2.46 ± 1.26	60.33	0.51
Gln	30.40 ± 5.60	20.94 ± 3.47	31.12	0.20
Pro	10.13 ± 1.68	10.29 ± 4.16	1.50	0.97
Gly	-6.90 ± 3.84	-14.25 ± 4.72	106.61	0.27
Ala	11.99 ± 2.25	4.65 ± 1.95	61.27	0.05
Cit	10.72 ± 2.15	9.38 ± 2.25	12.51	0.68
Val	29.40 ± 4.93	23.30 ± 4.84	20.74	0.41
Cys	2.21 ± 1.41	-0.02 ± 0.57	100.94	0.19
Met	3.32 ± 0.77	1.92 ± 1.04	42.05	0.32
Ile	17.82 ± 3.60	12.12 ± 2.28	32.00	0.23
Leu	23.76 ± 4.22	18.33 ± 3.43	22.86	0.36
Tyr	4.74 ± 1.04	2.19 ± 0.84	53.69	0.11
Phe	4.70 ± 1.19	1.75 ± 0.60	62.85	0.07
Trp	2.44 ± 0.85	2.81 ± 0.64	14.98	0.74
Orn	11.37 ± 1.83	11.97 ± 0.81	5.28	0.77
Lys	15.49 ± 2.70	7.25 ± 1.71	53.15	0.04
His	5.85 ± 1.51	3.78 ± 0.95	35.43	0.29
Arg	17.97 ± 4.36	8.42 ± 3.08	53.12	0.12

Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference.



Figures 3.10. Umbilical uptakes of amino acids significantly altered by treatment as assessed by $^{3}H_{2}0$ transplacental diffusion at 130 dGA. Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference. Ala, alanine; Asn, asparagine; Glu, glutamate; Gln, glutamine; Ile, isoleucine; Leu, leucine; Tyr, tyrosine. *P≤0.05, #P≤0.10, when CSH RNAi pregnancies are compared with control RNAi pregnancies.

Table 3.9. Relative umbilical uptakes of individual amino acids (μmol/min/kg fetus).

Amino acid	CON RNAi (n = 4)	CSH RNAi (n = 4)	% change	P-value
Tau	-0.03 ± 0.09	-0.42 ± 0.17	1474.97	0.08
Asp	-0.09 ± 0.10	-0.05 ± 0.10	47.20	0.78
Thr	2.58 ± 0.20	2.30 ± 0.93	10.64	0.78
Ser	-0.41 ± 0.34	-0.34 ± 1.03	18.19	0.95
Asn	1.61 ± 0.10	1.21 ± 0.17	24.50	0.09
Glu	-3.12 ± 0.21	-2.12 ± 0.60	32.00	0.17
Gln	8.58 ± 0.46	6.76 ± 1.25	21.22	0.22
Pro	1.77 ± 0.45	1.69 ± 0.43	4.70	0.90
Gly	3.38 ± 0.23	3.06 ± 1.02	9.44	0.77
Ala	4.25 ± 0.17	3.16 ± 0.83	25.61	0.24
Cit	0.42 ± 0.24	0.29 ± 0.30	30.20	0.75
Val	4.66 ± 0.15	3.98 ± 0.95	14.55	0.51
Cys	0.13 ± 0.03	0.06 ± 0.13	56.42	0.61
Met	0.96 ± 0.08	0.69 ± 0.25	27.96	0.35
Ile	2.76 ± 0.14	2.21 ± 0.35	19.95	0.20
Leu	4.44 ± 0.19	3.60 ± 0.51	18.94	0.17
Tyr	1.57 ± 0.09	1.21 ± 0.19	23.03	0.14
Phe	1.61 ± 0.12	1.29 ± 0.29	19.65	0.35
Trp	0.39 ± 0.08	0.19 ± 0.13	49.91	0.25
Orn	0.15 ± 0.10	0.13 ± 0.25	14.38	0.94
Lys	2.71 ± 0.27	2.36 ± 0.40	12.65	0.50
His	0.75 ± 0.05	0.57 ± 0.16	24.76	0.30
Arg	2.77 ± 0.28	2.37 ± 0.49	14.52	0.50

Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference.

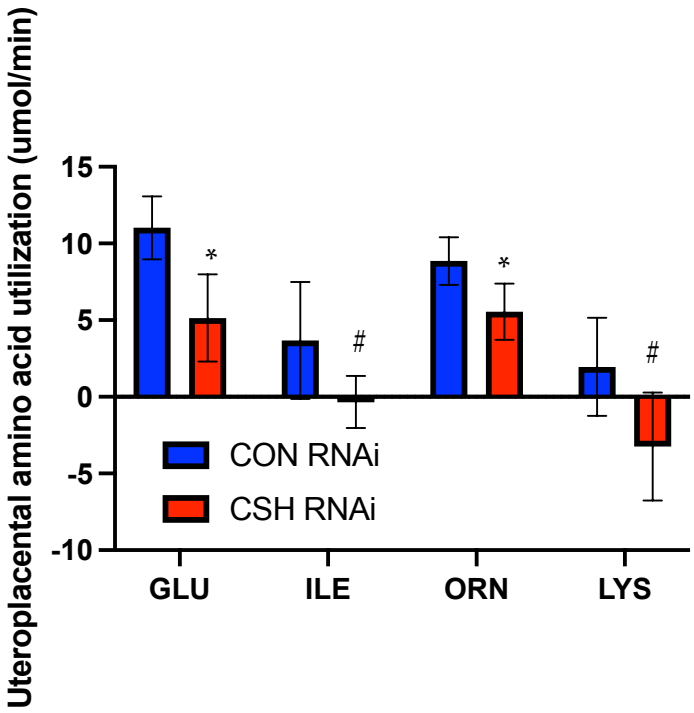


Figure 3.11. Uteroplacental utilization of amino acids significantly altered by treatment as assessed by $^{3}H_{2}0$ transplacental diffusion at 130 dGA. Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference. Glu, glutamate; Ile, isoleucine Lys, Lysine; Orn, ornithine. *P≤0.05, #P≤0.10, when CSH RNAi pregnancies are compared with control RNAi pregnancies.

Table 3.10. Relative uteroplacental utilization of individual amino acids (μmol/min/kg placenta).

Amino acid	CON RNAi (n = 4)	CSH RNAi (n = 4)	% change	P-value
Tau	0.82 ± 1.35	0.00 ± 1.47	99.92	0.70
Asp	-2.23 ± 1.05	-1.12 ± 0.59	49.45	0.40
Thr	6.23 ± 5.11	-2.25 ± 4.30	136.04	0.25
Ser	32.36 ± 5.48	21.78 ± 9.55	32.69	0.37
Asn	-3.65 ± 2.15	-3.66 ± 0.81	0.21	1.00
Glu	24.37 ± 2.19	14.20 ± 4.39	41.73	0.08
Gln	-20.26 ± 5.79	-21.92 ± 5.62	8.21	0.84
Pro	4.24 ± 3.89	2.48 ± 7.55	41.42	0.84
Gly	-41.92 ± 6.74	-41.64 ± 10.39	0.68	0.98
Ala	-15.00 ± 3.35	-17.92 ± 2.59	19.49	0.52
Cit	15.80 ± 3.08	11.57 ± 3.74	26.79	0.42
Val	12.29 ± 4.46	4.16 ± 8.06	66.14	0.41
Cys	2.58 ± 2.32	-0.32 ± 2.01	112.54	0.38
Met	-2.38 ± 1.50	-2.71 ± 1.06	13.85	0.86
Ile	7.29 ± 3.35	0.04 ± 1.67	99.50	0.10
Leu	3.21 ± 4.46	-2.15 ± 2.16	166.78	0.32
Tyr	-4.84 ± 1.11	-6.67 ± 0.68	37.75	0.21
Phe	-5.74 ± 2.00	-7.55 ± 0.72	31.52	0.43
Trp	0.83 ± 1.16	2.65 ± 0.78	218.76	0.24
Orn	20.08 ± 3.31	17.57 ± 4.34	12.51	0.66
Lys	4.01 ± 3.20	-7.52 ± 2.62	287.65	0.03
His	3.43 ± 1.93	1.04 ± 1.30	69.56	0.35
Arg	9.06 ± 6.75	-3.45 ± 6.33	138.10	0.22

Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference.

Total nutrient uptakes

With the transplacental diffusion technique, it is possible to calculate substrate specific carbon supply to both the uterus and the fetus (**Table 2**). Furthermore, it is possible to calculate the total carbon and nitrogen available for catabolic processes. As summarized in **Table 11** uterine carbon uptakes from amino acids (P = 0.004), glucose (P = 0.04), and lactate (P = 0.22) were reduced in CSH RNAi pregnancies. This resulted in a 60% reduction (P = 0.04) in total carbon available for uptake in CSH RNAi pregnancies. Uterine nitrogen uptake was also reduced (P = 0.001) by 61% in CSH RNAi pregnancies.

Umbilical carbon uptakes from amino acids (P = 0.13; **Table 11**), glucose (P = 0.02), and lactate (P = 0.03) were reduced in CSH RNAi fetuses leading to a 42% reduction (P = 0.04) in total carbon uptake. Umbilical nitrogen uptake was numerically lower (P = 0.13) in CSH RNAi fetuses. No umbilical nutrient to oxygen quotients differed between treatments (**Table 11**).

Table 3.11. Total nutrient uptakes (μmol/min)

	CON RNAi	CSH RNAi	% change	P-value
Uterine	**(n = 4)**	**(n = 4)**		
Sum of uterine amino acid carbon uptake	1006.29 ± 112.94	405.94 ± 71.71	59.66	0.004
Sum of uterine glucose carbon uptake	2562.06 ± 340.25	1412.67 ± 266.44	44.86	0.04
Sum of lactate carbon uptake	402.52 ± 56.07	-241.24 ± 462.00	159.93	0.22
Sum of uterine carbon uptake	3970.88 ± 491.12	1577.36 ± 783.96	60.28	0.04
Sum of uterine nitrogen uptake	318.94 ± 28.48	123.25 ± 18.19	61.36	0.001
Umbilical	**(n = 4)**	**(n = 4)**		
Sum of umbilical amino acid carbon uptake	840.11 ± 65.12	512.89 ± 172.70	38.95	0.13
Sum of umbilical glucose carbon uptake	807.81 ± 75.88	430.07 ± 96.37	46.76	0.02
Sum of umbilical lactate carbon uptake	369.47 ± 17.90	226.57 ± 48.32	38.68	0.03
Sum of total fetal carbon uptake	2017.39 ± 113.97	1169.53 ± 295.20	42.03	0.04
Sum of umbilical nitrogen uptake	262.04 ± 19.78	159.64 ± 54.50	39.08	0.13
Umbilical glucose:oxygen quotient	0.61 ± 0.06	0.50 ± 0.04	17.88	0.15
Umbilical lactate:oxygen quotient	0.28 ± 0.01	0.27 ± 0.01	4.58	0.46
Umbilical amino acid:oxygen quotient	0.68 ± 0.03	0.63 ± 0.11	7.96	0.66
Total umbilical nutrient quotient	1.58 ± 0.06	1.40 ± 0.12	11.22	0.24

Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference.

130 dGA hormones

Uterine concentrations of insulin, insulin-like growth factor 1 (IGF1), cortisol, and estradiol-17β were not impacted by RNAi treatment, as summarized in **Table 12**. In contrast, umbilical artery IGF1 was reduced ($P \leq 0.01$; Table 12) by 48% in CSH RNAi fetuses. Umbilical artery concentrations of insulin, cortisol and estradiol were not statistically impacted by CSH RNAi.

Table 3.12. Concentrations of maternal or fetal hormones.

130 dGA hormones	CON RNAi	CSH RNAi	% change	P-value
Maternal (uterine)	**(n = 4)**	**(n = 4)**		
Uterine artery insulin, ng/mL	0.92 ± 0.17	0.77 ± 0.10	15.92	0.49
Uterine artery IGF1, ng/mL	297.91 ± 45.18	260.24 ± 25.74	12.64	0.50
Uterine artery cortisol, ng/mL	126.53 ± 12.95	96.27 ± 29.07	23.91	0.38
Uterine vein estradiol, pg/mL	7.32 ± 2.34	4.42 ± 1.81	39.71	0.36
Fetal (umbilical)	**(n = 4)**	**(n = 4)**		
Umbilical artery insulin, ng/mL	1.12 ± 0.15	0.69 ± 0.23	38.63	0.16
Umbilical artery IGF1, ng/mL	140.89 ± 9.30	72.85 ± 14.94	48.29	0.01
Umbilical artery cortisol, ng/mL	11.98 ± 4.14	58.62 ± 29.83	389.25	0.17
Umbilical vein estradiol, pg/mL	5.59 ± 1.99	6.59 ± 0.75	17.99	0.65

Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference.

DISCUSSION

To describe the progression of CSH RNAi induced IUGR, we set out to document the physiological ramifications of CSH RNAi in a cohort of IUGR pregnancies. We hypothesized that CSH RNAi would reduce both fetal and maternal blood flow, resulting in reductions in global nutrient uptake. Unfortunately, due to fetal demise and catheter failures, complete studies were only accomplished on n=4 pregnancies per group, somewhat limiting our statistical power. Additionally, while fetal sex was equally distributed (one female, three males) in each treatment, fetal sex could not be included in our statistical model. Regardless, results from this study directly support this hypothesis.

By using Doppler ultrasonography, we established that by 90 dGA, umbilical blood flow was reduced in CSH RNAi pregnancies. It is somewhat surprising, however, that the decrease in blood flow was not accompanied by a corresponding increase in vascular resistance, a hallmark of placental insufficiency (Galan et al., 2005; Regnault et al., 2002).This might suggest that CSH is not acting in a way that reduces placental vascularity, which would have likely resulted in increased vascular resistance, but in a way that modulates the size of major vessels such as the umbilical artery, which was reduced in our current study. While not statistically different (P=0.11), the ~60% increase in cotyledonary NOS3 in the CSH RNAi near term (130 dGA) can perhaps, in part, explain why there was not an increase in vascular resistance. This is supported by an ex vivo study of placental perfusion from human fetal growth-restricted pregnancies, where flow mediated vasodilation was reduced but NOS3 was still elevated (Jones et al., 2015). This combination of normal vascular resistance with decreased umbilical blood flow appears to be a unique distinction between CSH RNAi induced IUGR and

other forms of placental insufficiency investigated in sheep. For example, in hyperthermia induced IUGR pregnancies, umbilical blood flow is reduced, and vascular resistance is increased (Galan et al., 1998; Galan et al., 2005). Those pregnancies also have reduced umbilical artery NOS3 mRNA concentrations (Arroyo et al., 2010).

Near term (130 dGA) uterine and umbilical blood flow was assessed by the $^{3}H_2O$ transplacental diffusion technique. Total umbilical blood and plasma flows were significantly reduced in CSH RNAi pregnancies, as well as relative umbilical blood flows. This suggests the decrease in umbilical blood flow was somewhat independent of both fetal weight and placental weight. While the reduction (42%) in uterine blood flow in CSH RNAi pregnancies was not statistically different, it agrees with the statistically significant reduction in uterine blood flow reported earlier for CSH RNAi pregnancies (Tanner et al., 2021a). In that study (Tanner et al., 2021a), uterine blood flow was reduced by only 24% with no change in uterine or fetal weights. Perhaps one driver of CSH RNAi induced IUGR is decreased uterine blood flow, which precedes a subsequent uterine mass reduction and ultimately prevents the uteroplacental unit from transporting adequate nutrients to sustain fetal growth.

With the noted decreases in blood flow, it is not surprising that fetal weights were reduced in this cohort of CSH RNAi pregnancies. Compared to a previous study that also reported near-term IUGR in CSH RNAi pregnancies, a similar degree of fetal growth restriction was observed with a 30% weight reduction in the current cohort vs. 32% in the Baker et al. (2016) study. It appears that CSH RNAi induced IUGR can be classified as asymmetric IUGR as the brain to fetal weight ratio was significantly elevated, a proven indicator of asymmetric fetal growth (Regnault et al., 2003). This

cohort of CSH RNAi pregnancies with IUGR also had reduced lower leg length, which was also observed in CSH RNAi pregnancies with normal fetal weights (Tanner et al., 2021a).

Similar to fetal weight reductions, the fetal livers of CSH RNAi pregnancies in this study were comparably reduced (35% vs. 36.5%) as in the Baker et al. (2016) study, suggesting the necessity of CSH in preserving liver mass and corresponding liver-mediated IGF1 production. A novel finding reported on this cohort of CSH RNAi pregnancies was proportionately smaller hearts, especially the left ventricles. In a sheep model of experimentally induced placental growth restriction (carunclectomy), left ventricular cardiomyocytes number was reduced in the offspring during adulthood, and was correlated to birth weight (Vranas et al., 2017). Fetal muscle weights were also significantly reduced in this CSH RNAi IUGR cohort, as has been documented in other sheep models of IUGR (Chang et al., 2019; Rozance et al., 2018). In this current study, this effect does appear to be a function of fetal weight reduction, as normalizing each muscle relative to fetal mass removes the differences except for the flexor digitorum superficialis.

In the current cohort, there was a 21% reduction in placental weight, which wasn't statistically different from the control pregnancies. While it can certainly be argued that CSH RNAi did induce metabolic, hemodynamic and growth perturbations to these pregnancies, it is also accurate that this was a less severe placental growth restriction compared to the 52% placental mass reduction in the Baker et al. (2016) study. While difficult to truly assess the differences between the two studies, one possible explanation includes variability of robustness in the activity of the RNAi over

gestation. If the RNAi is more robustly expressed during key timepoints in placentation, it is possible that the degree of placental mass reduction would be more severe. Based on placental efficiency, the smaller placentas in the Baker et al. study (2016) could be argued to be more efficient, as they produced approximately the same degree of fetal growth restriction as the current study. Thus, it is possible that the impacts of CSH RNAi yield consistent degrees of reduced fetal growth in the IUGR phenotype, which could suggest similar placental dysfunction between the two studies.

One truly novel insight into the current cohort provided was the potential impacts of CSH on uterine size, as it was not assessed in the Baker et al. (2016) study. There is some evidence during early pregnancy that CSH can impact the uterus. In ovariectomized ewes that have been hormonally manipulated to mimic early pregnancy, CSH infusion acts on the endometrial glands (Spencer et al., 1999). Through the intrauterine infusion of exogenous recombinant CSH from 16–25 days post estrus, endometrial gland density increased (Spencer et al., 1999). A similar effect occurred when a separate treatment of growth hormone (GH) was infused over the same timeframe, suggesting that both CSH and GH may act on the early-pregnancy endometrium in a similar fashion (Spencer et al., 1999). This also may suggest a potential role for CSH, working through the same receptor to support uterine modifications for successful pregnancy outcomes.

The direct vs. indirect impacts of CSH on uterine mass are quite difficult to tease apart. In sheep, pregnancy increases nitric oxide, which has been shown to stimulate uterine blood flow and increase NOS3 in the uterine vascular endothelium (Magness et al., 1997). As re-ported by Tanner et al. (2021a), CSH RNAi results in similar reductions

in both uterine blood flow (24%) and caruncular NOS3 (24%), even in the absence of IUGR or uterine weight reductions. In the present cohort of CSH RNAi induced IUGR pregnancies, similar degrees of reductions in uterine blood flow (42%) and NOS3 (38%) were also observed. It is plausible that this relationship could influence the perfusion of the uterus, partially ex-plaining the reduced uterine weights. This is supported by the global decrease in the uterine uptakes of oxygen, glucose, and amino acids.

Through the application of the $^{3}H_2O$ transplacental diffusion technique, the uptake of substrates from maternal circulation and their partitioning to uterus, fetus and placenta can be directly assessed. Pregnancies perturbed by IUGR often experience global re-ductions in the uptakes of all of the key substrates including oxygen, glucose, and amino acids. This study again demonstrated this relationship with global reductions in the uptakes of all three substrates by all aspects of uteroplacental circulation. It is worth noting that the uterine uptakes of substrates (Table 2) really represent the quantity of substrate the entire uteroplacental unit (uterus and placenta) is taking up from maternal circulation. When the umbilical uptake of the substrate is accounted for, the difference between uterine (uteroplacental) and umbilical uptakes represents what the uteroplacental unit is utilizing. A novel but not entirely unexpected finding is the global reduction in oxygen uptake by the CSH RNAi induced IUGR pregnancies. Oxygen uptake is considered a flow limited substrate (Barry and Anthony, 2008; Carter, 1989) and blood flow was reduced in this cohort of CSH RNAi induced IUGR pregnancies. Other sheep models of placental insufficiency IUGR are also characterized by uteroplacental reductions in oxygen uptakes (Regnault et al., 2003; Regnault et al., 2007). Kingdom and Kaufmann (1997) described several modalities for

examining pregnancy hypoxia and their respective etiologies. It appears that CSH deficiency results in placental hypoxia, where maternal blood is adequately oxygenated (as evidenced by no differences in maternal oxygen content, see Table 6) but oxygen cannot adequately diffuse across the placenta (Kingdom and Kaufmann, 1997), as evidenced by reduced fetal uptakes and uteroplacental utilization. In this case, it appears that reduced oxygen uptake is likely originating from reduced blood flow, resulting in a smaller uterine mass which cannot transport as much oxygen from maternal circulation. Functionally, this means both the fetus and placenta had less oxygen available for oxidative metabolism and, not surprisingly, their growth was reduced.

While we have previously demonstrated that CSH RNAi results in reductions to the fraction of glucose transferred to the fetus, even in cases of normal fetal weights (Tanner et al., 2021a), the effects on glucose uptake in this cohort of CSH RNAi induced IUGR pregnancies were far more severe. As glucose is considered the primary substrate for fetal oxidative metabolism in both humans and sheep (Battaglia and Meschia, 1986), it is not surprising that reductions in glucose uptake and utilization by the uteroplacental unit and the fetus in this cohort led to IUGR. Even when umbilical glucose uptake was normalized to fetal weight, glucose uptake was still reduced, suggesting a direct impact of CSH on umbilical glucose uptake as it is not just a function of fetal mass. CSH RNAi also reduced umbilical lactate uptake, leading to further reductions in carbon sources for the fetus.

Placental glucose transport is dependent on several factors, including the maternofetal glucose gradient, the availability of transporters to shuttle glucose across

the placenta, the oxidative needs of the placenta, and placental mass (Barry and Anthony, 2008; Hay et al., 1990). In the current study, there were no significant changes to placental SLC2A1 or SLC2A3 concentrations. This fits with the lack of SLC2A1 changes in the human placentae of IUGR pregnancies (Jansson et al., 1993). Ultimately, this suggests that the reductions in glucose uptake and utilization by the uteroplacental unit are likely due to decreases in blood flow and uteroplacental mass.

One clear distinction between IUGR and normal weight CSH RNAi phenotypes is the impact on uteroplacental (placental) glucose utilization. In CSH RNAi pregnancies with normal fetal weights, uteroplacental glucose utilization was increased but the fetal uptakes of glucose were not altered (Tanner et al., 2021a). This suggests that with reduced CSH, the placenta requires additional glucose to support appropriate fetal growth and transfer of nutrients to the fetus. This might hint at a potential compensatory mechanism in the normal weight pregnancies that was not sufficient to maintain fetal growth in our CSH RNAi IUGR pregnancies. In the more severe IUGR CSH RNAi phenotype, it is likely that this compensatory mechanism is overridden, possibly being preceded by greater proportional reductions in blood flow leading to a smaller uterus, unable to transfer enough glucose from maternal circulation to support adequate placental function and, therefore, fetal growth.

Amino acids are also considered an important oxidative substrate for fetal development (Barry and Anthony, 2008). In the current cohort of CSH RNAi pregnancies with IUGR, the uterine uptakes of eight essential amino acids (including all three branch-chain amino acids) were reduced along with eight nonessential amino acids. This resulted in substantial reductions in carbon (60%) and nitrogen (61%) taken

up by the uterus (see Table 11). This could contribute to the explanation of why the uteri containing CSH RNAi pregnancies were smaller. In normal weight CSH RNAi pregnancies, only the uterine uptake of taurine and glycine were reduced (Tanner et al., 2021a). It is interesting that neither of these amino acids were statistically reduced in CSH RNAi IUGR pregnancies. Regardless of these variations, it does appear that uterine uptakes of amino acids were more profoundly impacted in cases of CSH RNAi IUGR.

The decreased uterine uptakes of amino acids also resulted in fewer amino acids available for fetal uptake in CSH RNAi pregnancies, with reduced uptakes of both iso-leucine and leucine and five non-essential amino acids, including glutamine. Because the concentration of amino acids is usually higher in fetal circulation, the transport of amino acids is dependent on energy availability due to active transport mechanisms necessary to transport against the concentration gradient (Hay, 1991). In CSH RNAi pregnancies with IUGR, there was reduced placental utilization of both glutamate and ornithine, as well as limited uteroplacental utilization of isoleucine and lysine. In both sheep and humans, there is no net placental transfer of glutamate (Lemons et al., 1976; Schneider et al., 1979). The fetus takes up glutamine from uterine circulation, and the fetal liver converts it to glutamate to supply the placenta (Battaglia, 2000). Thus, the decreased utilization of glutamate by the CSH RNAi uteroplacental unit is likely due to the decreased umbilical uptakes of glutamine, leading to the reduced production of glutamate from by the fetal liver.

One of the key postulated roles for CSH in regulating fetal growth is the stimulation of the insulin-like growth factors (IGFs; Handwerger, 1991). This is

supported by suggestive evidence in humans of parallel increases in IGF1 and CSH in maternal circulation (Breuer, 1969). In hypophysectomized rats, ovine CSH was infused which stimulated IGF1 secretion (Hurley et al., 1977). Furthermore, in pregnant rats and sheep, plasma IGF1 concentrations are maintained after hypophysectomy until delivery of the placenta (Handwerger, 1991; Daughaday and Kapadia, 1978). Based on that evidence, Handwerger hypothesized that CSH stimulated IGF1 in both maternal and fetal circulation (Handwerger, 1991). Interestingly, in our CSH RNAi pregnancies (Baker et al., 2016; Ali et al., 2020; Tanner et al., 2021a), maternal concentrations of IGF1 were not influenced by CSH RNAi, which differs from what was originally hypothesized.

However, our data does support the proposed actions of CSH on IGF1 secretion in the fetus. Handwerger suggested CSH likely stimulated fetal IGF1 and IGF2 production, as evidenced by the treatment of rat embryonic fibroblasts with ovine CSH (Adams et al., 1983). We have directly and repeatedly demonstrated that CSH RNAi causes reductions in umbilical IGF1 concentrations, in both the IUGR (Baker et al., 2016 and the current cohort) and normal weight phenotypes (Ali et al., 2020). Furthermore, CSH is also likely acting in a paracrine fashion on the placenta to impact placental IGFs, as evidenced by CSH RNAi reducing placental mRNA concentrations of IGF1 and IGF2 (Jeckel et al., 2018). This is relevant to the current study, as one of the roles of the IGFs includes the placental transport of glucose and amino acids (Kniss et al., 1994). Because of the direct effects of CSH on the secretion of IGF1 in fetal circulation, it is not surprising that CSH RNAi results in reduced placental glucose and amino acid transfer, as well as subsequent IUGR.

The IGFs are not the only hormone postulated to be impacted by CSH. CSH has been postulated to increase maternal insulin secretion and impair glucose tolerance (Handwerger, 1991). Ex vivo, CSH has been demonstrated to stimulate insulin secretion by isolated pancreatic islet cells (Martin and Friesen, 1969). However, our in vivo data do not support this, as in both the CSH RNAi IUGR (Baker et al., 2016 and current study) and normal weight phenotypes (Ali et al., 2020), maternal circulating insulin concentrations are not altered. Furthermore, maternal glucose concentrations are not impacted by CSH RNAi in either the IUGR (current study) or normal weight (Tanner et al., 2021a) phenotypes. Maternal glucose and insulin concentrations were also unaltered in a model of CSH infusion into the late gestation sheep (Oliver et al., 1995). In the fetus, however, CSH does appear to contribute to insulin concentration. In previous (Baker et al., 2016) and current cohorts of CSH RNAi fetuses with IUGR, umbilical insulin concentrations were reduced by 48% and 39%, respectively. It would be very interesting to follow a cohort of CSH RNAi offspring into adulthood to examine adult glucose homeostasis and insulin sensitivity, as CSH RNAi results in both hypoglycemia and likely hypoinsulinemia. Overall, our in vivo data support the postulated roles of CSH in fetal circulation, but not maternal.

CONCLUSIONS

While CSH has been previously demonstrated as necessary for adequate fetal growth, the specific role of CSH in modulating fetal growth has yet to be determined. In this current study, we examined the physiological ramifications of CSH deficiency in cases of IUGR during late gestation. These data suggest that CSH is not only important

for uterine blood flow and uteroplacental glucose utilization, but it also facilitates adequate umbilical blood flow necessary for the uptakes of oxygen, oxidative substrates, and hormones necessary to support fetal growth. Additionally, the current study demonstrated a novel role of CSH is supporting uterine growth to facilitate adequate transfer of nutrients.

CHAPTER IV: MATERNAL HYPERGLYCEMIA INCREASES UTEROPLACENTAL OXYGEN UTILIZATION BUT FAILS TO RESTORE FETAL GLUCOSE CONCENTRATIONS IN CHORIONIC SOMATOMAMMOTROPIN RNA INTERFERENCE PREGNANCIES

INTRODUCTION

Intrauterine growth restriction (IUGR) is a significant human health concern, impacting 6-8% of pregnancies worldwide (Brar and Rutherford, 1988; Pollack and Divon, 1992). IUGR results from the inability of the fetus to reach its growth potential *in utero*, reducing perinatal survival and increasing the risk of metabolic disease in adulthood (Gagnon, 2003; Regnault et al., 2002). While the etiologies responsible for IUGR are vast and varied, they can broadly be classified as maternal, fetal, or placental in origin. Of these, the most frequent origin is placental resulting from structural or functional abnormalities (Anthony et al., 2003). More specifically, placental insufficiency is responsible for approximately 60% of growth restricted pregnancies barring fetal malformations (Ghidini, 1996).

A study by Daikoku et al. (1979) found 60% of mothers that gave birth to IUGR infants had reduced circulating chorionic somatomammotropin (CSH) from 36 weeks to term. In fact, a single low (<4 ug/mL) maternal blood sample during late gestation (35 weeks of gestation) was predictive of a 30% increased risk of neonatal hypoxia and fetal distress (Letchworth and Chard, 1972). The causal relationship between CSH and IUGR was demonstrated using lentiviral mediated RNA interference (RNAi) in sheep (RNAi; Baker et al., 2016; Jeckel et al., 2018; Tanner et al., 2021b). Despite the

demonstrated link between CSH and fetal growth, the exact biological function of CSH remains to be fully determined.

To begin to directly assess the physiological ramifications of CSH, recent studies (Tanner et al., 2021a; Tanner et al., 2021b) combined the use of CSH RNAi *in vivo* with the transplacental diffusion technique to measure changes to placental and fetal physiology. Using this approach, the physiological responses of CSH RNAi pregnancies of differing severities including IUGR (Tanner et al., 2021b) and normal weight pregnancies (Tanner et al., 2021a) have been assessed. These divergent phenotypes have also been observed in human pregnancies with either low maternal CSH (Daikoku et al., 1979) or CSH gene loci disruptions (Rygaard et al., 1998). Regardless of fetal weigh reductions, one of the primary biological impacts of CSH appears to be regulating uteroplacental glucose utilization (Tanner et al., 2021a, b). In CSH RNAi pregnancies characterized by IUGR, uteroplacental glucose utilization decreases dramatically (Tanner et al., 2021b). However, it appears that in less severe CSH RNAi phenotypes, a compensatory mechanism responds by increasing uteroplacental glucose utilization to maintain placental function and support fetal growth, despite other perturbations (Tanner et al., 2021a). Based on this evidence, the objective of this study was to test how artificially increasing maternal glucose concentrations, thereby increasing the maternal-fetal glucose gradient, will impact placental glucose utilization and transport in a cohort of CSH RNAi pregnancies. Thus, we hypothesized that CSH RNAi pregnancies would have diminished umbilical glucose uptake despite elevated maternal glucose concentrations, demonstrating perturbations in uteroplacental glucose transfer.

MATERIALS & METHODS

All animal procedures were approved by the Colorado State University Institutional Animal Care and Use Committee (Protocol # KP 6), the Institutional Biosafety Committee (18-029B) and the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee (Protocol #00714).

Lentiviral generation

Lentiviral generation of hLL3.7 tg6 (target 6; CSH RNAi) and hLL3.7 NTS (scramble control/non-targeting sequence; control RNAi) are as described previously (Baker et al., 2016; Jeckel et al., 2018; Tanner et al., 2021a, b). Briefly, both the NTS and tg6 sequences (Baker et al., 2016; Jeckel et al., 2018; Tanner et al., 2021a, b) were cloned into the LL3.7 vector, and all subsequent virus generation and titering were completed in accordance with our previously described procedures (Baker et al., 2016).

Generation of CSH RNAi pregnancies

All ewes (Dorper breed composition) were group housed in pens at the Colorado State University Animal Reproduction and Biotechnology Laboratory and provided access to hay, trace mineral, and water in order to meet or slightly exceed their National Research Council (2007) requirements. All animal procedures were as previously described (Baker et al., 2016; Jeckel et al., 2018; Tanner et al., 2021). In summary, fully expanded and hatched blastocysts were collected by flushing the uteri at 9 days post conception after estrous synchronizing and breeding. Each blastocyst was infected with 100,000 transducing units of either control RNAi or CSH RNAi and surgically transferred

(Baker et al., 2016; Jeckel et al., 2018, Tanner et al., 2021a, Tanner et al., 2021b) into a synchronized recipient ewe. Each recipient ewe was monitored daily for return to standing estrus and confirmed pregnant at 50 days of gestational age (dGA) by ultrasound (ALKOA SSD-500V, Wallingford, CT). Using these methods, a total of 17 pregnancies were generated (n = 8 Control RNAi; n = 9 CSH RNAi).

Doppler velocimetry

At 90 dGA, all pregnancies (n = 17) underwent Doppler velocimetry using a Mindray M7 Premium (Mindray Medical Equipment, Mahwah, NJ) with a convex C5-2s probe (2.1-5.1 MHz) abdominal transducer by a single technician. Methods were as previously described in Tanner et al. (2021b). Briefly, after confirming fetal viability, fetal binocular distance (cm), biparietal circumference (cm), abdominal circumference (cm), femur (cm) and tibia length (cm) were recorded and averaged across 3 independent measurements. Pulse-waved Doppler velocimetry measurements of the umbilical artery were performed with color flow Doppler with an angle of isonation of 30 degrees (Galan et al., 1998, Galan et al., 2005, Tanner et al., 2021b). All Doppler indices of perfusion were recorded on 3 independent sets of 3 cardiac cycles (minimum of 9 cardiac waveforms) and averaged (Galan et al., 1998, Galan et al., 2005, Tanner et al., 2021b). All calculations of blood flow and resistance were as described in Tanner et al. (2021b).

Surgical instrumentation of fetus and ewe

At approximately 115 dGA, pregnant recipient ewes were transported to the University of Colorado Anschutz Medical Campus, Perinatal Research Center (Aurora,

CO). Animals had access to *ad libitum* alfalfa pellets (Standlee Hay, Kimberly, ID) and water. All animals underwent surgical placement of fetal and maternal catheters at 126 dGA, to determine blood flow and nutrient flux as previously described (Bonds et al., 1980; Jones et al., 2019; Brown 2015; Hay et al., 1981; Regnault et al., 2003; Tanner et al., 2021a, b). Briefly, the following catheters were placed: fetal descending aorta (representing umbilical artery blood), fetal femoral vein, and umbilical vein, maternal femoral artery (representing uterine artery blood), maternal femoral vein, and uterine vein. Due to one ewe morbidity (control RNAi) and two fetal demises (1 control RNAi, 1 CSH RNAi), a total of 14 pregnancies were fully studied (n= 6 control RNAi; n= 8 CSH RNAi).

Baseline studies and blood flow calculations

At 136 dGA, uterine and umbilical blood flows were determined by the steady state $^{3}H_2O$ transplacental diffusion technique as summarized previously (Tanner et al., 2021a; Cilvik et al., 2021). Briefly, samples were simultaneously collected from maternal femoral artery (A), uterine vein (V), umbilical vein (γ) and fetal descending aorta (α) simultaneously every 20 minutes and averaged across 4 draws for analysis of blood biochemistry, nutrient content, $^{3}H_2O$ and hormone concentrations (Tanner et al., 2021a).

Uterine and umbilical blood flows were calculated by the steady state diffusion technique described previously (Meschia et al., 1966). Uterine, umbilical, and uteroplacental utilization of oxygen, glucose, lactate, and amino acids were calculated by the $^{3}H_2O$ transplacental diffusion technique (Meschia et al., 1980) and reported as an

average of draws one through four. All other calculations were described extensively in Tanner et al. (2021a, b).

Hyperglycemic clamp study and tissue collection

To test the impact of altering the maternal-fetal glucose gradient on uteroplacental glucose utilization and transfer, ewes were made hyperglycemic by a 3mL bolus of 70% dextrose (wt:vol in saline), followed by a constant infusion of 70% dextrose at a rate of 16 mL/hour for 170 minutes. This procedure was titrated to keep the plasma glucose concentration over 7.0 $mmol \cdot L^{-1}$ (glucose concentrations are ~2.8 $mmol \cdot L^{-1}$ in euglycemic pregnant ewes; Carver et al., 1996). After 90 minutes of dextrose infusion, blood samples were simultaneously drawn from all four vessels every 20 minutes for 80 minutes. One pregnancy in each treatment was excluded from the hyperglycemic clamp analyses for failure to reach hyperglycemic glucose levels, thus leaving 13 pregnancies to be studied (n= 5 control RNAi ; n= 7 CSH RNA). The two pregnancies that failed to reach hyperglycemic glucose levels were still included in all baseline measurements.

Following the last draw at 170 minutes after initiation of the infusion, all ewes and fetuses were euthanized (n = 14), and tissues were harvested at 130 dGA as previously described by Tanner et al. (2021a). Briefly, after trimming, placentomes were selected from each placenta and separated into cotyledonary (fetal) and caruncular (maternal) components then snap frozen in liquid N and stored at -80ºC. Fetal weight and dissected organ weights were recorded, and tissues were snap frozen in liquid nitrogen.

Ponderal index and fetal brain: liver weight ratios were calculated as previously described (Tanner et al., 2021a).

Biochemical analysis of blood samples

Whole blood O_2 content, O_2 saturation (SO_2), partial pressure of oxygen (PO_2), partial pressure of carbon dioxide (PCO_2), pH and hematocrit measurements were analyzed by an ABL 800 Blood Gas analyzer as previously described (Tanner et al., 2021a, b). Plasma glucose and lactate were measured by a Yellow Springs Instrument 2900 (YSI Incorporated, Yellow Springs, OH) as described previously (Tanner et al., 2021a). Baseline plasma amino acids were measured by HPLC (Tanner et al., 2021a, b). Maternal (uterine) and fetal (umbilical) concentrations of insulin were assessed prior to commencement of the baseline study by enzyme-linked immunosorbent assay (ALPCO Immunoassays 80-INSOV-E01) as described previously (Andrews et al., 2015; Benjamin et al., 2017; Cilvik et al., 2021; Tanner et al., 2021b). Umbilical artery insulin concentrations were measured across the baseline study and under hyperglycemic conditions.

Statistical analysis

Data were analyzed by Student's t-test in GraphPad Prism (8.3.1). As previously described, due to one ewe morbidity (control) and a fetal demise in each treatment group, a total of 6 control RNAi (3 females, 3 males) and 8 CSH RNAi (3 females, 5 males) pregnancies were included in this analysis. While all 8 CSH RNAi pregnancies had fetuses that weighed 2 standard deviations below the mean weight of the control

fetuses, a subset (n = 5; 2 females, 3 male) of CSH RNAi fetuses also had placental weights that fell 2 standard deviations below the mean of the control placentas. We classified this subgroup as CSH RNAi placental insufficiency with intrauterine growth restriction (PI- IUGR) to examine the effects of CSH RNAi during placental insufficiency. This is consistent with the Baker et al. (2016) study where pregnancies were classified as IUGR based on the same criteria. Statistical significance was set at $P \leq 0.05$ and a statistical tendency at $P \leq 0.10$. Data are reported as the mean ± standard error of the mean (SEM). The data figures are presented as scatter plots, with the horizontal line representing the mean, and the capped vertical lines representing the SEM. Variability in the n included in each scattergram is due to one or more catheters failing to draw during the baseline and hyperglycemic studies.

RESULTS

90 dGA Doppler measures and velocimetry

As determined by Doppler ultrasound at 90 dGA and summarized in **Table 1**, fetal binocular distance (cm), biparietal circumference (cm), abdominal circumference (cm), and tibia length (cm) did not differ between treatments. Fetal femur length (cm) however, was reduced (P = 0.05) by 8% in CSH RNAi pregnancies.

Umbilical artery blood flow (mL/min), vessel area (cm^2), pulsatility indices (PI), and fetal heart rate (bpm) did not differ between treatments. However, umbilical artery resistance indices (RI) and the systolic: diastolic (S/D) ratios were both reduced (P = 0.03), indicating less vascular resistance in CSH RNAi pregnancies. CSH RNAi

pregnancies characterized by PI-IUGR did not have uniquely impacted growth or hemodynamic parameters at 90 dGA ultrasounds and followed the same patterns as the full CSH RNAi cohort (**Table 1**) .

Table 4.1. Doppler velocimetry and fetal measures for CON RNAi vs CSH RNAi and CON vs CSH RNAi PI-IUGR at 90 dGA.

	CON RNAi vs. CSH RNAi						CON RNAi vs. CSH RNAi PI-IUGR			
	CON RNAi (n=8)	**CON RNAi SEM**	**CSH RNAi (n=9)**	**CSH RNAi SEM**	**P-value**	**% Difference**	**CSH RNAi- PI IUGR (n=5)**	**PI IUGR SEM**	**P-Value**	**% Difference**
Binocular distance, cm	4.89	0.07	4.72	0.13	0.27	3.51	4.88	0.16	0.93	0.27
Biparietal circumference, cm	15.89	0.37	15.21	0.36	0.24	4.27	15.29	0.51	0.38	3.74
Abdominal circumference, cm	20.95	0.48	20.76	0.36	0.99	0.91	20.87	0.55	0.92	0.39
Femur Length, cm	4.94	0.08	4.55	0.13	0.05	8.03	4.53	0.21	0.07	8.34
Tibia Length, cm	3.20	0.1	3.11	0.11	0.63	2.64	3.10	0.20	0.67	3.06
Vessel Area, cm^2	0.14	0.01	0.13	0.02	0.91	8.49	0.14	0.03	1.00	0.03
Peak Systolic Velocity, cm/s	54.07	2.66	51.74	2.40	0.31	4.30	51.35	3.94	0.58	5.02
End Diastolic Velocity, cm/s	15.44	0.66	17.29	1.11	0.14	11.99	17.34	0.99	0.14	12.30
Umbilical artery blood flow, mL/min	120.17	5.59	113.58	11.08	0.86	5.48	121.03	17.94	0.96	0.71
Pulsatility Index	2.02	0.05	1.85	0.12	0.20	8.27	1.87	0.20	0.40	7.10
Resistance Index	0.71	0.01	0.66	0.02	0.03	6.98	0.65	0.03	0.05	8.14
Systolic: Diastolic	3.62	0.11	3.14	0.19	0.03	13.43	3.06	0.26	0.05	15.55
Fetal heart rate, bpm	189.73	3.64	194.30	6.76	0.62	2.41	200.47	11.73	0.32	5.66

CSH, chorionic somatomammotropin; CON, control; RNAi, RNA interference; PI-IUGR, placental insufficiency- intrauterine growth restriction

136 dGA fetal and uteroplacental measures

Fetal mass was reduced (P = 0.02; **Figure 1**) by 21% in CSH RNAi pregnancies. As summarized in **Table 2**, fetal measures of growth were impacted by CSH RNAi with the fetal brain: liver weight ratio, an indicator of asymmetric fetal growth increased (P = 0.02) by 45%, while crown-rump length (cm) tended to be reduced (P = 0.08). However, fetal ponderal index and lower leg length did not differ between treatments. Fetal liver weights were reduced (P = 0.01) by 28% in CSH RNAi pregnancies, with right liver lobes especially being impacted (P = 0.004). Other fetal organs such kidneys (P = 0.01) and hearts (P = 0.03) were reduced in CSH RNAi pregnancies, with the spleen also tending (P = 0.09) to be smaller. However, fetal pancreas, lung, adrenal glands, perirenal adipose tissue, and brain weights were not impacted by treatment. On a relative basis, the brain to fetal weight ratio (P = 0.02) was increased, along with a tendency (P = 0.07) for the pancreas: fetal weight ratio to be increased. Placental weight was reduced (P = 0.003; **Figure 1**) by 37% in CSH RNAi pregnancies, along with both uterine (P = 0.02; **Figure 1**) and uteroplacental weight (P = 0.004). As placental weights were reduced more severely than fetal weight, it is unsurprising that placental efficiency was elevated (P = 0.03) in CSH RNAi pregnancies, however, neither fetal membrane weights nor placentome number were impacted.

While all CSH RNAi pregnancies produced fetal weights that fell 2 standard deviations below the mean weight of the control animals, a subset (n = 5) also produced pregnancies with placental weight reductions following the same criteria. Due to the severity of placental growth restriction (52%; P = 0.00004; **Figure 1**), this group was analyzed against the control pregnancies to examine the effects of CSH RNAi in cases

of placental insufficiency (PI-IUGR). This greater degree of placental growth restriction resulted in 30% reductions (P = 0.001; **Figure 1**) in fetal weight, along with reduced fetal crown rump length (P = 0.01; **Table 2**) and lower leg length (P = 0.004). Additionally, the greater severity and asymmetry of this PI-IUGR phenotype was emphasized by the fetal brain to liver weight ratio which was increased (P = 0.002) by 68%. While the same fetal organs were impacted as noted in the full CSH RNAi group, the degree of growth reductions were greater in the PI-IUGR group. Fetal liver weights were reduced (P = 0.0003) by 41%, kidney (P = 0.002) by 25%, heart (P = 0.001) by 26% and spleen (P = 0.01) by 42% in CSH RNAi PI-IUGR fetuses. Unlike the full CSH RNAi group, CSH RNAi PI-IUGR fetuses also tended (P ≤ 0.10) to have reduced fetal pancreas and lung weights, as well as elevated liver: fetal weight and adrenal: fetal weight ratios. Like the full CSH RNAi group, both uterine (P = 0.04; **Figure 1**) and uteroplacental weights (P = 0.004) were reduced, but uniquely to CSH RNAi PI-IUGR, the placentome number also tended (P = 0.09) to be reduced.

Table 4.2. Fetal and uteroplacental measures recorded at necropsy for CON vs CSH RNAi and CON vs CSH RNAi PI-IUGR at 136 dGA.

	CON RNAi vs. CSH RNAi						CON RNAi vs. CSH RNAi PI-IUGR			
	CON RNAi (n=6)	CON RNAi SEM	CSH RNAi (n=8)	CSH RNAi SEM	P-value	% Difference	CSH RNAi- PI IUGR (n=5)	PI IUGR SEM	P-Value	% Difference
Crown-rump length, cm	51.38	0.71	48.49	1.19	0.08	5.64	46.88	1.37	0.01	8.76
Ponderal index	3.44	0.16	3.17	0.12	0.21	7.77	3.09	0.13	0.15	10.14
Lower leg length, cm	33.37	0.42	32.09	0.94	0.29	3.83	30.82	0.48	0.004	7.63
Pancreas, g	3.91	0.17	3.73	0.28	0.63	4.54	3.39	0.16	0.06	13.40
Pancreas: fetal wight	0.0008	0.0000	0.0010	0.0001	0.07	23.94	0.0011	0.0001	0.05	28.61
Liver, g	125.16	3.32	90.46	9.85	0.01	27.73	74.22	9.04	0.0003	40.70
Liver: fetal wight	0.0271	0.0008	0.0245	0.0014	0.17	9.68	0.0231	0.0018	0.06	14.83
L liver lobe, g	37.24	1.35	29.20	3.86	0.11	21.60	22.68	2.92	0.001	39.10
R liver lobe, g	88.84	3.14	61.98	6.09	0.004	30.23	52.36	6.29	0.0005	41.07
Kidneys, g	23.04	0.92	18.59	0.88	0.01	19.32	17.31	0.89	0.002	24.86
Perirenal adipose tissue, g	9.80	1.12	10.07	0.79	0.85	2.76	10.51	1.19	0.69	7.19
Spleen, g	7.68	0.74	5.71	0.71	0.09	25.70	4.48	0.44	0.01	41.64
Adrenal glands, g	0.42	0.02	0.44	0.04	0.64	6.08	0.48	0.06	0.28	15.22
Adrenals: fetal wight	0.0001	0.0000	0.0001	0.0000	0.20	49.13	0.0002	0.0000	0.07	81.12
Brain, g	51.80	1.11	50.48	1.60	0.55	2.55	49.37	2.38	0.36	4.70
Brain: fetal wight	0.0112	0.0002	0.0143	0.0010	0.02	27.63	0.0157	0.0011	0.002	40.44
Brain: liver	0.42	0.01	0.60	0.06	0.02	45.40	0.70	0.07	0.002	67.86
Lungs, g	137.56	10.36	123.79	13.74	0.47	10.01	106.17	13.44	0.10	22.82
Heart, g	31.37	1.31	25.78	1.60	0.03	17.81	23.26	0.56	0.001	25.83
Placental efficiency	9.07	0.47	11.82	0.84	0.03	30.26	13.04	0.96	0.004	43.72
Fetal membrane, g	635.83	84.81	416.26	91.87	0.12	34.53	391.96	144.99	0.17	38.35
Placentomes, #	70.50	5.42	63.38	3.71	0.30	10.11	58.00	1.79	0.09	17.73
Uteroplacental weight, g	1912.41	80.07	1322.32	126.81	0.004	30.86	1201.30	175.47	0.004	37.18

CSH, chorionic somatomammotropin; CON, control; RNAi, RNA interference; PI-IUGR, placental insufficiency- intrauterine growth restriction

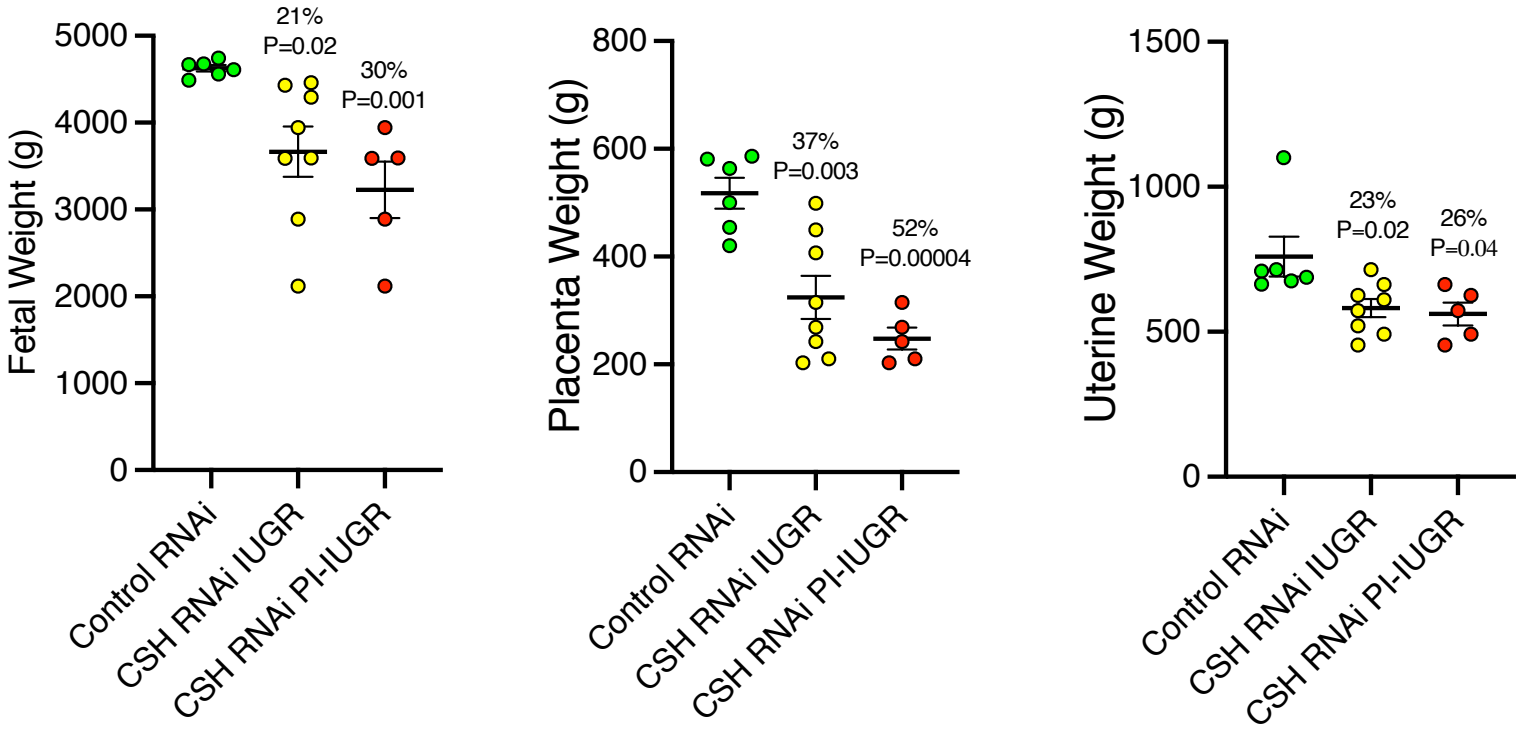


Figure 4.1: Measures of fetal weight: (a) placental weight; (b) and uterine weight; (c) at 136 dGA. Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference; PI-IUGR; placental insufficiency intrauterine growth restriction

Blood flow during basal conditions

As assessed by the $^{3}H_{2}0$ transplacental diffusion and Fick principle technique near term (136 dGA), uterine blood (P = 0.02; **Figure 2**) and plasma (P = 0.04) flow (mL/min) were reduced by 34% in CSH RNAi pregnancies. However, on a relative (mL/min/kg uterus, fetus, or placenta) neither uterine blood nor plasma flow differed between treatments. Umbilical blood (P = 0.02; **Figure 2**) and plasma (P = 0.01) flow (mL/min) were reduced by 28% and 33%, respectively, in CSH RNAi pregnancies. This impact was also observed somewhat independent of fetal weight as relative (mL/min/kg fetus) plasma flow (P = 0.02) was reduced and relative umbilical blood flow tended (P = 0.07) to be reduced in CSH RNAi pregnancies. Neither umbilical blood flow relative to placental weight (mL/min/100 g placenta) nor the uterine: umbilical blood flow ratio differed between treatments. While uterine hematocrit was not altered by treatment, umbilical vein hematocrit tended (P = 0.06) to be elevated in CSH RNAi fetuses.

In CSH RNAi pregnancies characterized by PI-IUGR, uterine blood (P = 0.03; **Figure 1**) and plasma flow (mL/min; P = 0.04) were each reduced by 41%, but not on a relative (mL/min/kg) basis. Umbilical blood flow (mL/min) was reduced (P = 0.004; **Figure 2**) by 42% in CSH RNAi PI-IUGR fetuses, and umbilical plasma flow was reduced (P = 0.004) by 48%. Like the full cohort of CSH RNAi pregnancies, relative (mL/min/kg fetus) plasma flow (P = 0.02) was reduced, and umbilical blood tended to be reduced (P = 0.07) in CSH RNAi PI-IUGR fetuses.

Table 4.3. Baseline uterine and umbilical blood flow for CON RNAi vs CSH RNAi and CON RNAi vs CSH RNAi PI-IUGR at 136 dGA.

	CON RNAi vs. CSH RNAi						CON RNAi vs. CSH RNAi PI-IUGR			
	CON RNAi (n=6)	CON RNAi SEM	CSH RNAi (n=8)	CSH RNAi SEM	P-value	% Difference	CSH RNAi-PI IUGR (n=5)	PI IUGR SEM	P-Value	% Difference
Uterine measurements										
Plasma flow, mL/min	1696.26	355.01	1119.31	243.07	0.04	34.01	1003.77	279.10	0.04	40.82
Blood flow, mL/min/kg fetus	533.54	109.05	454.34	91.01	0.24	14.84	457.36	113.31	0.37	14.28
Plasma flow, mL/min/kg fetus	365.39	77.20	313.05	62.67	0.30	14.32	314.82	77.94	0.43	13.84
Blood flow, mL/min/kg uterus	3603.17	743.53	2925.00	625.71	0.22	18.82	2644.01	706.21	0.15	26.62
Plasma flow, mL/min/kg uterus	2466.96	525.77	2016.93	431.08	0.27	18.24	1822.34	486.54	0.18	26.13
Blood flow, mL/min/100g placenta	462.60	92.06	529.58	112.57	0.41	14.48	576.79	147.65	0.23	24.69
Umbilical measurements										
Plasma flow, mL/min	556.25	29.57	370.95	82.20	0.01	33.31	289.30	103.47	0.004	47.99
Plasma flow, mL/min/kg fetus	120.34	6.77	95.36	18.81	0.02	20.76	87.62	28.13	0.02	27.20
Blood flow, mL/min/100g placenta	168.79	16.18	174.53	35.15	0.80	3.40	184.88	60.16	0.57	9.53
Uterine: Umbilical Flow	3.06	0.65	2.81	0.68	0.60	7.92	2.82	0.93	0.69	7.65
Umbilical vein hematocrit	0.35	0.01	0.40	0.08	0.06	15.61	0.43	0.14	0.06	22.53
Uterine artery hematocrit	0.32	0.01	0.31	0.04	0.54	2.59	0.31	0.07	0.49	3.72

CSH, chorionic somatomammotropin; CON, control; RNAi, RNA interference; PI-IUGR, placental insufficiency- intrauterine growth restriction

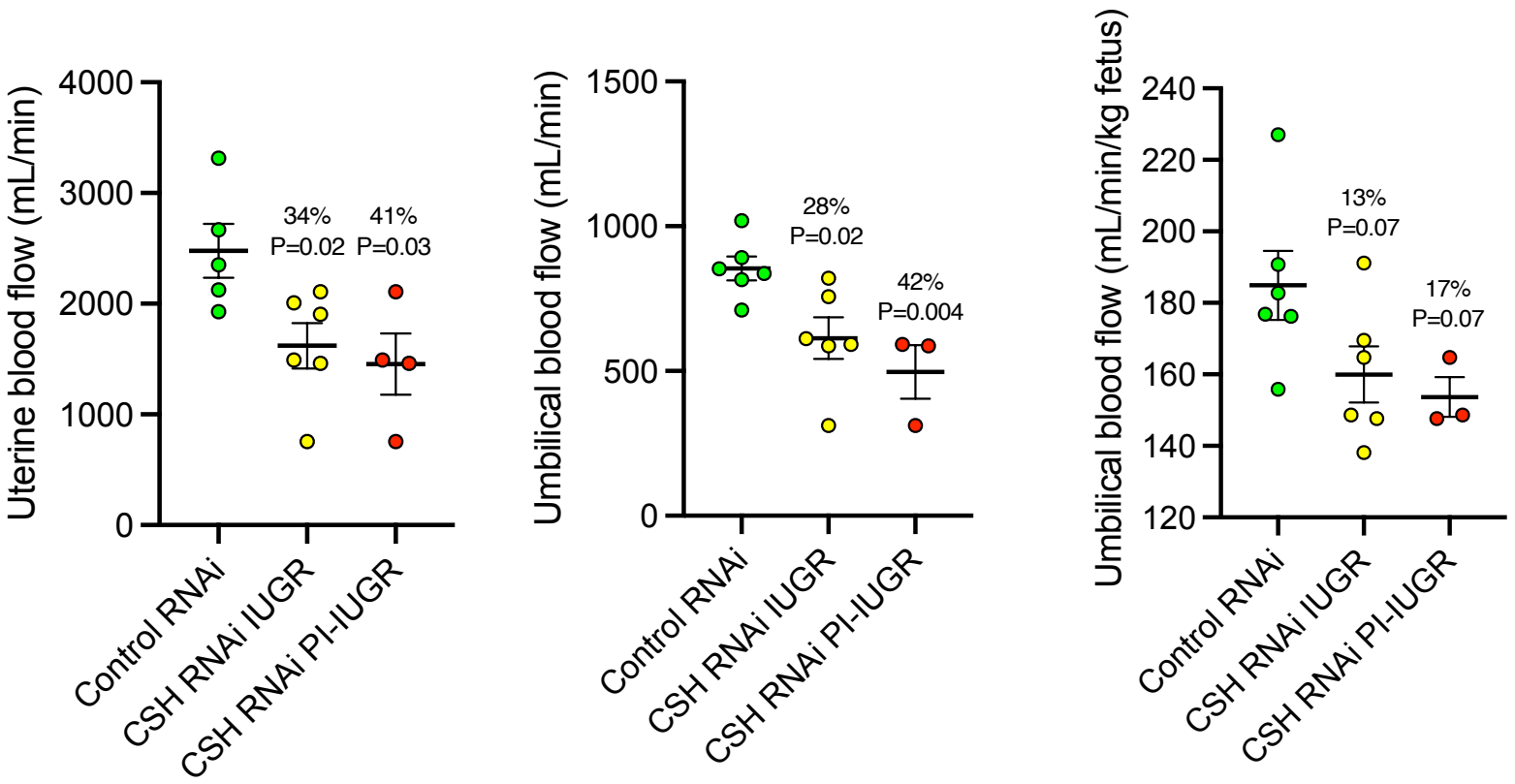


Figure 4.2: Measures of uterine blood flow (mL/min); (a) umbilical blood flow (mL/min); (b) and relative umbilical blood flow (mL/min/kg fetus); (c) at 136 dGA as assessed by the $^{3}H_{2}0$ transplacental diffusion technique. Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference; PI-IUGR; placental insufficiency intrauterine growth restriction.

Oxygen transfer during basal conditions

Under basal conditions, uterine oxygen uptake (mmol/min) was reduced (P = 0.005; **Figure 3**) by 34% in CSH RNAi pregnancies, but not on a relative basis (mmol/min/kg uterus; **Table 4**). While umbilical oxygen uptake (mmol/min) was not statistically different (P = 0.11; **Figure 3**), it was still numerically lower by 21%. Furthermore, uteroplacental oxygen utilization (mmol/min) was decreased (P = 0.03; **Figure 3**) by 41% in CSH RNAi pregnancies, although not on a relative basis (mmol/min/kg placenta). The fraction of uterine oxygen uptake utilized by the placenta or transferred to the fetus did not differ under basal conditions.

In CSH RNAi pregnancies characterized by PI-IUGR, uterine oxygen uptake (mmol/min) was decreased (P = 0.005; **Figure 3**) by 40%, following a similar pattern to the full CSH RNAi cohort. However, the impacts of CSH RNAi in PI-IUGR pregnancies were emphasized by significant reductions (26%) in relative (mmol/min/kg uterus) uterine oxygen uptake (P = 0.03; **Table 4**) pointing to greater dysfunction in oxygen uptake independent of uterine mass. CSH RNAi PI-IUGR fetuses also exhibited reduced (40%) umbilical oxygen uptake (P = 0.02; **Figure 3**) but not relative to fetal weight (mmol/min/kg fetus). Uteroplacental oxygen utilization (mmol/min) tended (P = 0.06) to be reduced in CSH RNAi PI-IUGR pregnancies, but not relative to placental weight (mmol/min/kg placenta).

Table 4.4. Baseline nutrient uptake and utilization for CON vs CSH RNAi and CON vs CSH RNAi PI-IUGR at 136 dGA.

	CON RNAi vs. CSH RNAi						CON RNAi vs. CSH RNAi PI-IUGR			
	CON RNAi (n=6)	CON RNAi SEM	CSH RNAi (n=8)	CSH RNAi SEM	P-value	% Difference	CSH RNAi- PI IUGR (n=5)	PI IUGR SEM	P-Value	% Difference
Oxygen										
Uterine O2 uptake, mmol/min/kg uterus	4.37	0.81	3.56	0.75	0.12	18.36	3.24	0.81	0.03	25.88
Umbilical O2 uptake, mmol/min/kg fetus	0.33	0.02	0.32	0.06	0.61	5.07	0.29	0.10	0.35	12.57
Uteroplacental O2 utilization, mmol/min/kg placenta	2.84	0.54	2.80	0.80	0.95	1.43	3.44	1.23	0.36	21.25
Uterine fraction of O2 utilized by placenta	0.51	0.10	0.43	0.26	0.33	14.23	0.49	0.16	0.79	3.37
Uterine fraction of O2 transferred to fetus	0.49	0.09	0.57	0.26	0.33	14.64	0.51	0.17	0.79	3.46
Glucose										
Uterine artery glucose concentrations, mmol/L	3.31	0.07	3.32	0.11	0.93	0.39	3.38	0.15	0.64	2.26
Uterine glucose uptake, µmol/min/kg uterus	612.35	121.83	516.90	107.10	0.24	15.59	477.06	122.78	0.16	22.09
Umbilical artery glucose concentrations, mmol/L	1.02	0.04	0.77	0.08	0.03	24.16	0.66	0.12	0.01	35.16
Umbilical uptake, µmol/min/kg fetus	29.79	2.32	25.39	5.08	0.18	14.77	24.34	8.26	0.25	18.28
Uterine artery to umbilical artery glucose gradient, mmol/L	2.29	0.08	2.64	0.17	0.12	15.56	2.7387	0.6356	0.04	20.05
Uteroplacental utilization, µmol/min/kg placenta	550.88	117.64	613.03	173.92	0.69	11.28	689.87	269.16	0.48	25.23
Uterine fraction of glucose utilized by placenta	0.69	0.13	0.67	0.16	0.75	2.10	0.68	0.22	0.90	1.06
Uterine fraction of glucose transferred to fetus	0.31	0.06	0.33	0.08	0.75	4.59	0.32	0.11	0.90	2.31
Lactate										
Uterine lactate export, µmol/min	143.58	35.41	79.89	21.06	0.07	44.36	84.02	28.64	0.15	41.48
Uterine lactate export, µmol/min/kg uterus	209.45	52.66	139.64	33.74	0.16	33.33	147.63	45.13	0.30	29.52
Umbilical lactate uptake, µmol/min	148.77	11.55	86.30	22.21	0.01	41.99	72.95	26.12	0.01	50.96
Umbilical lactate uptake, µmol/min/kg fetus	32.14	2.40	22.51	5.27	0.05	29.96	22.22	7.27	0.04	30.86
Uteroplacental lactate production, µmol/min	292.03	57.20	173.02	47.21	0.02	40.75	176.89	61.49	0.03	39.43
Uteroplacental lactate production, µmol/min/kg placenta	546.97	107.07	550.79	151.46	0.97	0.70	656.80	217.40	0.26	20.08
Uterine fraction of lactate utilized by placenta	0.48	0.10	0.54	0.13	0.45	12.52	0.59	0.19	0.24	22.95
Uterine fraction of lactate transferred to fetus	0.52	0.11	0.46	0.11	0.45	11.63	0.41	0.13	0.24	21.32

CSH, chorionic somatomammotropin; CON, control; RNAi, RNA interference; PI-IUGR, placental insufficiency- intrauterine growth restriction

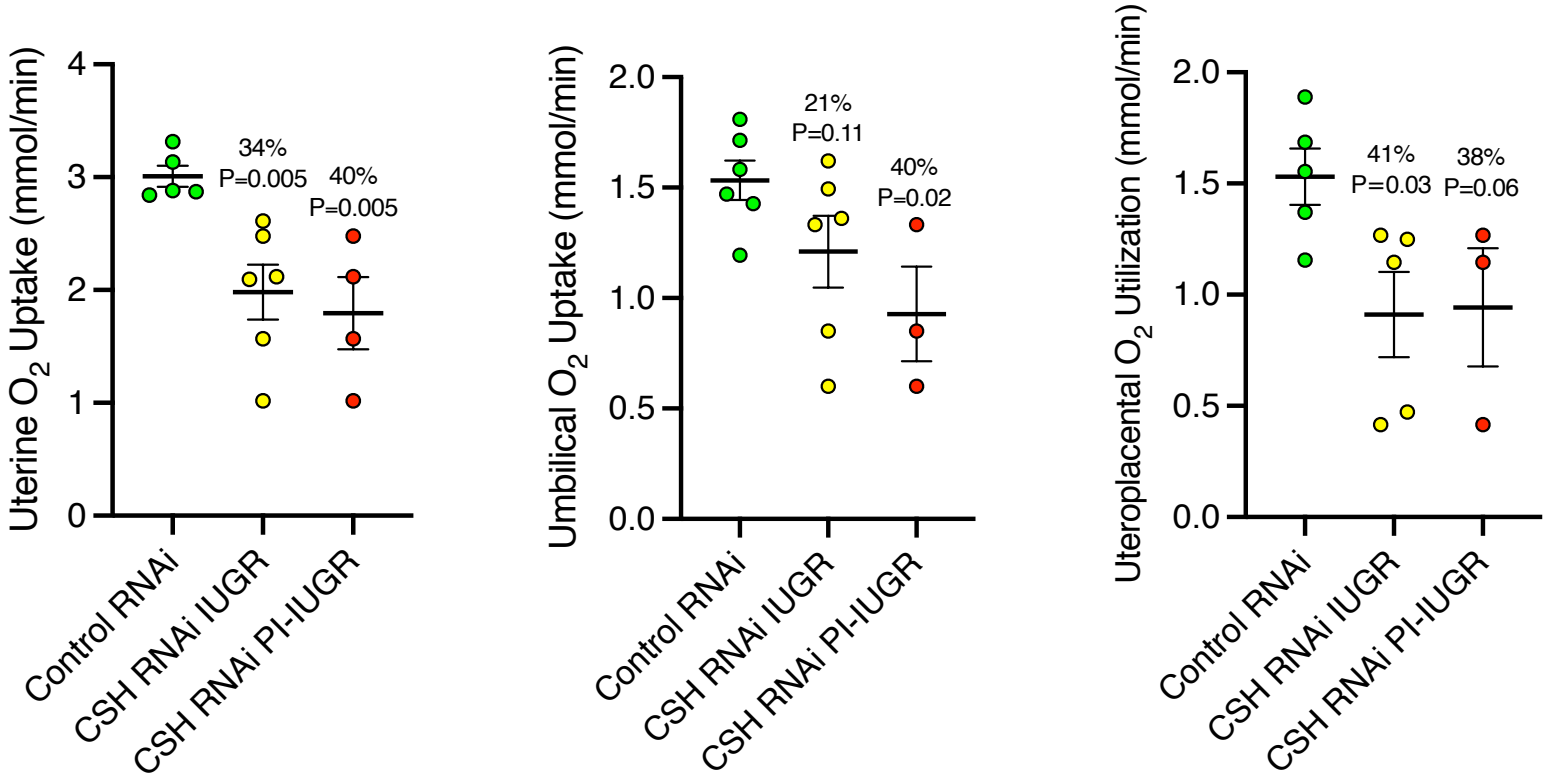


Figure 4.3: Measures of uterine oxygen uptake (mmol/min); (a) umbilical oxygen uptake (mmol/min); (b) and uteroplacental oxygen utilization (mmol/min); (c) at 136 dGA as assessed by the $^{3}H_{2}0$ transplacental diffusion technique. Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference; PI-IUGR; placental insufficiency intrauterine growth restriction.

Blood biochemistry during basal conditions

As summarized in **Table 5**, the only uterine blood biochemical measure which was altered in CSH RNAi pregnancies was uterine artery chloride concentrations (meq/L) which tended (P = 0.09) to be reduced. However, in ewes with CSH RNAi PI-IUGR pregnancies, not only was uterine artery chloride (meq/L) concentrations reduced (P = 0.03) but potassium concentrations (meq/L) also tended (P = 0.08) to be suppressed and methemoglobin (Met Hb%) levels tended (P = 0.06) to be increased. Additionally, uterine vein sodium (meq/L) was reduced (P = 0.05; **Table 5**) in ewes with CSH RNAi PI-IUGR pregnancies.

Fetal blood biochemistry (**Table 6**) was also impacted by CSH RNAi as umbilical arterial pO_2 (mmHg) and calcium concentrations (meq/L) both decreased ($P \leq 0.05$) in CSH RNAi pregnancies. Furthermore, umbilical artery oxyhemoglobin (O_2 Hb %, P = 0.07) and oxygen saturation (SO_2 %; P = 0.07) both tended to be decreased in CSH RNAi fetuses. Umbilical venous pO_2 (mmHg), oxyhemoglobin (O_2 Hb %) and oxygen saturation (SO_2 %) all were decreased ($P \leq 0.05$; **Table 6**) in CSH RNAi fetuses, while hemoglobin (tHb mmol/L; P = 0.05) was elevated. Fetuses from PI-IUGR CSH RNAi also experienced similar reductions ($P \leq 0.05$) to the full CSH RNAi cohort in umbilical arterial pO_2 (mmHg), oxyhemoglobin (O_2 Hb %), oxygen saturation (SO_2 %), and calcium (meq/L). Uniquely, the PI-IUGR fetuses also tended ($P \leq 0.10$) to have elevated umbilical artery pH and HCO^{3-}. Umbilical vein blood biochemistry of PI-IUGR fetuses was similarly impacted to the entire cohort of CSH RNAi pregnancies.

Table 4.5. Uterine baseline blood gases in CON vs CSH RNAi and CON vs PI-IUGR at 136 dGA.

	CON RNAi vs. CSH RNAi						CON RNAi vs. CSH RNAi PI-IUGR			
	CON RNAi (n=6)	CON SEM	CSH RNAi (n=8)	CSH RNAi SEM	P-Value	% Difference	CSH RNAi-PI IUGR (n=5)	PI IUGR SEM	P-Value	% Difference
Uterine Artery (A)										
pH $^{37.9C}$	7.45	0.01	7.46	0.01	0.26	0.15	7.46	0.01	0.30	0.13
pCO_2 $^{37.9C}$ mmHg	36.42	0.98	34.89	0.77	0.25	4.20	34.09	1.07	0.16	6.39
pO_2 $^{37.9C}$ mmHg	80.05	10.62	87.37	5.02	0.53	9.14	93.76	1.70	0.31	17.12
HCO^3-	24.38	0.61	24.06	0.65	0.74	1.33	23.37	0.65	0.33	4.18
tHb mmol/L	6.33	0.22	6.30	0.13	0.90	0.53	6.22	0.15	0.70	1.87
O_2 Hb %	91.42	0.56	91.86	0.58	0.62	0.48	92.34	0.82	0.37	1.00
SO_2 %	96.70	0.47	96.54	0.67	0.86	0.17	96.93	0.93	0.83	0.23
Met Hb %	1.31	0.06	1.20	0.05	0.20	8.33	1.14	0.07	0.06	13.52
O_2 ct mmol/L	5.93	0.21	5.89	0.12	0.88	0.63	5.86	0.15	0.81	1.18
Na^+ meq/L	146.29	0.52	147.19	0.98	0.49	0.61	146.80	1.41	0.75	0.35
K^+ meq/L	4.20	0.04	4.08	0.07	0.19	3.07	4.02	0.08	0.08	4.50
Ca^{2+} meq/L	2.63	0.03	2.66	0.06	0.78	0.87	2.58	0.05	0.43	2.00
CL^- meq/L	113.00	0.57	111.28	0.66	0.09	1.52	110.60	0.91	0.03	2.12
Uterine Vein (V)										
pH $^{37.9C}$	7.43	0.01	7.43	0.01	0.15	0.10	7.42	0.01	0.27	0.14
pCO_2 $^{37.9C}$ mmHg	39.80	1.16	40.09	0.81	0.98	0.74	39.81	1.22	1.00	0.03
pO_2 $^{37.9C}$ mmHg	54.86	6.44	55.67	4.42	0.54	1.47	60.01	3.17	0.56	9.39
HCO^3-	25.56	0.65	25.28	0.70	0.43	1.10	24.91	0.77	0.56	2.51
tHb mmol/L	6.38	0.25	6.28	0.14	0.67	1.52	6.26	0.21	0.74	1.94
O_2 Hb %	72.55	1.04	71.55	0.88	0.52	1.38	71.86	1.30	0.70	0.95
SO_2 %	75.54	1.12	74.40	0.94	0.50	1.50	74.71	1.41	0.67	1.10
Met Hb %	1.21	0.07	1.16	0.06	0.72	4.27	1.12	0.07	0.43	7.54
O_2 ct mmol/L	4.70	0.18	4.55	0.13	0.52	3.10	4.56	0.19	0.64	2.93
Na^+ meq/L	145.75	0.32	145.96	0.99	0.28	0.14	144.63	0.31	0.05	0.77
K^+ meq/L	4.19	0.06	4.08	0.09	0.18	2.43	4.00	0.11	0.17	4.42
Ca^{2+} meq/L	2.58	0.04	2.60	0.09	0.41	1.11	2.50	0.05	0.26	3.00
CL^- meq/L	111.50	0.36	111.96	0.99	0.80	0.41	110.56	0.66	0.24	0.84

CSH, chorionic somatomammotropin; CON, control; RNAi, RNA interference; PI-IUGR, placental insufficiency- intrauterine growth restriction

Table 4.6. Umbilical baseline blood gases in CON vs CSH RNAi and CON vs PI-IUGR at 136 dGA.

	CON RNAi vs. CSH RNAi						CON RNAi vs. CSH RNAi PI-IUGR			
	CON RNAi (n=6)	CON SEM	CSH RNAi (n=8)	CSH RNAi SEM	P-value	% Difference	CSH RNAi-PI IUGR (n=5)	PI IUGR SEM	P-Value	% Difference
Umbilical artery (α)										
pH $^{37.9C}$	7.36	0.01	7.37	0.01	0.35	0.17	7.38	0.01	0.08	0.35
pCO_2 $^{37.9C}$ mmHg	51.18	1.40	51.58	0.96	0.82	0.78	51.37	1.56	0.93	0.38
pO_2 $^{37.9C}$ mmHg	26.75	0.25	22.88	1.32	0.02	14.46	21.61	2.08	0.02	19.23
HCO^3-	27.29	0.35	28.43	0.76	0.23	4.17	29.19	1.16	0.10	6.97
tHb mmol/L	7.04	0.18	7.89	0.49	0.16	12.05	8.20	0.87	0.15	16.52
O_2 Hb %	53.42	1.65	42.77	4.73	0.07	19.94	38.14	7.67	0.05	28.59
SO_2 %	54.63	1.67	43.74	4.85	0.07	19.93	38.96	7.84	0.04	28.68
Met Hb %	0.30	0.05	0.42	0.07	0.20	40.48	0.44	0.12	0.26	47.92
O_2 ct mmol/L	3.85	0.09	3.30	0.33	0.17	14.19	3.02	0.56	0.11	21.51
Na^+ meq/L	140.50	0.66	141.18	0.47	0.43	0.48	140.50	0.40	1.00	0.00
K^+ meq/L	3.70	0.09	3.78	0.09	0.53	2.34	3.86	0.09	0.28	4.51
Ca^{2+} meq/L	2.93	0.04	2.79	0.04	0.05	4.71	2.74	0.06	0.03	6.22
CL^- meq/L	107.13	1.02	106.32	1.48	0.68	0.75	104.88	2.16	0.34	2.10
Umbilical vein (γ)										
pH $^{37.9C}$	7.39	0.01	7.41	0.01	0.16	0.22	7.42	0.01	0.15	0.29
pCO_2 $^{37.9C}$ mmHg	44.16	1.21	44.36	1.06	0.91	0.44	44.02	1.84	0.95	0.33
pO_2 $^{37.9C}$ mmHg	38.30	2.68	27.39	2.26	0.01	28.50	26.83	2.65	0.03	29.96
HCO^3-	25.78	0.36	26.96	1.07	0.35	4.57	27.10	1.79	0.40	5.11
tHb mmol/L	6.95	0.17	8.05	0.44	0.05	15.85	8.30	0.79	0.08	19.50
O_2 Hb %	80.89	1.61	65.94	5.15	0.03	18.48	61.61	8.81	0.03	23.83
SO_2 %	83.69	1.50	68.30	5.41	0.03	18.40	63.26	9.09	0.03	24.42
Met Hb %	0.03	0.10	0.29	0.20	0.28	1071.43	0.12	0.14	0.60	375.00
O_2 ct mmol/L	5.65	0.13	5.22	0.35	0.30	7.72	4.97	0.62	0.22	12.12
Na^+ meq/L	140.92	0.77	142.14	1.20	0.44	0.87	142.00	2.13	0.60	0.77
K^+ meq/L	3.68	0.09	3.75	0.09	0.57	2.02	3.89	0.08	0.16	5.66
Ca^{2+} meq/L	3.00	0.03	2.97	0.06	0.72	0.94	2.93	0.10	0.45	2.41
CL^- meq/L	108.92	1.17	107.29	1.44	0.42	1.50	106.00	2.26	0.26	2.68

CSH, chorionic somatomammotropin; CON, control; RNAi, RNA interference; PI-IUGR, placental insufficiency- intrauterine growth restriction

Glucose transfer during basal conditions

Under basal conditions, uterine artery glucose concentrations (mmol/L) did not differ between treatments. Uterine glucose uptake (μmol/min) was reduced (P = 0.03; **Figure 4**) by 31% in CSH RNAi pregnancies, but not on a relative basis (μmol/min/kg uterus). Umbilical artery glucose concentrations were reduced (P = 0.03) in CSH RNAi fetuses. Umbilical glucose uptake (μmol/min) was reduced (P = 0.03; **Figure 4**) by 29% in CSH RNAi pregnancies, but not when normalized to fetal mass (μmol/min/kg fetus). While not statistically different (P = 0.12; **Figure 4**), uteroplacental glucose utilization (μmol/min) was numerically reduced by 31% in CSH RNAi pregnancies. Relative uteroplacental glucose utilization (μmol/min/ kg placenta) was not different between treatments. The fraction of uterine glucose uptake utilized by the placenta or transferred to the fetus did not differ under basal conditions. CSH RNAi pregnancies characterized by PI-IUGR, had similar patterns for reduced glucose uptake and utilization to all CSH RNAi pregnancies (**Table 4**).

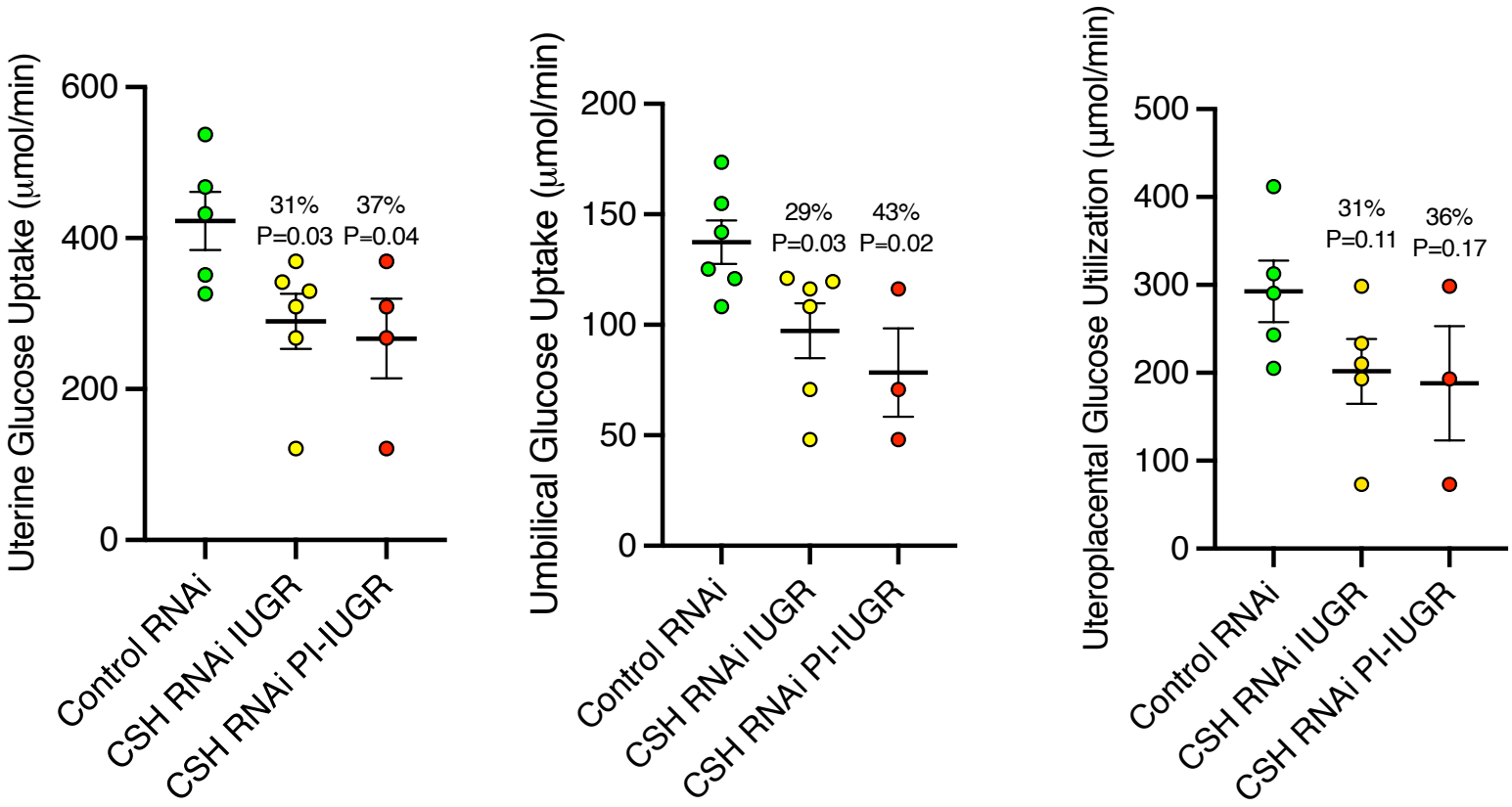


Figure 4.4: Measures of uterine glucose uptake (µmol/min); (a) umbilical glucose uptake (µmol/min); (b) and uteroplacental glucose utilization (µmol/min); (c) at 136 dGA as assessed by the $^{3}H_{2}0$ transplacental diffusion technique. Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference; PI-IUGR; placental insufficiency intrauterine growth restriction.

Lactate transfer during basal conditions

The uterine export of lactate (μmol/min) under basal conditions tended (P = 0.07; **Table 4**) to be reduced in CSH RNAi pregnancies, although not on a relative basis (μmol/min/kg uterus). This resulted in reduced (P ≤ 0.05; **Table 4**) umbilical lactate (μmol/min) and relative lactate uptake (μmol/min/kg fetus) in CSH RNAi pregnancies. Uteroplacental lactate production (μmol/min) was also reduced (P = 0.02; **Table 4**) in CSH RNAi pregnancies. On a relative basis however, uteroplacental lactate production (μmol/min/kg placenta) did not differ between treatments. Nor were the fractions of uterine lactate utilized by the placenta or transferred to the fetus impacted by treatment.

CSH RNAi pregnancies with IUGR followed similar patterns. While uterine lactate export was not significantly impacted (P = 0.15; **Table 4**) in CSH RNAi PI-IUGR pregnancies, they were numerically reduced by a similar degree to the full cohort of CSH RNAi pregnancies (41% PI-IUGR vs. 44% CSH RNAi). Likewise, uteroplacental lactate production was reduced (P = 0.03; **Table 4**) by similar amounts to the full CSH RNAi cohort (39% PI-IUGR vs. 41% CSH RNAi). Furthermore, both umbilical lactate and relative lactate uptake were reduced (P ≤ 0.05; **Table 4**) in PI-IUGR fetuses, mirroring the pattern of reductions observed in the full CSH RNAi group.

Amino acid transfer during basal conditions

One of the greatest distinctions between the full CSH RNAi cohort and the subgroup with placental insufficiency are amino acids uptakes and utilization. In the full CSH RNAi cohort, the only uterine amino acid uptake (μmol/min; **Table 7**) impacted was threonine (P = 0.04), which was reduced by 49% in contrast to control pregnancies, and no amino

acids were impacted on a relative basis (μmol/min/kg uterus; **Table 8**). However, in CSH RNAi pregnancies characterized by PI-IUGR, the uterine uptakes of threonine, serine, isoleucine, and leucine were all reduced ($P \leq 0.05$; **Table 7**), and the uptakes of glutamine and ornithine both tended ($P \leq 0.10$) to be reduced. On a relative basis (μmol/min/kg uterus; **Table 8**), the uptakes of threonine and isoleucine were both reduced ($P \leq 0.05$), and the uptakes of serine and leucine both tended ($P \leq 0.10$) to be reduced. It appears that in cases of CSH RNAi induced PI-IUGR, the uterine uptakes of more amino acids are impacted.

The umbilical uptakes (μmol/min; **Table 9**) of glutamine, leucine, phenylalanine, and lysine were all reduced ($P \leq 0.05$) in all CSH RNAi fetuses, and the uptakes of tyrosine and tryptophan tended to be reduced ($P \leq 0.10$). On a relative basis (μmol/min/kg fetus; **Table 10**), the umbilical uptake of phenylalanine was reduced ($P = 0.004$) and the uptake of leucine ($P = 0.10$) tended to be reduced in CSH RNAi fetuses. In CSH RNAi fetuses characterized by PI-IUGR, the umbilical uptakes (μmol/min; **Table 9**) of asparagine, glutamine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, and lysine were reduced ($P \leq 0.05$), and the uptakes of glycine, methionine, histidine, and arginine tended ($P \leq 0.10$) to be reduced. Additionally, the production of glutamate in CSH RNAi fetuses with PI-IUGR was reduced ($P = 0.03$) by 69%. On a relative basis (μmol/min/kg fetus; **Table 10**), the uptakes of asparagine, alanine, leucine, tyrosine, and phenylalanine were reduced ($P \leq 0.05$), and the uptake of glutamine tended ($P = 0.07$) to be reduced. Furthermore, the relative production (μmol/min/kg fetus) of glutamate by the fetal liver tended ($P = 0.06$) to be reduced in CSH RNAi fetuses with PI-IUGR.

The uteroplacental utilization (μmol/min; **Table 11**) of alanine and phenylalanine was greater in all CSH RNAi pregnancies, and the utilization of both asparagine and glycine tended to be greater ($P \leq 0.10$). On a relative basis (μmol/min/kg placenta; **Table 12**), the uteroplacental utilization of asparagine, alanine, and phenylalanine tended ($P \leq 0.10$) to be greater in CSH RNAi pregnancies. However, the utilization of aspartate tended ($P = 0.08$) to be reduced in CSH RNAi pregnancies. In CSH RNAi pregnancies characterized by PI-IUGR, only phenylalanine utilization was greater than control pregnancies, and both absolute (μmol/min; **Table 11**) and relative asparagine utilization (μmol/min/kg placenta; **Table 12**) tended ($P = 0.08$) to be greater.

Table 4.7. Baseline uterine amino acid uptakes (μmol/min) at 136 dGA.

	CON RNAi vs. CSH RNAi						CON RNAi vs. CSH RNAi PI-IUGR			
	CON RNAi (n=6)	CON RNAi SEM	CSH RNAi (n=8)	CSH RNAi SEM	P-Value	% Difference	CSH RNAi-PI IUGR (n=5)	PI IUGR SEM	P-Value	% Difference
TAU	-0.98	1.96	-1.52	1.15	0.83	55.73	-2.08	1.13	0.69	113.18
ASP	-0.59	0.70	-1.54	0.48	0.30	160.54	-1.08	0.54	0.64	83.23
THR	13.89	3.25	7.10	2.02	0.04	48.87	5.31	1.76	0.02	61.76
SER	12.03	2.75	8.92	2.82	0.38	25.90	5.32	1.53	0.02	55.81
ASN	2.91	1.43	4.42	1.89	0.57	52.11	2.68	1.64	0.92	7.99
GLU	-3.39	1.66	0.60	1.29	0.11	117.69	-0.63	0.86	0.24	81.37
GLN	27.66	7.95	17.24	4.78	0.21	37.69	11.51	3.62	0.09	58.40
PRO	13.21	5.21	10.86	3.20	0.69	17.80	6.37	1.63	0.29	51.77
GLY	-9.31	8.15	0.53	4.77	0.36	105.73	-6.93	3.65	0.83	25.54
ALA	7.06	3.16	6.15	3.18	0.85	12.93	0.91	1.34	0.16	87.09
CIT	12.27	9.81	11.72	5.86	0.96	4.47	2.73	3.26	0.47	77.78
VAL	25.20	7.30	20.04	5.76	0.54	20.46	12.46	3.43	0.13	50.54
CYS	-0.63	0.98	1.15	0.82	0.24	282.84	0.34	0.47	0.49	154.75
MET	3.57	1.62	2.34	0.84	0.50	34.60	1.47	0.92	0.34	58.96
ILE	16.85	3.45	12.30	3.26	0.21	27.02	8.20	1.92	0.004	51.35
LEU	20.13	4.18	15.30	3.99	0.27	24.01	10.96	2.72	0.01	45.59
TYR	4.38	2.05	3.23	1.68	0.69	26.35	1.35	1.59	0.33	69.20
PHE	3.78	1.19	3.50	1.50	0.89	7.33	1.72	0.61	0.17	54.39
TRP	3.80	1.45	3.27	0.67	0.70	13.86	2.98	0.70	0.63	21.68
ORN	14.79	4.17	10.22	2.74	0.29	30.88	6.85	1.94	0.10	53.69
LYS	13.30	4.40	11.45	3.82	0.75	13.85	6.18	2.23	0.20	53.49
HIS	5.83	1.76	3.55	0.88	0.19	39.16	2.63	0.75	0.12	54.87
ARG	9.69	8.03	14.93	4.71	0.59	54.05	9.05	2.41	0.95	6.66

CSH, chorionic somatomammotropin; CON, control; RNAi, RNA interference; PI-IUGR, placental insufficiency- intrauterine growth restriction

Table 4.8. Baseline relative (µmol/min /kg uterus) uterine amino acid uptakes at 136 dGA.

	CON RNAi vs. CSH RNAi						CON RNAi vs. CSH RNAi PI-IUGR			
	CON RNAi (n=6)	CON RNAi SEM	CSH RNAi (n=8)	CSH RNAi SEM	P-Value	% Difference	CSH RNAi- PI IUGR (n=5)	PI IUGR SEM	P-Value	% Difference
TAU	-1.56	2.88	-2.52	2.12	0.81	61.29	-3.70	2.18	0.62	137.24
ASP	-0.84	1.02	-2.91	0.89	0.17	246.34	-2.19	1.06	0.43	160.10
THR	20.12	4.72	12.68	3.37	0.12	36.97	9.81	3.08	0.05	51.27
SER	17.51	4.05	16.13	4.75	0.80	7.88	10.20	3.03	0.09	41.77
ASN	4.12	2.05	7.40	3.08	0.44	79.71	4.40	2.64	0.94	6.86
GLU	-4.92	2.39	1.16	2.45	0.15	123.67	-1.30	1.58	0.30	73.63
GLN	40.14	11.69	30.68	8.19	0.45	23.58	20.71	5.89	0.15	48.41
PRO	19.10	7.55	19.70	5.68	0.95	3.15	11.94	3.09	0.43	37.48
GLY	-13.93	12.22	0.93	9.02	0.40	106.69	-12.89	6.95	0.95	7.47
ALA	10.03	4.57	10.56	5.60	0.95	5.29	1.22	2.44	0.18	87.88
CIT	17.17	14.39	21.53	11.00	0.83	25.41	5.37	6.62	0.55	68.71
VAL	36.15	10.46	35.95	10.01	0.99	0.57	22.93	6.34	0.27	36.58
CYS	-0.97	1.46	2.15	1.57	0.24	322.91	0.58	0.87	0.47	160.54
MET	5.28	2.38	4.06	1.40	0.66	23.13	2.48	1.42	0.39	52.97
ILE	24.37	4.97	22.14	5.64	0.69	9.19	15.15	3.48	0.02	37.86
LEU	29.07	5.98	27.39	6.71	0.79	5.79	20.16	4.80	0.06	30.64
TYR	6.37	2.98	5.92	2.90	0.92	6.99	2.91	3.07	0.48	54.28
PHE	5.50	1.75	6.10	2.47	0.86	10.94	3.17	1.19	0.30	42.28
TRP	5.59	2.13	6.08	1.29	0.82	8.70	5.63	1.41	0.99	0.70
ORN	21.29	5.96	18.55	4.95	0.68	12.90	12.69	3.62	0.21	40.41
LYS	19.22	6.37	20.55	7.03	0.89	6.93	10.97	3.77	0.30	42.91
HIS	8.40	2.53	6.43	1.59	0.44	23.42	4.83	1.30	0.22	42.50
ARG	14.41	11.74	27.63	9.00	0.40	91.71	17.07	4.95	0.86	18.46

CSH, chorionic somatomammotropin; CON, control; RNAi, RNA interference; PI-IUGR, placental insufficiency- intrauterine growth restriction

Table 4.9. Baseline umbilical amino acid uptakes (μmol/min) at 136 dGA.

	CON RNAi vs. CSH RNAi						CON RNAi vs. CSH RNAi PI-IUGR			
	CON RNAi (n=6)	CON RNAi SEM	CSH RNAi (n=8)	CSH RNAi SEM	P-Value	% Difference	CSH RNAi- PI IUGR (n=5)	PI IUGR SEM	P-Value	% Difference
TAU	-0.33	0.59	-0.33	0.40	0.99	1.60	-1.11	0.59	0.45	231.54
ASP	-0.55	0.59	0.67	0.46	0.15	222.43	0.25	0.11	0.39	146.31
THR	11.45	3.10	6.05	1.51	0.13	47.13	4.63	1.70	0.18	59.58
SER	0.38	2.82	-1.84	1.72	0.53	586.55	-2.15	0.98	0.56	669.37
ASN	5.16	0.49	3.39	1.10	0.16	34.29	1.52	0.63	0.003	70.66
GLU	-17.86	2.91	-10.80	3.41	0.14	39.55	-5.58	2.29	0.03	68.78
GLN	37.95	4.27	24.31	5.93	0.05	35.95	15.96	6.03	0.02	57.93
PRO	12.25	1.64	9.37	2.24	0.24	23.49	9.89	3.92	0.49	19.24
GLY	13.78	2.87	9.15	2.71	0.25	33.65	4.55	1.54	0.07	67.00
ALA	14.70	2.20	8.78	2.70	0.11	40.27	4.15	2.02	0.02	71.78
CIT	1.66	1.87	1.29	0.48	0.85	22.24	0.87	0.61	0.78	47.68
VAL	20.49	3.05	13.47	3.50	0.12	34.24	8.33	3.05	0.04	59.34
CYS	0.56	0.20	0.38	0.12	0.47	31.26	0.23	0.08	0.29	59.46
MET	4.74	0.87	2.90	0.79	0.13	38.73	2.10	0.84	0.09	55.64
ILE	10.93	1.11	8.29	2.14	0.23	24.10	5.47	1.95	0.02	49.90
LEU	19.30	0.77	12.85	3.10	0.02	33.44	9.32	3.29	0.001	51.71
TYR	7.71	0.78	4.80	1.37	0.07	37.76	3.18	1.10	0.01	58.79
PHE	8.76	0.32	4.19	1.16	0.002	52.22	2.76	0.98	0.00003	68.49
TRP	2.35	0.39	1.35	0.35	0.07	42.51	0.88	0.33	0.04	62.33
ORN	1.91	1.77	0.22	0.78	0.42	88.35	0.07	0.36	0.51	96.16
LYS	11.61	1.16	7.56	1.72	0.03	34.88	6.09	2.31	0.03	47.53
HIS	3.62	0.50	2.43	0.77	0.21	33.05	1.72	0.87	0.09	52.49
ARG	12.20	1.74	8.60	2.00	0.14	29.51	7.10	2.45	0.10	41.80

CSH, chorionic somatomammotropin; CON, control; RNAi, RNA interference; PI-IUGR, placental insufficiency- intrauterine growth restriction

Table 4.10. Baseline relative (μmol/min/kg fetus) umbilical amino acid uptakes at 136 dGA.

	CON RNAi vs. CSH RNAi						CON RNAi vs. CSH RNAi PI-IUGR			
	CON RNAi (n=6)	CON RNAi SEM	CSH RNAi (n=8)	CSH RNAi SEM	P-Value	% Difference	CSH RNAi- PI IUGR (n=5)	PI IUGR SEM	P-Value	% Difference
TAU	-0.07	0.13	-0.10	0.10	0.87	40.29	-0.30	0.15	0.32	322.74
ASP	-0.12	0.13	0.17	0.11	0.13	246.84	0.09	0.04	0.30	179.33
THR	2.47	0.68	1.60	0.37	0.25	35.22	1.50	0.53	0.37	39.52
SER	0.09	0.61	-0.46	0.40	0.49	620.05	-0.60	0.25	0.47	775.47
ASN	1.11	0.10	0.87	0.26	0.36	21.65	0.54	0.24	0.03	51.57
GLU	-3.87	0.65	-2.63	0.76	0.21	32.16	-1.62	0.61	0.06	58.28
GLN	8.20	0.91	6.21	1.34	0.13	24.23	5.00	1.77	0.07	38.98
PRO	2.66	0.37	2.87	0.95	0.84	7.83	3.71	1.76	0.45	39.61
GLY	2.98	0.62	2.32	0.61	0.43	22.34	1.49	0.51	0.15	50.09
ALA	3.17	0.47	2.14	0.62	0.18	32.58	1.21	0.55	0.04	61.76
CIT	0.36	0.41	0.36	0.13	0.99	1.47	0.32	0.19	0.95	11.34
VAL	4.44	0.67	3.46	0.80	0.28	22.08	2.66	0.94	0.13	39.99
CYS	0.12	0.04	0.10	0.03	0.64	19.29	0.07	0.02	0.45	41.29
MET	1.03	0.19	0.76	0.19	0.29	26.43	0.66	0.25	0.28	35.48
ILE	2.36	0.25	2.14	0.49	0.60	9.61	1.73	0.59	0.17	26.70
LEU	4.18	0.17	3.34	0.73	0.10	20.12	2.93	0.98	0.01	29.89
TYR	1.67	0.18	1.23	0.31	0.18	26.46	0.98	0.32	0.03	41.09
PHE	1.89	0.07	1.07	0.27	0.004	43.30	0.86	0.29	0.0001	54.46
TRP	0.51	0.08	0.35	0.08	0.16	30.70	0.29	0.10	0.14	43.32
ORN	0.42	0.38	0.07	0.19	0.45	82.70	0.05	0.11	0.54	88.09
LYS	2.52	0.27	2.01	0.44	0.20	20.04	1.97	0.72	0.32	21.88
HIS	0.79	0.11	0.63	0.19	0.46	19.87	0.54	0.25	0.33	30.99
ARG	2.64	0.38	2.31	0.51	0.53	12.40	2.32	0.81	0.63	12.07

CSH, chorionic somatomammotropin; CON, control; RNAi, RNA interference; PI-IUGR, placental insufficiency- intrauterine growth restriction

Table 4.11. Baseline uteroplacental amino acid utilization (μmol/min) at 136 dGA.

	CON RNAi vs. CSH RNAi						CON RNAi vs. CSH RNAi PI-IUGR			
	CON RNAi (n=6)	CON RNAi SEM	CSH RNAi (n=8)	CSH RNAi SEM	P-Value	% Difference	CSH RNAi-PI IUGR (n=5)	PI IUGR SEM	P-Value	% Difference
TAU	-0.72	2.19	-0.55	1.09	0.95	23.21	-0.40	1.05	0.93	44.02
ASP	-0.08	0.89	-2.22	0.90	0.16	2728.48	-0.92	0.53	0.57	1066.81
THR	3.19	2.65	0.93	1.58	0.54	70.82	0.58	2.34	0.59	81.81
SER	12.19	3.55	10.40	2.79	0.63	14.66	7.55	2.50	0.31	38.03
ASN	-2.27	1.25	2.20	1.29	0.06	197.05	2.56	1.67	0.08	212.92
GLU	12.25	4.13	12.13	4.88	0.99	1.00	5.56	2.09	0.25	54.64
GLN	-9.76	3.63	-3.43	3.55	0.31	64.79	-2.63	5.14	0.35	73.08
PRO	2.35	5.00	1.95	2.93	0.95	17.29	-3.56	2.53	0.48	251.42
GLY	-22.40	7.91	-5.96	3.90	0.10	73.39	-9.42	4.80	0.28	57.94
ALA	-7.87	2.77	-0.34	0.98	0.03	95.64	-1.94	1.12	0.16	75.37
CIT	11.30	8.98	13.31	5.90	0.87	17.83	3.96	3.60	0.61	64.95
VAL	5.89	5.29	9.34	2.96	0.60	58.68	6.28	3.07	0.96	6.72
CYS	-1.12	0.89	1.09	0.78	0.15	197.38	0.48	0.43	0.28	143.06
MET	-0.54	1.43	-0.36	0.81	0.93	33.12	-0.25	1.35	0.91	53.48
ILE	6.53	1.88	4.78	1.61	0.46	26.74	2.89	1.60	0.20	55.77
LEU	1.10	2.15	3.36	1.81	0.51	203.90	1.73	2.46	0.88	56.60
TYR	-2.72	1.65	-2.17	1.23	0.82	20.08	-2.99	2.00	0.93	10.10
PHE	-4.94	1.22	-0.69	0.66	0.01	86.02	-1.50	0.66	0.04	69.64
TRP	1.44	1.59	1.91	0.57	0.80	32.71	1.94	0.71	0.84	35.09
ORN	13.42	4.35	11.06	3.14	0.63	17.58	7.91	2.74	0.35	41.04
LYS	2.61	3.76	6.37	3.02	0.51	144.22	1.97	1.74	0.92	24.42
HIS	2.55	1.67	1.40	0.92	0.60	45.29	1.14	1.19	0.62	55.25
ARG	-1.54	6.54	7.34	3.62	0.32	576.00	2.69	2.40	0.69	274.35

CSH, chorionic somatomammotropin; CON, control; RNAi, RNA interference; PI-IUGR, placental insufficiency- intrauterine growth restriction

Table 4.12. Baseline relative (µmol/min/kg placenta) uteroplacental amino acid utilization at 136 dGA.

	CON RNAi vs. CSH RNAi						CON RNAi vs. CSH RNAi PI-IUGR			
	CON RNAi (n=6)	CON RNAi SEM	CSH RNAi (n=8)	CSH RNAi SEM	P-Value	% Difference	CSH RNAi-PI IUGR (n=5)	PI IUGR SEM	P-Value	% Difference
TAU	-1.16	4.14	-1.31	2.98	0.98	12.57	-0.46	3.64	0.93	60.03
ASP	0.02	1.57	-6.00	2.28	0.08	24252.54	-3.56	2.03	0.26	14451.65
THR	6.85	5.25	3.91	5.13	0.75	42.96	3.88	8.17	0.80	43.44
SER	23.69	7.37	30.20	7.11	0.37	27.49	28.64	9.23	0.61	-20.87
ASN	-4.58	2.47	7.49	4.18	0.07	263.66	9.52	6.20	0.08	307.94
GLU	23.43	7.97	30.84	9.92	0.55	31.62	20.27	7.27	0.78	13.48
GLN	-18.51	6.76	-7.23	10.69	0.49	60.94	-6.23	17.03	0.55	66.34
PRO	4.72	9.77	-0.81	9.60	0.75	117.24	-16.32	12.03	0.29	445.76
GLY	-41.77	15.42	-21.14	11.10	0.33	49.38	-32.56	15.05	0.70	22.05
ALA	-14.76	5.00	-2.40	2.79	0.07	83.77	-6.83	3.61	0.30	53.74
CIT	20.93	17.84	35.28	13.79	0.57	68.60	19.17	14.86	0.95	8.37
VAL	10.42	9.80	28.50	8.95	0.21	173.56	26.51	12.86	0.39	154.40
CYS	-2.20	1.77	2.90	1.75	0.11	231.47	2.24	1.69	0.19	201.71
MET	-0.57	2.67	-0.69	2.74	0.98	22.29	-0.27	4.61	0.96	52.94
ILE	12.21	3.43	14.12	4.55	0.72	15.62	12.22	6.45	1.00	0.11
LEU	1.83	4.10	10.51	5.79	0.33	473.84	8.64	9.04	0.55	371.92
TYR	-4.50	3.21	-6.45	3.97	0.75	43.55	-9.48	6.49	0.53	110.89
PHE	-8.94	2.05	-2.67	1.94	0.06	70.12	-5.51	2.21	0.19	38.37
TRP	3.25	3.16	6.02	1.81	0.49	85.12	7.46	2.71	0.42	129.18
ORN	26.08	9.20	31.67	7.67	0.56	21.40	29.87	9.92	0.77	14.51
LYS	5.30	7.60	16.00	6.63	0.36	201.83	8.25	6.38	0.82	55.76
HIS	4.62	3.21	4.53	2.86	0.99	1.87	5.24	4.43	0.92	13.44
ARG	-2.37	11.62	19.76	8.45	0.20	932.94	12.88	9.82	0.47	643.13

CSH, chorionic somatomammotropin; CON, control; RNAi, RNA interference; PI-IUGR, placental insufficiency- intrauterine growth restriction

Total nutrient uptakes and quotients during basal conditions

As summarized in **Table 13**, the uterine uptakes of nitrogen and carbon from amino acids did not differ by treatment. However, uterine carbon uptake (μmol/min) from glucose was reduced (P = 0.03) by 31% in CSH RNAi pregnancies, and uterine carbon uptake from lactate tended (P = 0.07) to be reduced by 44%. Together, this resulted in a 35% reduction (P = 0.05) in total carbon uptake by the uteri of CSH RNAi pregnancies, although not on a relative basis (μmol/min/kg uterus). In CSH RNAi PI-IUGR pregnancies, the reduction in uterine carbon uptake was reduced (P = 0.02) by 38% on an absolute basis (μmol/min) and tended (P = 0.07) to be reduced by 22% on a relative basis (μmol/min/kg uterus). This suggest that decreases in uterine carbon uptake in cases of CSH RNAi PI-IUGR was not completely due to uterine mass.

While umbilical uptakes (μmol/min; **Table 13**) of nitrogen and carbon from amino acids were not significantly impacted by treatment, the uptakes of carbon from glucose (P = 0.03) and lactate (P = 0.01) were reduced by 29% and 42% respectively in CSH RNAi fetuses. This resulted in a 32% reduction (P = 0.01) in total umbilical carbon uptake (μmol/min) in CSH RNAi fetuses. These reductions in total carbon uptake were somewhat independent of fetal mass as they tended (P = 0.08) to be reduced on a relative basis (μmol/min/kg fetus). While none of the individual nutrient: oxygen quotients (amino acids, glucose, lactate) statistically differed by treatment, the fetal total nutrient quotient (the number of carbon: oxygen molecules taken up by the umbilicus) was reduced (P = 0.01) by 18% in CSH RNAi fetuses. In CSH RNAi fetuses with PI-IUGR, the uptake of carbon from glucose (P = 0.02), lactate (P = 0.01), and amino acids (P = 0.06) were reduced by 43%, 51%, and 48% respectively. This produced a 44%

reduction (P= 0.005) in umbilical carbon uptake in CSH RNAi PI-IUGR fetuses, and a tendency (P = 0.06) for the total nutrient quotient to be reduced.

Table 4.13. Total uterine and umbilical nutrient uptakes (μmol/min) and nutrient quotients at 136 dGA.

	CON RNAi vs. CSH RNAi IUGR						CON RNAi vs. CSH RNAi PI-IUGR			
	CON RNAi (n=6)	CON SEM	CSH RNAi (n=8)	CSH RNAi SEM	P-value	% Difference	CSH RNAi-PI IUGR (n=5)	PI IUGR SEM	P-Value	% Difference
Uterine										
Amino acid carbon uptake, umol/min	1535.63	476.46	933.27	273.86	0.28	39.23	933.27	256.76	0.28	39.23
Glucose carbon uptake, umol/min	2537.71	507.35	1739.19	377.10	0.03	31.47	1601.19	451.07	0.04	36.90
Lactate uptake, umol/min	430.73	106.23	239.66	63.18	0.07	44.36	252.05	85.93	0.15	41.48
Total carbon uptake, umol/min	4504.08	900.27	2935.59	905.80	0.05	34.82	2786.51	686.93	0.02	38.13
Relative carbon uptake, umol/min/kg fetus	6521.19	1300.50	5175.52	1550.97	0.14	20.64	5070.54	1145.44	0.07	22.25
Total nitrogen uptake, umol/min	388.65	155.19	520.37	166.39	0.57	33.89	288.17	90.75	0.63	25.85
Umbilical										
Amino acid carbon uptake, umol/min	204.46	23.91	137.02	33.70	0.12	32.98	105.97	43.43	0.06	48.17
Glucose carbon uptake, umol/min	824.88	59.09	583.90	113.03	0.03	29.21	470.22	171.26	0.02	43.00
Lactate uptake, umol/min	446.31	34.64	258.91	63.11	0.01	41.99	218.86	78.37	0.01	50.96
Total carbon uptake, umol/min	1475.66	73.57	1002.66	214.87	0.01	32.05	830.36	290.09	0.005	43.73
Relative carbon uptake, umol/min/kg fetus	319.39	17.30	265.22	53.35	0.08	16.96	262.62	87.69	0.16	17.78
Total nitrogen uptake, umol/min	62.52	8.44	49.25	11.12	0.26	21.23	42.96	15.47	0.21	31.29
Amino acid: oxygen quotient	0.66	0.07	0.54	0.11	0.18	18.90	0.52	0.17	0.23	21.80
Glucose: oxygen quotient	0.54	0.03	0.49	0.09	0.15	9.95	0.50	0.16	0.39	7.18
Lactate: oxygen quotient	0.30	0.04	0.21	0.05	0.11	28.14	0.23	0.08	0.29	21.38
Total nutrient quotient	1.50	0.07	1.24	0.24	0.01	17.52	1.25	0.40	0.06	16.46

CSH, chorionic somatomammotropin; CON, control; RNAi, RNA interference; PI-IUGR, placental insufficiency- intrauterine growth restriction

Blood flow during maternal hyperglycemia

Uterine and umbilical blood flow were also assessed during the maternal hyperglycemic clamp to ensure accurate nutrient uptake and utilization measures. Due to the exclusion of two ewes that failed to reach hyperglycemic conditions and catheter patency issues, the CSH RNAi group could not be stratified by placental insufficiency. Thus, all measures are reported based on the full CSH RNAi cohort. During the maternal hyperglycemic clamp, uterine blood, and plasma flow (mL/min; **Table 14**) both tended ($P \leq 0.10$) to be reduced by 25% in CSH RNAi pregnancies. While neither uterine blood and plasma flow were altered relative to fetal or uterine weight (mL/min/kg), uterine blood flow relative to placental weight (mL/min/100 g placenta) tended ($P = 0.10$) to be increased by 24% in CSH RNAi pregnancies during the hyperglycemic clamp.

Umbilical plasma flow (mL/min; **Table 14**) was reduced ($P = 0.05$) in CSH RNAi fetuses during the maternal hyperglycemic clamp, and umbilical blood flow (mL/min) tended ($P = 0.06$) to be reduced by 34% and 30% respectively. While relative umbilical blood flow (mL/min/kg fetus or 100g placenta) did not differ between treatments, relative umbilical plasma flow (mL/min/kg fetus) tended ($P = 0.09$) to be reduced in CSH RNAi fetuses. Interestingly, unlike during basal conditions, the uterine: umbilical blood flow ratio was elevated ($P = 0.01$) by 21% in CSH RNAi pregnancies. Umbilical vein hematocrit was increased ($P = 0.05$) in CSH RNAi fetuses, similar to basal conditions.

Table 4.14. Uterine and umbilical blood flow during maternal hyperglycemic clamp (136 dGA).

	CON RNAi (n=5)	CON RNAi SEM	CSH RNAi (n=7)	CSH RNAi SEM	P-value	% Difference
Uterine measurements						
Blood Flow, mL/min	2090.02	470.43	1558.82	383.08	0.07	25.42
Plasma Flow, mL/min	1444.67	328.45	1079.05	266.52	0.09	25.31
Blood Flow, mL/min/kg fetus	449.84	101.26	446.53	98.34	0.88	0.74
Plasma Flow, mL/min/kg fetus	311.06	70.84	308.19	67.88	0.88	0.92
Blood Flow, mL/min/kg uterus	3046.35	683.34	2902.37	687.50	0.71	4.73
Plasma Flow, mL/min/kg uterus	2105.76	477.19	2006.38	476.41	0.73	4.72
Blood Flow, mL/min/100g placenta	396.59	89.74	492.45	112.94	0.10	24.17
Umbilical measurements						
Blood Flow, mL/min	824.70	55.45	575.95	148.15	0.06	30.16
Plasma Flow, mL/min	533.45	39.71	350.90	93.84	0.05	34.22
Blood Flow, mL/min/kg fetus	179.05	13.38	149.39	34.01	0.14	16.56
Plasma Flow, mL/min/kg fetus	115.81	9.38	89.97	21.07	0.09	22.32
Blood flow, mL/min/100g placenta	165.98	19.42	152.11	35.02	0.58	8.36
Uterine: Umbilical Flow	2.70	0.61	3.27	0.88	0.01	21.02
Umbilical vein hematocrit	0.35	0.01	0.40	0.09	0.05	13.09
Uterine artery hematocrit	0.31	0.01	0.32	0.05	0.82	1.13

CSH, chorionic somatomammotropin; CON, control; RNAi, RNA interference; PI-IUGR, placental insufficiency- intrauterine growth restriction

Oxygen transfer during maternal hyperglycemia

Neither uterine (mmol/min; **Figure 5**) or relative uterine (mmol/min/kg uterus; **Table 15**) uptakes of oxygen differed between treatments during the maternal hyperglycemic clamp. However, both umbilical (mmol/min; **Figure 5**) and relative umbilical (mmol/min/kg fetus; **Table 15**) uptakes of oxygen were reduced ($P \leq 0.05$) by 31% and 17% respectively in CSH RNAi fetuses. This is partially explained by the increased ($P = 0.05$; **Figure 5**) relative uteroplacental oxygen utilization (mmol/min/kg) in CSH RNAi pregnancies which was elevated by 84%, but absolute (mmol/min) uteroplacental oxygen utilization did not change. This is further evidenced by the 52% increase ($P = 0.05$; **Figure 6**) in the fraction of uterine oxygen uptake utilized by the placenta in CSH RNAi pregnancies during maternal hyperglycemia. This led to a 24% fractional decrease ($P = 0.05$; **Figure 6**) in transfer of oxygen to the fetus.

Table 4.15. Nutrient uptake and utilization under hyperglycemic clamp conditions at 136 dGA.

	CON RNAi (n=5)	CON SEM	CSH RNAi (n=7)	CSH RNAi SEM	P-value	% Difference
Oxygen						
Relative uterine O2 uptake, mmol/min/kg uterus	3.46	0.79	3.49	0.83	0.96	0.71
Relative umbilical O2 uptake, mmol/min/kg fetus	0.36	0.02	0.30	0.07	0.03	16.97
Uteroplacental O2 utilization, mmol/min	0.77	0.23	0.97	0.28	0.46	24.88
Glucose						
Uterine artery glucose concentrations, mmol/L	8.02	0.28	7.88	0.49	0.84	1.67
Relative uterine glucose uptake, µmol/min/kg uterus	870.67	201.27	929.56	276.30	0.83	6.76
Umbilical artery glucose concentrations, mmol/L	2.49	0.19	2.10	0.45	0.38	15.36
Relative umbilical uptake, µmol/min/kg fetus	52.74	4.54	44.60	10.00	0.17	15.44
Uterine artery to umbilical artery glucose gradient, µmo/L	5.53	0.19	6.18	0.97	0.04	11.76
Uteroplacental utilization, µmol/min	367.58	97.09	403.67	135.78	0.79	9.82
Lactate						
Uterine lactate export, µmol/min	147.16	33.60	74.78	24.94	0.03	49.18
Relative uterine lactate export, µmol/min/kg uterus	214.54	48.89	139.42	45.41	0.16	35.02
Umbilical lactate uptake, µmol/min	147.98	13.21	85.76	22.38	0.01	42.04
Relative umbilical lactate uptake, µmol/min/kg fetus	32.12	3.05	22.06	5.08	0.02	31.33
Uteroplacental lactate production, µmol/min	284.35	64.56	157.27	48.81	0.01	44.69
Relative uteroplacental lactate production, µmol/min/kg placenta	541.56	126.10	481.57	149.14	0.62	11.08

CSH, chorionic somatomammotropin; CON, control; RNAi, RNA interference; PI-IUGR, placental insufficiency- intrauterine growth restriction.

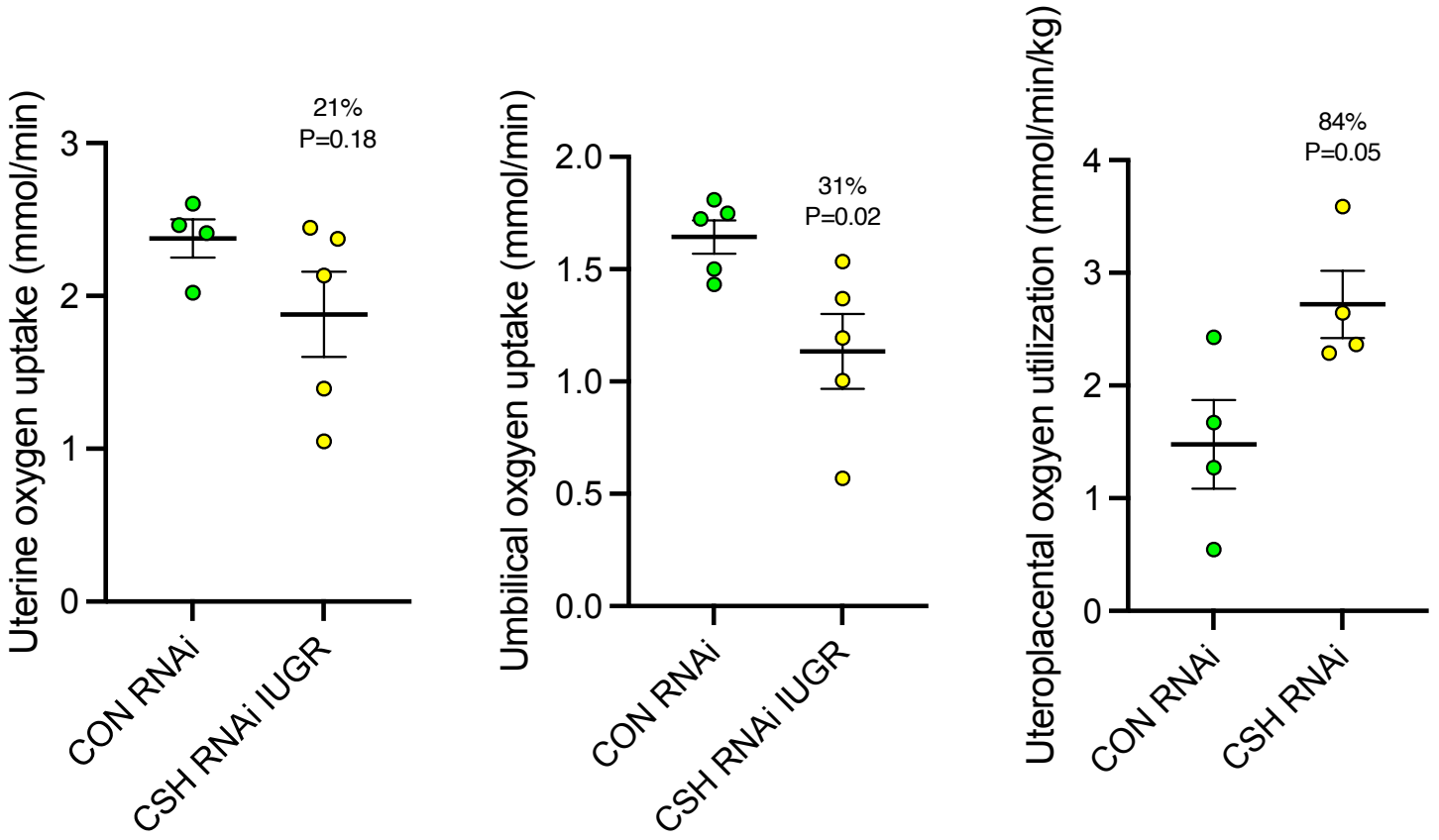


Figure 4.5: Measures of uterine oxygen uptake (mmol/min); (a) umbilical oxygen uptake (mmol/min); (b) and relative uteroplacental oxygen uptake (mmol/min/kg placenta); (c) under maternal hyperglycemic conditions at 136 dGA. Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference; PI-IUGR; placental insufficiency intrauterine growth restriction.

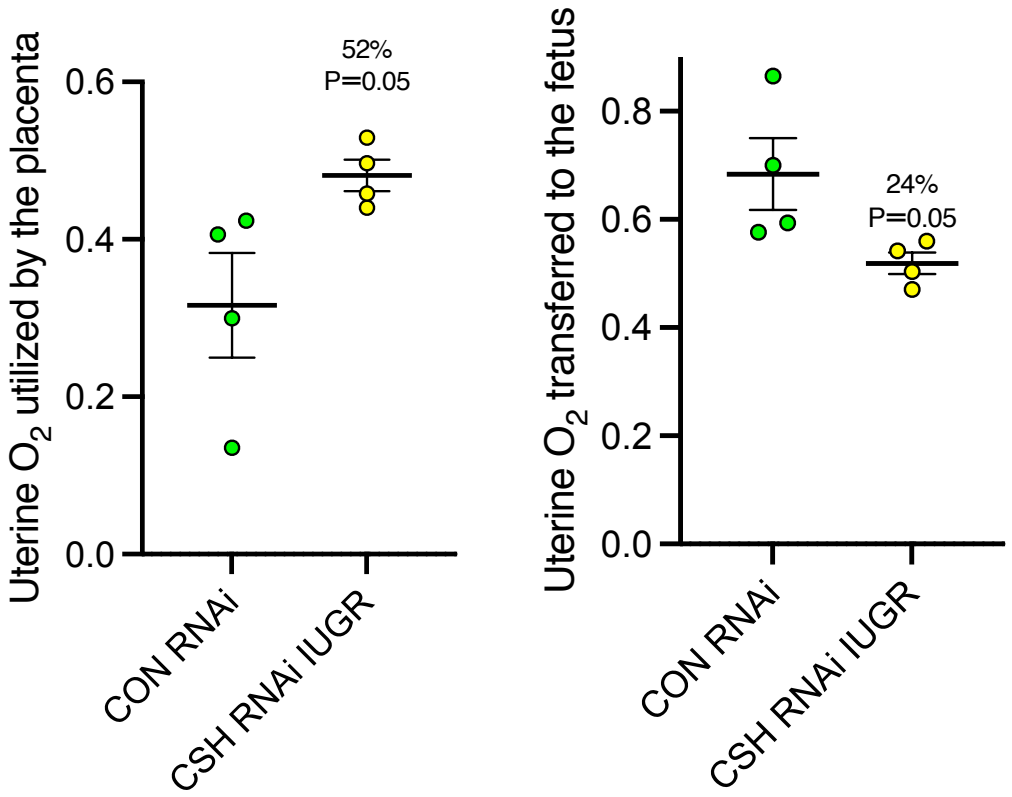


Figure 4.6: Measures of the fraction of uterine oxygen uptake utilized by the placenta (mmol/min); (a) and fraction of uterine oxygen uptake transferred to the fetus (mmol/min); (b) under maternal hyperglycemic conditions at 136 dGA. Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference; PI-IUGR; placental insufficiency intrauterine growth restriction.

Blood biochemistry during maternal hyperglycemia

In response to the hyperglycemic clamps, uterine artery blood gases did not experience additional hyperglycemic specific responses, but chloride concentrations (meq/L) were still reduced ($P = 0.05$; **Table 16**) in ewes carrying CSH RNAi pregnancies. Uterine vein methemoglobin (Met Hb%) also tended ($P = 0.09$; **Table 16**) to be reduced in CSH RNAi pregnancies.

Maternal hyperglycemia continued to suppress ($P = 0.03$; **Table 16**) umbilical artery pO_2 (mmHg) in CSH RNAi fetuses and tended ($P = 0.08$) to increase umbilical artery hemoglobin (tHb mmol/L). Furthermore, umbilical vein pO_2 (mmHg), oxyhemoglobin (O_2 Hb%), and oxygen saturation (SO_2 %) all tended ($P \leq 0.10$; **Table 16**) to be reduced in CSH RNAi fetuses, whereas umbilical vein hemoglobin (tHb mmol/L) tended ($P = 0.07$) to increase.

Table 4.16. Uterine blood gases during maternal hyperglycemia CON vs CSH RNAi at 136 dGA.

	CON RNAi (n=5)	CON RNAi SEM	CSH RNAi (n=7)	CSH RNAi SEM	P-Value	% Difference
Uterine Artery (A)						
pH $^{37.9C}$	7.46	0.01	7.48	0.01	0.23	0.27
pCO_2 $^{37.9C}$ mmHg	35.25	1.05	36.40	1.21	0.63	3.25
pO_2 $^{37.9C}$ mmHg	76.36	12.91	91.55	0.94	0.31	19.89
HCO^3-	24.04	0.68	25.11	0.83	0.94	4.43
tHb mmol/L	6.13	0.25	6.46	0.14	0.71	5.28
O_2 Hb %	91.76	0.60	89.16	1.34	0.30	2.83
SO_2 %	96.59	0.67	95.77	0.90	0.31	0.86
Met Hb %	1.44	0.07	1.21	0.05	0.25	15.83
O_2 ct mmol/L	5.72	0.23	5.98	0.15	0.78	4.57
Na^+ meq/L	146.19	0.91	150.95	3.13	0.29	3.26
K^+ meq/L	4.18	0.05	4.09	0.07	0.45	2.04
Ca^{2+} meq/L	2.66	0.01	2.79	0.05	0.16	4.62
CL^- meq/L	112.75	0.66	110.30	0.88	0.05	2.17
Uterine Vein (V)						
pH $^{37.9C}$	7.43	0.01	7.43	0.01	0.95	0.07
pCO_2 $^{37.9C}$ mmHg	40.21	1.29	39.82	0.64	0.95	0.98
pO_2 $^{37.9C}$ mmHg	52.69	7.52	61.93	1.09	0.34	17.54
HCO^3-	25.41	0.78	25.58	0.53	0.98	0.69
tHb mmol/L	6.25	0.27	6.36	0.13	0.95	1.70
O_2 Hb %	69.72	2.55	72.39	0.65	0.47	3.84
SO_2 %	75.59	0.11	75.30	0.75	0.47	0.38
Met Hb %	1.30	0.02	1.14	0.05	0.09	12.50
O_2 ct mmol/L	4.64	0.20	4.66	0.12	0.73	0.54
Na^+ meq/L	144.69	0.05	145.25	0.73	0.36	0.39
K^+ meq/L	4.21	0.03	4.11	0.07	0.30	2.23
Ca^{2+} meq/L	2.60	0.02	2.70	0.08	0.46	3.95
CL^- meq/L	112.00	0.36	109.31	0.82	0.18	2.40

CSH, chorionic somatomammotropin; CON, control; RNAi, RNA interference; PI-IUGR, placental insufficiency- intrauterine growth restriction

Table 4.17. Umbilical blood gases during maternal hyperglycemia CON vs CSH RNAi at 136 dGA.

	CON RNAi (n=5)	CON RNAi SEM	CSH RNAi (n=7)	CSH RNAi SEM	P-Value	% Difference
Umbilical artery (α)						
pH $^{37.9C}$	7.35	0.01	7.35	0.01	0.94	0.05
pCO_2 $^{37.9C}$ mmHg	52.67	1.50	53.66	0.67	0.81	1.87
pO_2 $^{37.9C}$ mmHg	26.31	0.43	21.18	1.55	0.03	19.48
HCO^3-	27.30	0.31	28.16	0.69	0.77	3.16
tHb mmol/L	7.15	0.20	8.04	0.30	0.08	12.41
O_2 Hb %	47.42	2.81	34.55	5.21	0.15	27.13
SO_2 %	48.39	2.89	35.29	5.32	0.15	27.06
Met Hb %	0.39	0.07	0.61	0.11	0.44	55.45
O_2 ct mmol/L	3.39	0.16	2.69	0.37	0.28	20.54
Na^+ meq/L	140.55	0.76	141.19	0.62	0.28	0.45
K^+ meq/L	3.53	0.11	3.65	0.11	0.72	3.55
Ca^{2+} meq/L	2.94	0.04	2.80	0.07	0.49	4.77
CL^- meq/L	108.05	1.31	106.13	1.63	0.52	1.78
Umbilical vein (γ)						
pH $^{37.9C}$	7.39	0.01	7.40	0.01	0.87	0.13
pCO_2 $^{37.9C}$ mmHg	44.95	1.63	45.97	1.27	0.87	2.28
pO_2 $^{37.9C}$ mmHg	37.04	3.12	27.74	1.96	0.06	25.11
HCO^3-	25.66	0.36	26.94	0.80	1.00	5.00
tHb mmol/L	7.10	0.21	8.12	0.27	0.07	14.35
O_2 Hb %	76.06	3.27	57.08	5.53	0.09	24.95
SO_2 %	78.82	3.66	59.20	5.83	0.09	24.89
Met Hb %	0.27	0.21	0.52	0.14	0.59	95.75
O_2 ct mmol/L	5.41	0.19	4.58	0.34	0.23	15.43
Na^+ meq/L	140.65	1.26	139.38	2.41	0.64	0.91
K^+ meq/L	3.51	0.11	3.59	0.11	0.63	2.35
Ca^{2+} meq/L	3.02	0.05	2.96	0.09	0.98	1.87
CL^- meq/L	109.65	1.50	105.06	1.35	0.15	4.18

CSH, chorionic somatomammotropin; CON, control; RNAi, RNA interference; PI-IUGR, placental insufficiency- intrauterine growth restriction

Glucose transfer during maternal hyperglycemia

Following 90 minutes of dextrose infusion, when steady-state maternal hyperglycemia (>7.0 mmol·L^{-1} glucose) had been achieved, uterine artery glucose concentrations (mmol/L) did not statistically differ between treatments (**Table 15**). Neither uterine (µmol/min; **Figure 7**) and relative uterine (µmol/min/kg uterus) glucose uptakes differed between treatments. This contrasts with uterine glucose uptake during baseline which was suppressed in CSH RNAi pregnancies. Umbilical glucose uptakes however, tended (P = 0.06; **Figure 7**) to be reduced (µmol/min) in CSH RNAi by 29% but not on a relative basis (µmol/min/kg fetus; **Table 15**). The uterine artery to umbilical artery glucose gradient was elevated (P = 0.04) in CSH RNAi pregnancies, suggestive of dysfunction in glucose transport across the placenta. Uteroplacental glucose utilization (µmol/min) did not differ between treatments. While relative uteroplacental glucose utilization (µmol/min/kg placenta; **Table 15**) was not statistically different (P = 0.20; **Figure 7**), it was numerically elevated by 51% in CSH RNAi pregnancies, hinting at greater glucose utilization by the placenta on a relative basis.

Placental glucose transport is summarized in **Figure 8,** examining the relationship between umbilical glucose uptake (y-axis, µmol/min) and the maternal-fetal glucose gradient represented by the uterine artery to umbilical artery glucose gradient (x-axis, mmol/L). For control RNAi pregnancies, the linear regression equation is y = 29.54 X + 74.15, with an x-intercept of -2.51 mmol/L. For CSH RNAi pregnancies, the linear regression equation is y= 19.05X + 52.40, with an x-intercept of -2.75 mmol/L. The overall slopes did not statistically differ from each other (P = 0.34); however, the elevations were different (P = 0.003) with CSH RNAi having a lower y-intercept

compared to control RNAi. This suggests different maximal glucose transport during maternal hyperglycemia.

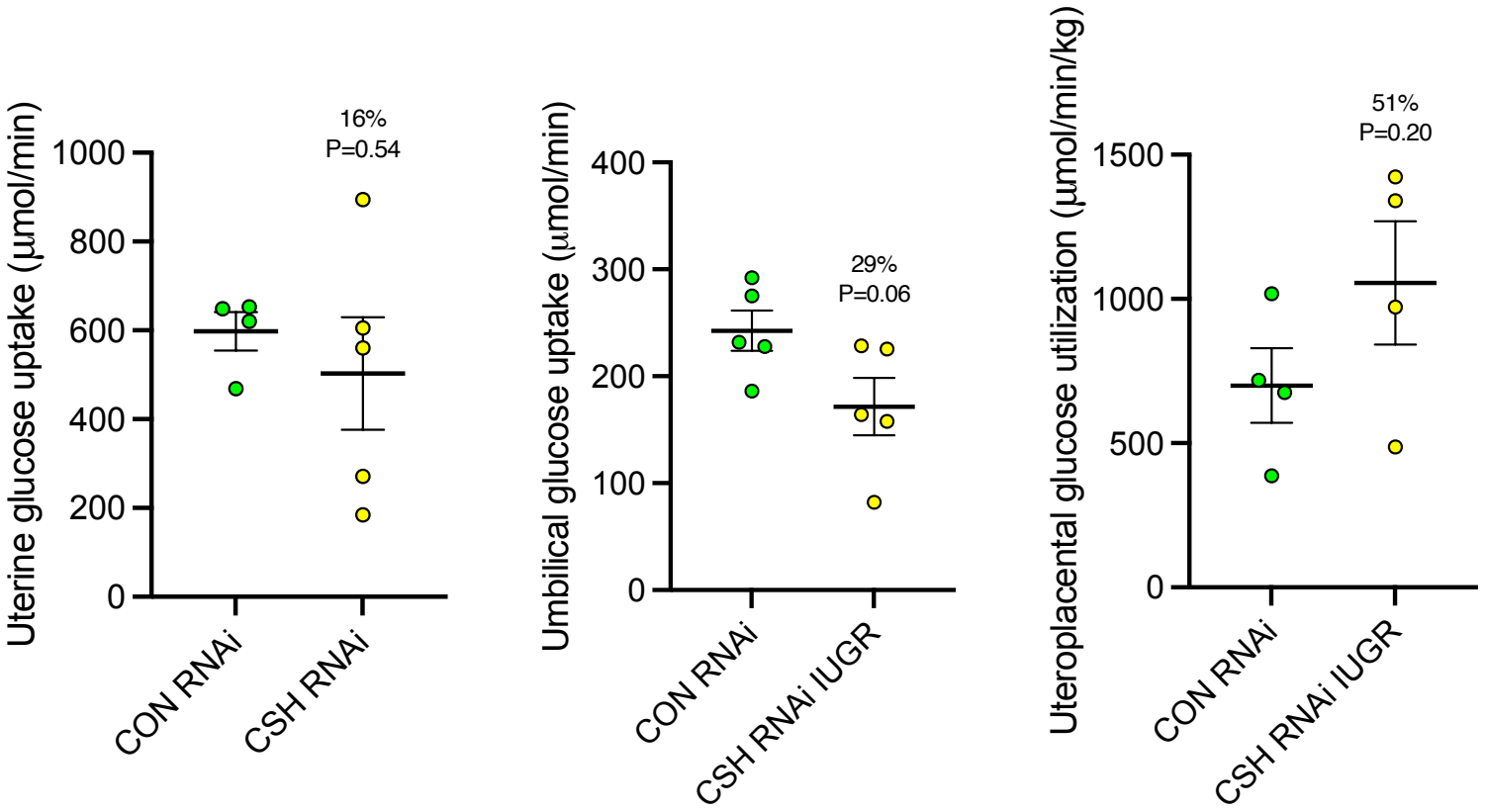


Figure 4.7: Measures of uterine glucose uptake (µmol/min); (a) umbilical glucose uptake (µmol/min); (b) and relative uteroplacental glucose uptake (µmol/min/kg placenta); (c) under maternal hyperglycemic conditions at 136 dGA. Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference; PI-IUGR; placental insufficiency intrauterine growth restriction.

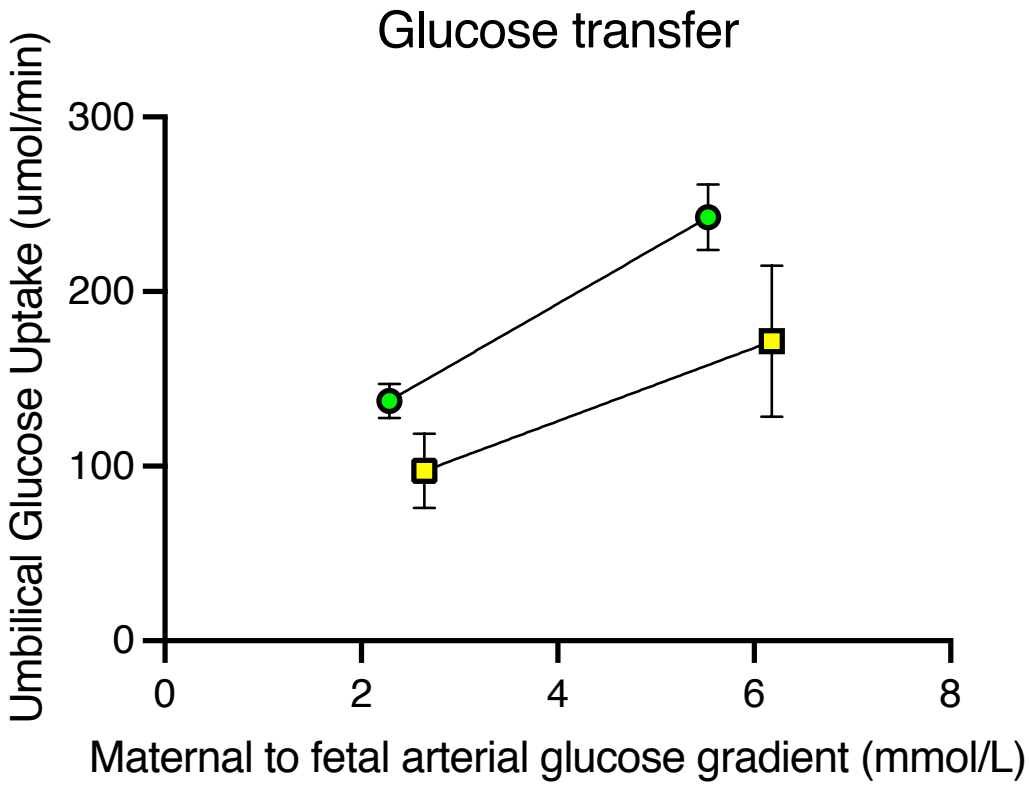


Figure 4.8: Simple linear regression of glucose transfer across the placenta in Control vs. CSH RNAi pregnancies

Lactate transfer during maternal hyperglycemia

In response to maternal hyperglycemia, uterine lactate export was elevated (P = 0.03; **Table 15**) in CSH RNAi by 49% but not on a relative basis (μmol/min/kg uterus; **Table 15**). Unsurprisingly, umbilical absolute (μmol/min) and relative (μmol/min/kg fetus) lactate uptakes were reduced (P ≤ 0.05) by 42% and 31% respectively in CSH RNAi fetuses. Uteroplacental lactate production (μmol/min) was also reduced (P = 0.01) by 45% in CSH RNAi pregnancies but was not altered relative to placental weight (μmol/min/kg placenta).

Insulinemic responses to basal and hyperglycemic conditions

While neither uterine artery nor vein insulin concentrations (ng/mL; **Figure 9**) were statistically different between treatments during baseline conditions, they were numerically elevated by 30% and 46% respectively. These numerical relationships were also reflected in uterine artery and vein insulin concentrations in CSH RNAi PI-IUGR pregnancies but remained statistically similar to controls.

Baseline umbilical insulin concentrations were reduced (P = 0.01; **Figure 9**) by 51% in CSH RNAi pregnancies, and by 58% in CSH RNAi PI-IUGR fetuses (P = 0.01). Interestingly, during maternal hyperglycemic clamp conditions, there was a tendency (P = 0.10; **Figure 9**) for insulin to remain suppressed by similar degrees to baseline conditions (51% vs 51%).

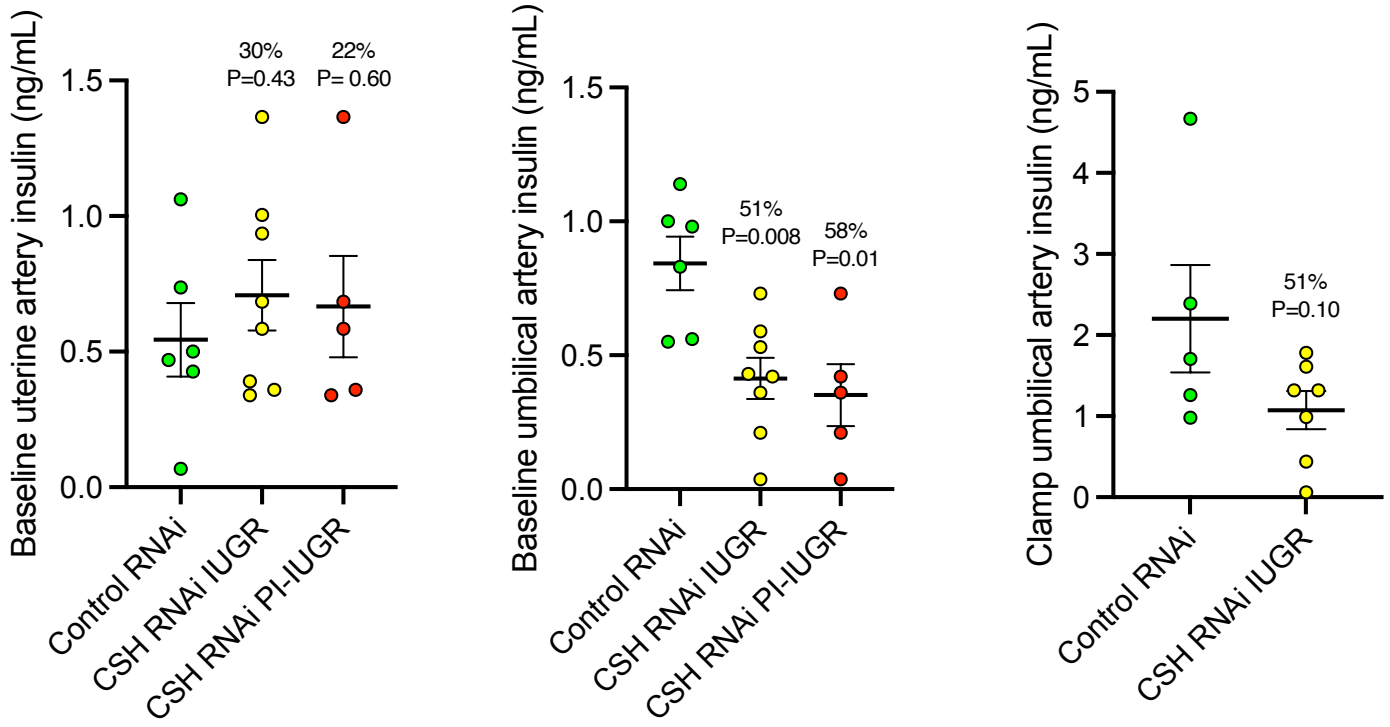


Figure 4.9: Measures of baseline uterine artery insulin (ng/mL); (a) baseline umbilical artery insulin (ng/mL); (b) and umbilical artery insulin under maternal hyperglycemic conditions (ng/mL); (c) at 136 dGA. Data are shown as means ± SEM for all pregnancies in each treatment group. CSH, chorionic somatomammotropin; RNAi, RNA interference; PI-IUGR; placental insufficiency intrauterine growth restriction.

DISCUSSION

Previously, we demonstrated that CSH RNAi alters *in vivo* uteroplacental glucose uptake and utilization, suggesting a likely role of CSH in regulating placental function (Tanner et al., 2021a, b). Based on this evidence, we set out to investigate the ramifications of artificially increasing maternal glucose concentrations in late gestation on CSH RNAi pregnancies. We hypothesized that despite elevated maternal glucose concentrations, umbilical glucose uptakes would still be depressed in CSH RNAi pregnancies, demonstrating dysfunction in placental metabolism and glucose transfer. The results from this investigation largely support this hypothesis.

In the current cohort, high maternal glucose concentrations failed to restore umbilical glucose uptake in the CSH RNAi fetus. This was evidenced by reduced umbilical glucose uptakes during maternal hyperglycemia in CSH RNAi fetuses, as well as an increased maternal-fetal glucose gradient (uterine artery - umbilical artery gradient), a hallmark of dysfunction in placental glucose transfer. Because placental glucose transfer is characterized by saturable, carrier-mediated transport, a higher glucose concentration in maternal circulation should facilitate greater transfer across the placenta (Widdas et al., 1952; Hay, 1991) barring placental dysfunction. A multiplicity of factors can impact placental glucose transfer including uterine and umbilical blood flow, the maternal-fetal glucose gradient, the availability of placental glucose transporters, placental mass, and placental metabolic demands (Barry and Anthony, 2008; Hay et al., 1990).

During basal conditions, uterine glucose uptake was suppressed in CSH RNAi pregnancies. However, during maternal hyperglycemia, the uterine glucose uptakes in

CSH RNAi pregnancies increased to similar levels as control pregnancies. This suggests that during maternal hyperglycemia, the high concentrations of glucose in maternal circulation resulted in similar uterine uptakes of glucose despite the reductions in uterine blood flow. This appears to indicate that reduced uterine blood flow is not the primary reason for the reduced umbilical glucose uptakes during the clamps, and points towards dysfunction in either glucose transporter availability, decreased placental mass or increased placental metabolism as explanations of the failure for fetal glucose concentrations to be restored.

A previous study (Tanner et al., 2021b) that investigated the *in vivo* physiology of CSH RNAi IUGR pregnancies described similar patterns to the current cohort of reduced uterine and umbilical glucose uptakes but documented no significant changes in placental SLC2A1 (GLUT1) or SLC2A3 (GLUT3) concentrations. This contrasts with Jeckel et al. (2018) that noted decreased SLC2A3 concentrations in CSH RNAi near-term (135 dGA) pregnancies, however that study was performed under fasted, anesthetized conditions as described in Baker et al. (2016) which may potentially explain the discrepancy. While it is possible that glucose transporter availability was impacted in the current cohort, the physiological data point towards increases in placental oxidative demands as the most robust explanation for why fetal glucose concentrations were not restored with elevated maternal glucose.

This is best illustrated by examining uteroplacental glucose and oxygen utilization relative to placental mass during the maternal hyperglycemia. It is worth noting that due to maternal or fetal catheter patency complications, only 4 of the 7 CSH RNAi animals had calculable uteroplacental substrate utilizations, somewhat limiting our sample size.

Despite this limitation, relative (μmol/min/kg placenta) uteroplacental glucose utilization rose by 51% (but not statistically, P = 0.20) during maternal hyperglycemia in CSH RNAi pregnancies. This dramatic increase in relative glucose utilization along with a concomitant 84% increase (P = 0.05) in relative uteroplacental oxygen utilization both point towards greater placental oxidation of substrates independent of placental mass. This could in part, explain why the fetal glucose concentrations were not restored by increasing the maternal glucose concentration which led to elevated uterine artery to umbilical artery glucose gradients in CSH RNAi pregnancies.

This is further supported by the two-point regression analysis of placental glucose transfer that saw reduced y-intercepts in CSH RNAi pregnancies, supporting the notion of reduced glucose transfer during hyperglycemia. Together, these data point towards a previously discussed (Tanner et al., 2021a) compensatory mechanism, activated during CSH RNAi, which appears to increase the placental utilization of oxidative substrates to support adequate fetal growth (Tanner et al., 2021a). However, in instances of CSH RNAi induced IUGR (Tanner et al., 2021b), where greater reductions in blood flow and nutrient uptake occur, it appears this compensatory mechanism may be deleterious as increasing placental oxidation fails to support adequate fetal growth. This may provide evidence to suggest under basal conditions, placental glucose utilization appeared to be reduced (P = 0.11) in CSH RNAi pregnancies, suggesting this compensatory mechanism is not active, but may still be triggered during periods of excess maternal nutrient availability. Fundamentally, these results support the potential role for CSH in regulating glucose transfer to the fetus by regulating placental metabolism.

While it appears that CSH RNAi results in reduced umbilical glucose uptake as a byproduct of increased uteroplacental oxidative demands, this differs from a different model of IUGR in the sheep. During maternal hyperthermia induced PI-IUGR (Thureen et al., 1992), placental glucose transfer is suppressed without substantial changes to uteroplacental glucose consumption or perfusion. This is evidenced by measuring uteroplacental glucose utilization, blood flow, and a linear regression analysis of umbilical glucose uptake (y-axis) in relation to maternal-fetal plasma arterial glucose concentrations (x-axis; Thureen et al., 1992). Due to limited changes in uptake and perfusion, along with non-parallel and statistically different regression lines, it was determined that fetuses from hyperthermic PI-IUGR fetuses had reduced glucose uptakes due to reduced placental glucose transfer capacity (Thureen et al., 1992). The distinctions between these two models support the idea that different placental perturbations may result in the similar fetal phenotype.

One unintended strength of this study was the opportunity to examine a single cohort of CSH RNAi pregnancies of various phenotypic severities. This provided novel insights into the impacts of CSH RNAi on blood biochemistry, fetal organ weights, and amino acid uptakes, as well as bolstered the repeatability of our previous findings (Tanner et al., 2021a; b). Despite the variation in phenotypic severity, the entire cohort of CSH RNAi pregnancies had fetal weights that fell 2 standard deviations below the mean of the control fetuses, resulting in an overall 21% fetal growth restriction and a 37% reduction in placental weight. A subset however, also had placental weights that fell 2 standard deviations below the mean of the control placentas, thus, were designated as CSH RNAi PI-IUGR pregnancies. This subset of CSH RNAi pregnancies

characterized by PI-IUGR had a greater degree of fetal growth restriction (30%), closely mirroring the degree of growth restriction (30-32%) previously reported (Baker et al., 2016; Tanner et al., 2021b). Furthermore, the CSH RNAi PI-IUGR fetuses had placental weights 52% smaller than control pregnancies, identical to the degree of placental growth restriction (52%) observed in the Baker et al. (2016) study. These PI-IUGR CSH RNAi pregnancies also had a similar degree of fetal liver weight reductions compared to the previously reported liver weight reductions (Baker et al., 2016). These results support the repeatability of this animal model, and further emphasizes the role of CSH in regulating fetal and placental growth.

One distinction between this cohort and other models of IUGR (Galan et al., 2005) was the impact of CSH RNAi on Doppler velocimetry at 90 dGA. In this study, decreased systolic: diastolic ratios and resistance indices were observed, both indicative of reduced vascular resistance but no changes to umbilical blood flow at 90 dGA were documented. This emphasizes the uniqueness of this CSH RNAi model in comparison to other IUGR models such as maternal hyperthermia induced PI-IUGR which is characterized by increased vascular resistance coupled with decreased umbilical blood flow (Galan et al., 2005). Interestingly, despite these hemodynamic distinctions, both IUGR models result in both fetal and placental insufficiency as well as altered nutrient uptakes. This supports the notion that no single IUGR model can capture the heterogenicity of IUGR and thus, a multiplicity of models are needed to begin to understand the vast etiologies and consequences of IUGR.

Interestingly, the Doppler velocimetry of this cohort also differs from the Tanner et al. (2021b) study which observed decreases in umbilical blood flow by 90 dGA during

Doppler ultrasound. While umbilical blood flow near term (130-135 dGA) was reduced in both the Tanner et al. (2021b) study and the current study, 90 dGA Doppler velocimetry was only able to document decreased umbilical blood in Tanner et al. (2021b). This may infer some variability in the timing of IUGR development between studies.

Another novel insight gained by stratifying the current cohort based on severity was organ specific growth restriction. In contrasts to previous reports (Tanner et al., 2021a, b), we found reductions in fetal heart, kidney, and pancreas weights in CSH RNAi PI-IUGR animals, along with a tendency for fetal lung and spleen weights to also be reduced. Notably, fetal pancreas, liver, adrenal, and brain weights relative to fetal weight were also impacted, suggesting organ specific impacts beyond reductions in fetal mass.

The current cohort also provided greater resolution into the impacts of CSH RNAi on both uterine and umbilical blood biochemistry (gases, pH, buffers, and electrolytes). Previously, no changes in uterine and umbilical blood gases exempting oxygen content had been reported (Tanner et al., 2021a, b), likely due to smaller sample sizes. All CSH RNAi fetuses had reduced umbilical pO_2, oxyhemoglobin, oxygen saturation, and Ca^{2+}, as well as elevated hemoglobin and hematocrit. The PI-IUGR fetuses also had elevated pH and HCO^{3-}, supportive of greater fetal distress. Uterine circulation of the full CSH RNAi cohort had limited blood biochemical changes, however, uterine circulation in CSH RNAi PI-IUGR pregnancies had reduced electrolytes (K^+, Cl^-, and Na^+). While the specific reasons for each of these changes remains to be determined, they are likely driven by substantial decreases in uterine and umbilical oxygen uptakes, especially in the CSH RNAi PI-IUGR fetuses. Changes in fetal blood gasses reflective of lower

oxygen uptakes have been documented in other sheep models of IUGR (Regnault et al., 2003) and thus, support this idea.

A key repeatable finding confirmed by the current study was the role of CSH on uterine growth as all severities of CSH RNAi pregnancies experienced reductions in uterine weight. This relationship supports the Tanner et al. (2021b) study, which suggested CSH may act to promote uterine growth via regulation of uterine blood flow. The strong relationship between CSH and uterine blood flow has been demonstrated across all physiological experiments on CSH RNAi pregnancies (Tanner et al., 2021a, b; current study) regardless of fetal weight. However, the degree of blood flow reductions appears to be enhanced based on the severity of CSH RNAi phenotype as evidenced by the current study. Furthermore, the effect of CSH RNAi on uterine blood flow disappears when normalized to uterine weight suggesting CSH may first be acting to promote blood flow leading to uterine growth and nutrient uptake to promote placental function and fetal growth.

The current study also supports previous findings (Tanner et al., 2021b) regarding the consequences of CSH RNAi on nutrient uptake and transport during maternal normoglycemic conditions. While the uterine and umbilical uptakes, as well as the uteroplacental utilization of both oxygen and glucose were reduced regardless of phenotypic severity in CSH RNAi pregnancies, PI-IUGR animals did experience more severe reductions compared to controls. Additionally, reductions in uterine oxygen uptakes occurred independent of uterine weight in PI-IUGR pregnancies. This may point towards greater reductions in uterine blood flow in this subgroup as oxygen is a blood flow limited substrate. Together, these data along with previous observations (Tanner et

al., 2021b) support the conclusion that reductions in blood flow led to reduced uterine weights resulting in diminished oxygen and glucose uptakes and utilization, which resulted in smaller fetuses in CSH RNAi pregnancies.

One of the most notable distinctions in nutrient uptake between the full CSH RNAi cohort and the PI-IUGR subgroup is the impact on amino acid uptakes. In the full CSH RNAi group, only the uterine uptake of threonine was reduced whereas in the PI-IUGR subgroup, the uterine uptakes of 3 essential and 3 non-essential amino acids were all reduced. While fewer uterine amino acids in total were impacted in this cohort compared to a previous study (Tanner et al., 2021b), the amino acids that were impacted were reduced similar to what was previously reported. In umbilical circulation, the PI-IUGR subgroup experienced reductions in 14 amino acids (7 essential and 7 non-essential) whereas the full CSH RNAi cohort only noted decreases in 6 amino acids (4 essential and 2 non-essential). A greater total number of umbilical amino acids were impacted than previously described (Tanner et al., 2021b). The impacts on uteroplacental amino acid utilization in this cohort were similar regardless of severity of phenotype.

While the exact biological role of CSH continues to be investigated, Handwerger (1991) postulated that one of the main functions of CSH was to act as a partitioning agent to regulate fetal nutrient supply, acting distinctively in the mother and the fetus. In the mother, Handwerger speculated that CSH increased late-gestation IGF1 concentrations (Hurley et al., 1977c, Daughaday and Kapadia, 1978) along with promoting glucose intolerance, proteolysis, and lipolysis to facilitate greater nutrient partitioning to the fetus (Handwerger, 1991). Our results do not support the postulated

maternal actions of CSH. In fact, while not statistically different, our data support increased insulin secretions. This is consistent with previous CSH RNAi studies that saw either limited changes (Tanner et al., 2021b) or numerical increases in maternal insulin concentrations (Baker et al., 2016). Furthermore, no changes to maternal nutrient availability have been documented (Tanner et al., 2021a, b; current study). While the high maternal CSH concentrations (~1000-fold excess of receptor K_d; Pratt et al., 1995) are perplexing, the actions of CSH in maternal circulation thus far appear to be limited to regulating uterine blood flow, although this merits further investigation.

In the fetus, Handwerger predicted that CSH stimulated IGF1 production to promote somatogenic activity (Handwerger, 1991). Our data largely support Handwerger's proposition pertaining to fetal physiology as we have previously and repeatedly demonstrated that CSH RNAi results in decreased IGF1 regardless of fetal weight reductions (Baker et al., 2016; Ali et al., 2020; Tanner et al., 2021b). We have also determined that CSH RNAi impairs umbilical amino acid uptake *in vivo* (Tanner et al., 2021b; current study), although this effect appears to be dependent on severity of growth restriction. The results from our recent studies also support the role of CSH in regulating umbilical blood flow (Tanner et al., 2021b; current study) and vascular resistance (current study), enhancing fetal insulin secretion (Tanner et al., 2021b; current study), and ultimately facilitating the uptake of all key oxidative substrates such as glucose, lactate, and amino acids (Tanner et al., 2021b; current study). These data stress the necessity of adequate CSH to promote adequate fetal growth and placental function.

CONCLUSIONS

Ultimately, based on the literature and the data presented in this study, it appears that two of the important biological roles of CSH are 1) promoting blood flow and nutrient uptake by the uteroplacental unit and 2) regulating uteroplacental oxidative metabolism. Furthermore, it appears that CSH acts in an endocrine fashion to promote fetal liver growth and IGF production, and likely, pancreatic growth and insulin production. When CSH deficiency occurs, deficiencies in blood flow and nutrient uptake occur, impacting both the fetus and the placenta and resulting in metabolic maladaptation. In the placenta, greater amounts of oxidative substrates are required to maintain function, resulting in fewer nutrients being available for transfer to the fetus. In the fetus, the liver and pancreas have reduced capacity to produce IGF1 and insulin respectively. Ultimately, these adaptations lead to the development of fetal and placental growth restriction.

CHAPTER V: CHORIONIC SOMATOMAMMOTROPIN DEFICIENCY PERTURBS THE PLACENTAL METABOLIC TRANSCIPTOME

INTRODUCTION

Chorionic somatomammotropin (CSH; aka placental lactogen) is a member of the growth hormone/prolactin family and is secreted by the binucleate cells of the ruminant placenta. While CSH is the most abundantly produced placental hormone, the exact biological functions of CSH continues to evade elucidation. Decreases of CSH in maternal circulation have been associated with IUGR in sheep (Lea et al., 2007) but until recently, no direct evidence existed to demonstrate the necessity of CSH for adequate fetal growth. To this end, our lab developed an approach to generate CSH deficient pregnancies in sheep using lentiviral-mediated RNA interference (RNAi) as described by Baker et al. (2016). Using this approach, we demonstrated CSH RNAi directly resulted in the development of intrauterine growth restriction (IUGR) at both 50 days of gestational age (dGA; Jeckel et al.,2018) and 135 dGA (near-term; Baker et al., 2016).

Interestingly, near term (135 dGA) two distinct phenotypes emerge in response to CSH RNAi: 1) pregnancies with IUGR and 2) pregnancies with normal fetal and placental weights (Baker et al., 2016; Ali et al., 2020). These two phenotypes resemble those documented in the human in response to CSH loci mutations/deletions (Daikoku et al, 1979 and Rygaard et al., 1998). Both CSH RNAi phenotypes displayed reduced umbilical insulin-like growth factor 1 (IGF1), as well as increased uterine artery to vein glucose gradients, an indicator of impaired glucose uptake (Ali et al., 2020). This led us

to believe that metabolic perturbations as a result of CSH RNAi were occurring regardless of changes in fetal and placental weight. This was supported by *in vivo* physiological experiments on CSH RNAi pregnancies without IUGR which exhibited reduced uterine blood flow and increased glucose utilization by the placenta, ultimately leading to reduced glucose transfer to the fetus (Tanner et al., 2021).

Thus, to investigate transcriptomic changes in CSH RNAi placentae, we employed RNA sequencing. We hypothesized that CSH RNAi would result in transcriptional pertubations of genes involved in metabolism and cell function in both IUGR and non-IUGR phenotypes. Therefore, the objective of this study was to characterize placental transcriptional pathways that are impacted due to CSH deficiency, in order to provide insight into altered placental metabolism observed *in vivo,* and to aid in describing the progression of IUGR in CSH deficient pregnancies.

MATERIALS AND METHODS

Creation of CSH RNAi pregnancies and tissue collections

All procedures were approved by the Colorado State University Institutional Animal Care and Use Committee (#14-5257A) and the Institutional Biosafety Committee (#11-034B and 13-043B). The generation of these CSH RNAi pregnancies was extensively described by Baker et al. (2016). Briefly, fully expanded and hatched blastocysts were collected at 9 dGA. Each blastocyst was infected with 100,000 transducing units of either control RNAi (scramble control; n= 8) or CSH RNAi (target 6/tg6; n =16) lentivirus. After 4 h of infection, a single washed blastocyst was surgically transferred into a synchronized recipient ewe. Placentomes were collected at terminal

surgery at 135 dGA and were processed by separating the fetal portions of the placenta (cotyledons) from the maternal portion of the placenta (caruncle) which were subsequently snap frozen in liquid N_2 and stored at -80°C. Selection of cotyledonary samples for transcriptomic analysis were selected based on four traits: 1) placental weight, 2) fetal weight, 3) uterine artery-vein glucose gradient, 4) umbilical artery IGF1 concentrations. The 5 samples that most distinctly clustered within each treatment (CON n=5; CSH-IUGR n = 5; CSH-NW n=5) based on SD from the treatment mean for the combination of aforementioned traits were selected for RNA sequencing.

RNA Sequencing, alignments and analysis

Frozen cotyledons were pulverized, and RNA was isolated using RNeasy Mini Kit (Ref# 74104; QIAGEN, Hilden, Germany) according to manufacturer instructions. Samples were submitted to the University of Colorado Genomics and Microarray Core (Aurora, CO) for sequencing. RNA integrity was assessed by Agilent TapeStation (Agilent Technologies, Inc, Boulder, CO) and all samples had a minimum RNA integrity number (RIN) of ≥ 9.0. NuGEN Universal mRNA library prep kit (cat # 0508; NuGEN Technologies, Inc., Redwood City, CA) was utilized according to manufacturer's instructions. Both automated clusters generation (150 bp) and RNAseq analysis were by the Illumina NovaSEQ6000 (100,000,000 paired end reads). Adapter sequences were trimmed with Trimmomatic (Bolger et al., 2014). Paired-end reads were mapped to the reference ovine genome (*Ovis aries*, Oar.ra_1.0, Baylor College of Medicine) using HISAT2 version 2.2.0 (Kim et al., 2015). Transcripts were assembled and gene counts were obtained through StringTie version 1.3.3 (Kovaka et al., 2019, Pertea et al., 2016,

Pertea et al., 2015). DESeq2 using fragment bias correction was utilized to determine differentially expressed genes (DEGs) between each CSH RNAi phenotype and controls (CON vs. CSH-IUGR and CON vs CSH- NW). The minimum alignment count was 15. The threshold for significance after multiple testing corrections (Q-value) was set to Q ≤ 0.10 (equivalent to P <0.00035). All genes that were false discovery rate protected (Q ≤ 0.10) were used for pathway analysis with DAVID 6.8 (Huang et al., 2009a and Huang et al., 2009b) and Ingenuity Pathway Analysis (IPA; QIAGEN Inc;https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis). The threshold for considering a pathway or ontology significant was P ≤ 0.05. Real-time qPCR validation of a subset of genes identified by DEG analysis is ongoing.

RESULTS

A total of 346 DEGs meeting a threshold of Q ≤ 0.10 were identified between CSH-IUGR vs CON, with 162 being up-regulated and 184 being down-regulated in CSH-IUGR pregnancies. Analysis of CSH-NW vs CON pregnancies revealed 96 DEGs, 40 of which were up-regulated and 56 were down-regulated in CSH-NW pregnancies. Thirty-six common DEGs were identified in both CSH-IUGR and CSH-NW pregnancies, indicating likely direct effects of CSH RNAi. Table 1 contains a list of DEGs impacted by CSH RNAi that could prove interesting to further investigate to test how CSH RNAi differentially impacts placental function in IUGR vs normal weight pregnancies.

Table 5.1. List of interesting differentially expressed genes common to all chorionic somatomammotropin RNA interference pregnancies (direct effects) or unique to phenotype (intrauterine growth restricted vs. normal weight).

Comparison	Gene	Category	Function	Direction	Q-value
CON vs CSH RNAi[1]	STARD8	Nutrient transporter	Lipid transfer	Up	0.001
	LOC101111573	Nutrient transporter	Cationic amino acid transporter	Down	0.102
	LRP11	Receptor	Low-density lipoprotein receptor	Up	0.057
	CD38	Immune function	Immune function	Up	0.031
	CXCL11	Immune & signaling	positive regulation of cAMP	Up	0.058
	CXCL9	Immune & signaling	positive regulation of cAMP	Up	0.019
	ANGPTL7	Vascular function	Angiopoietin related protein	Up	0.007
CON vs CSH-IUGR[2]	PFKP	Metabolic enzyme	Phosphofructokinase	Up	0.003
	PDK3	Metabolic enzyme	Pyruvate dehydrogenase kinase	Down	0.033
	LIPE	Metabolic enzyme	Lipase (hormone sensitive)	Up	0.043
	GOT2	Metabolic enzyme	Glutamic-Oxaloacetic Transaminase	Down	0.049
	LAP3	Metabolic enzyme	Leucine aminopeptidase	Down	0.066
	PDHB	Metabolic enzyme	Pyruvate dehydrogenase	Down	0.076
	PC	Metabolic enzyme	Pyruvate carboxylase	Up	0.103
	SLC35B1	Nutrient transporter	Galactose transport	Down	0.005
	SLC5A1	Nutrient transporter	Na+/Glucose cotransporter	Up	0.013
	SLC15A1	Nutrient transporter	Peptide transporter	Down	0.034
	SLC25A12	Nutrient transporter	Aspartate/ glutamate carrier	Down	0.040
	NOS2	Vascular function	Inducible nitric oxide synthase	Up	0.010
	PTGIS	Vascular function	Prostaglandin 12 synthase	Down	0.082
CON vs CSH-NW[3]	PAPPA2	Hormonal regulator	Insulin-like growth factor regulator	Down	0.032
	DIO2	Hormone activator	Deiodinase for thyroid hormone	Up	0.057
	ENO4	Metabolic enzyme	Glycolysis	Down	0.100

[1] Comparison between control (n=5) and all chorionic somatomammotropin RNA interference pregnancies (n=10)
[2]Comparison between control (n=5) and chorionic somatomammotropin RNA interference pregnancies with intrauterine growth restriction (n=5)
[3]Comparison between control (n=5) and chorionic somatomammotropin RNA interference pregnancies with normal fetal and placental weights (n=5)

DAVID 6.8 functional annotation and ontological analysis of pathways revealed numerous categories impacted in response to CSH RNAi with IUGR. The categories presented in Table 2 describe pathways or ontologies (n=127; $P<0.05$) that could potentially contribute to the progression of IUGR including: extracellular exosomes (n=46), metabolic pathways (n=27), extracellular space (n=17), protein processing in the endoplasmic reticulum (n=11), carbon metabolism (n=6), cellular responses to hypoxia (n=5), regulation of cell proliferation (n=5), lysine degradation (n=4), cAMP regulation in metabolic processes (n=3), and negative regulation of protein catabolism (n=3).

Table 5.2. DAVID 6.8 Functional annotation of pathways and ontologies impacted by chorionic somatomammotropin deficiency in pregnancies with intrauterine growth restriction.

DAVID Category	Biological role	Genes	Up	Down	P-value
KEGG_PATHWAY	Protein processing in endoplasmic reticulum	11	3	8	0.0002
GOTERM_CC_DIRECT	Extracellular exosome	46	20	26	0.001
GOTERM_BP_DIRECT	Positive regulation of cAMP metabolic process	3	3	0	0.001
GOTERM_BP_DIRECT	Cellular response to hypoxia	5	3	2	0.003
GOTERM_BP_DIRECT	Regulation of cell proliferation	5	5	0	0.02
GOTERM_CC_DIRECT	Extracellular space	17	10	7	0.03
KEGG_PATHWAY	Carbon metabolism	6	3	3	0.03
KEGG_PATHWAY	Metabolic pathways	27	8	19	0.05
GOTERM_BP_DIRECT	Negative regulation of protein catabolic process	3	2	1	0.05
KEGG_PATHWAY	Lysine degradation	4	2	2	0.05

Ingenuity Pathway Analysis revealed functional categories similarly disrupted (P≤0.01; Table 3) by CSH RNAi regardless of fetal weight included 1) hematological development and function, 2) cell death and survival, 3) cell-to-cell signaling, and 4) immune cell trafficking. By comparing CON vs CSH-IUGR, four canonical pathways) were identified (P≤0.01; Table 3) including eicosanoid signaling, 3-phosphoinositidine biosynthesis, and protein ubiquitination pathways. The cell functions disrupted (P≤0.01) in CSH-IUGR pregnancies included cellular movement, protein synthesis, lipid metabolism, and tissue morphology. In CON vs CSH-NW pregnancies, the canonical pathways implicated (P≤0.01; Table 3) included the mitotic roles of polo-like kinases and signaling by Rho family GTPases. Cell functions uniquely disrupted (P≤0.01) by CSH RNAi in pregnancies with normal weights included cell functions post-translational modification, cell signaling, and connective tissue development.

Table 5.3. Ingenuity Pathway Analysis of canonical pathways, cell functions, & physiological systems impacted by chorionic somatomammotropin RNA interference.

Contrast	Category	Name	Molecules impacted	P-value
CON vs CSH RNAi[1]	Physiological systems	Hematological development & function	41	0.013
	Cell functions	Cell death & survival	34	0.014
	Cell functions	Cell-to-cell signaling	33	0.011
	Physiological systems	Immune cell trafficking	20	0.011
CON vs CSH-IUGR[2]	Canonical pathways	Eicosanoid Signaling	3	0.005
	Canonical pathways	3-phosphoinositide biosynthesis	6	0.008
	Canonical pathways	Protein ubiquitination pathway	8	0.009
	Cell functions	Cellular movement	24	0.011
	Cell functions	Protein synthesis	50	0.007
	Cell functions	Lipid metabolism	40	0.011
	Physiological systems	Tissue morphology	17	0.011
CON vs CSH-NW[3]	Canonical pathways	Mitotic roles of polo-like kinase	3	0.001
	Canonical pathways	Signaling by Rho GTPases	4	0.007
	Cell functions	Post-translational modification	7	0.012
	Cell functions	Cell signaling	7	0.010
	Physiological systems	Connective tissue development	11	0.015

[1]Comparison between control (n=5) and all chorionic somatomammotropin RNA interference pregnancies (n= 10)

[2]Comparison between control (n=5) and chorionic somatomammotropin RNA interference pregnancies with intrauterine growth restriction (n=5)

[3]Comparison between control (n=5) and chorionic somatomammotropin RNA interference pregnancies with normal fetal and placental weights (n=5)

DISCUSSION

We hypothesized CSH RNAi would result in perturbation of placental metabolism and cell function, identified through transcriptomic alterations to genes in both IUGR and non-IUGR phenotypes. The results from the functional analyses support this hypothesis as CSH RNAi placentae possessed DEGs and functional groups that indicate disrupted metabolism and cell function. By examining common DEGs between both CSH RNAi phenotypes, it is possible to examine the direct effects of CSH RNAi on the placental transcriptome. The 36 commonly impacted DEGs included genes involved in lipid and amino acid transfer, and positive regulation of cAMP. Additionally, both CSH RNAi phenotypes saw changes with functional groups associated with hematological development and function, cell death and cell-to-cell signaling, thus indicating direct impacts of CSH RNAi on those pathways regardless of phenotype. Taken together, these DEGs and pathways support the idea that CSH RNAi placenta do differ from normal pregnancies. This is further supported by the altered physiology of both CSH RNAi phenotypes including reduced umbilical IGF1 and increased uterine artery-vein glucose gradients (Ali et al., 2020). Furthermore, the direct impacts of CSH RNAi on hematological function is supported by reductions in uterine blood flow as well as caruncular endothelial nitric oxide synthase (NOS3) in CSH RNAi pregnancies (Tanner et al., 2021).

CSH RNAi pregnancies resulting in IUGR were more transcriptionally perturbed (346 DEGs) than CSH RNAi pregnancies with normal weights (90 DEGs). Furthermore, these pregnancies had increased metabolic transcriptional disruptions as evidenced by the impacted functional groups of carbon and lipid metabolism, cAMP metabolic

processes, and protein catabolism and degradation. This is supported by the *in vivo* studies where these pregnancies experienced 32% and 52% reductions in fetal and placental weights, respectively, as well as reduced fetal liver weights (Baker et al., 2016). Interestingly, this RNAseq analysis revealed an increase in the Na+ dependent glucose cotransporter SLC5A1 in CSH RNAi pregnancies with IUGR. In the future, this could be examined as a potential compensatory mechanism as these placentae have been demonstrated to have decreased SLC2A1 (GLUT1), SLC2A3 (GLUT3) mRNA expression (Jeckel et al., 2018). Ultimately, these unique transcriptional disruptions to nutrient transporters, metabolic pathways, and metabolic enzymes could in part explain why some CSH RNAi pregnancies experience IUGR and some do not.

CONCLUSIONS

Chorionic somatomammotropin deficient pregnancies can result in fetal growth restriction, and sheep that have low birth weights often experience a high neonatal death loss. Often, those that survive have reduced postnatal growth potential which can impact the economic return of producers. This study examined placental transcriptomic alterations due to chorionic somatomammotropin deficiency to 1) identify novel genes and pathways that could explain the progression of growth restriction and 2) combine transcriptomic and physiological data to outline novel functions of chorionic somatomammotropin to be tested in the future. These data will provide insights to improve neonatal survival in livestock.

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