THESIS

INVESTIGATING THYROID HORMONE TRANSPORT AND REGULATION IN CHORIONIC SOMATOMAMMOTROPIN RNAI MODELS OF INTRAUTERINE GROWTH RESTRICTED PREGNANCIES

Submitted by

Anna Donovan

Department of Biomedical Sciences

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Master's Committee:

Advisor: Quint Winger

Russell Anthony
Caitlin Cadaret

ABSTRACT

INVESTIGATING THYROID HORMONE TRANSPORT AND REGULATION IN CHORIONIC SOMATOMAMMOTROPIN MODEL OF INTRAUTERINE GROWTH RESTRICTED PREGNANCIES

In vivo lentiviral mediated RNA interference of chorionic somatomammotropin (CSH) results in an intrauterine growth restricted (IUGR) phenotype in sheep. Abnormal levels of thyroid hormones (THs) 3,5,3′-triiodothyronine (T_3) and 3,5,3′,5′-tetraiodothyronine (T_4, thyroxine) and abnormal expression of placental deiodinase 2 and deiodinase 3 (DIO2/DIO3) mRNA have been implicated in IUGR. It has been reported that nutrient restricted models of IUGR pregnancies result in a decrease of thyroxine levels in maternal and fetal circulation. Transthyretin (TTR) is a TH binding molecule produced by trophoblast cells that preferentially binds T_4 and may shuttle T_4 through the placenta. It was our objective to better understand thyroid hormone transport during late gestation specifically in CSH RNAi models of IUGR pregnancies. We hypothesized that a reduction in placental CSH negatively impacts thyroid hormone transport from maternal circulation to fetal circulation due to perturbations in both thyroid hormone carrier protein activity and placental deiodinase enzyme expression.

The trophectoderm of hatched blastocysts (9 days gestational age; dGA) was infected with lentivirus expressing either a non-targeting sequence (NTS) shRNA to create control pregnancies, or a CSH shRNA to generate a CSH knockdown model of IUGR pregnancies. Uterine vein, uterine artery, umbilical vein, and umbilical artery serum was collected via

catheterization, and maternal and fetal tissue was harvested near term (~135 dGA) from growth restricted CSH RNAi pregnancies (n=4) and control pregnancies (n=4). TTR protein abundance was determined through western blot analysis, T_4 and T_3 levels were assessed using competitive ELISA assays, and DIO2 and DIO3 expression was measured using qRT-PCR. T_4 was reduced by 16% ($P \leq 0.05$) in CSH RNAi IUGR umbilical vein serum samples compared to control umbilical vein serum samples, and by 29% ($P \leq 0.05$) in CSH RNAi IUGR umbilical artery serum samples compared to control umbilical artery serum samples. TTR protein was 47% ($P \leq 0.10$) less abundant in CSH RNAi IUGR uterine artery serum compared to control serum and 64% ($P \leq 0.05$) less abundant in CSH RNAi IUGR uterine vein serum compared to control serum. In umbilical artery serum TTR protein abundance was 47% ($P \leq 0.05$) less abundant in CSH RNAi IUGR serum compared to control serum. Uterine TTR uptake tended to be reduced by 45% ($P \leq 0.10$) in CSH RNAi pregnancies compared to control pregnancies, and uteroplacental utilization tended to be reduced by 48% ($P \leq 0.10$) in CSH RNAi pregnancies compared to control pregnancies. Umbilical uptake was not measured in either control or CSH RNAi pregnancies. There was also no difference in TTR protein concentration in either maternal or fetal liver tissue when comparing CSH RNAi tissue to control tissue. Caruncle DIO2 mRNA expression was reduced by 60% ($P \leq 0.05$) in CSH RNAi caruncle samples when compared to control caruncle samples, with no significant change in DIO2 expression between CSH RNAi samples and control samples in the cotyledon. There was no difference in DIO3 expression between CSH RNAi samples and control samples in either the caruncle or cotyledon.

Our data suggests that in ovine pregnancies near term, a deficiency in CSH negatively impacts processes related to thyroid hormone transport into and throughout the placenta.

Dysregulation in thyroid hormone transport could be playing a role in negatively impacting placental physiology, thus aiding in the development of IUGR.

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The Placenta:

A developing fetus relies on the transfer of nutrients from maternal circulation to fetal circulation via the placenta. The placenta has been described as an apposition of parental and fetal tissue for the purpose of physiological exchange (Mossman, 1937), and while this definition is true, the placenta also acts as more than just a barrier of exchange (Barry & Anthony, 2008). It is just one organ but performs actions typical of multiple systems such as the lungs, kidney, liver, and the endocrine system to support fetal growth and development (Burton & Jauniaux, 2015; Barry & Anthony, 2008). Not only is the placenta the site of transfer of maternally supplied nutrients to the fetus, but it also utilizes nutrients and oxygen itself and produces functioning proteins and hormones that are sent to both maternal and fetal sides of circulation to maintain healthy pregnancies (Strauss et al., 1996; Wooding et al. 1997). If this organ is not properly functioning, depending on the severity of dysfunction, serious pregnancy disorders can arise leading to an increased likelihood of fetal death (Gagnon, 2003).

While the structure of the placenta looks vastly different between species of animals, the function is highly conserved. For the sake of ethical research, many different animal models are used to study physiological process occurring during pregnancy. Our lab works with sheep models to investigate nutrient exchange across the placenta, therefore for the scope of this review, we will characterize both sheep and human placentas

Human Placenta Characterization:

Human placentas are discoid in shape, meaning there is one large structure that is the single point of nutrient exchange between maternal circulation and fetal circulation (Grosser,

1909). Placentas are also characterized based on the layers between fetal and maternal blood. Human placentas are characterized as hemochorial meaning that the fetal trophoblast cells are in direct contact with maternal blood supply.

The placenta is derived from the trophectoderm layer of the blastocyst which consists of trophoblast cells. The three major subpopulations of trophoblasts are cytotrophoblasts (CTB), extra villous cytotrophoblast (EVT), and syncitiotrophoblast (STB) cells. The trophectoderm initially consists of CTBs which are mononucleated trophoblast cells located at the apical region of the villi (Rampersad et al., 2011). CTBs will begin to proliferate and fuse together to form a syncytium that sits on top of proliferating CTB cells (Frank, 2011). This layer is referred to as syncitiotrophoblast cells (STB) and are the cells in direct contact with maternal blood in humans, meaning that blood from maternal circulation will first encounter STBs before encountering CTBs. Villi formation continues, and capillary networks form. Cytotrophoblasts can also differentiate into extra villous trophoblast cells (Frank, 2011). These specialized trophoblast cells invade and remodel maternal spiral arteries leading to increased blood flow from maternal circulation to fetal circulation (Rampersad et al., 2011). If maternal arteries are not remodeled by EVTs, nutrient transfer is impaired which can lead to pregnancy disorders such as preeclampsia (Woelkers, 2011).

Sheep Placenta Characterization:

Unlike the human placenta, the sheep placenta is cotyledonary, meaning that there are multiple smaller sites of nutrient exchange from maternal circulation to fetal circulation rather than one larger site. These sites are formed in areas of endometrial tissue that are highly vascularized called caruncles (Stegeman, 1974). Around week four of gestation chorionic

epithelial tissue starts to interdigitate with these caruncles and create placentomes (Boshier, 1969). As gestation advances, this interdigitation continues and branched crypts develop. From these crypts, fetal villi develop which become the site of nutrient exchange at the maternal fetal interface (Steven, 1975). While it still is the trophectoderm that forms the placenta, sheep trophoblast cells are characterized differently from humans. Sheep do not have invasive trophoblast cells that compare to human EVTs, but there are binucleate cells that act like a syncytium between maternal and fetal circulation and are often compared to syncitiotrophoblast cells (Wooding, 1982).

While there are differences in human and sheep placenta anatomy, the two species have similar placental villi and vascular structure making sheep a good alternative to humans when studying nutrient exchange across the placenta (Leiser et al., 1997).

Fetal Thyroid Gland Development:

One of the substrates passed from maternal circulation to fetal circulation are thyroid hormones. The thyroid gland is an endocrine gland which sits at the base of the neck in humans and is responsible for the production of Thyroxine or 3,5,3',5'-tetraiodo-L-thyronine (T_4) and Triiodothyronine or 3,5,3'-triiodo-L-thyronine (T_3).

In humans, the thyroid gland begins to develop around 24 days after conception (Polk & Fisher, 2011). It has been reported that thyroglobulin, a glycoprotein structure necessary for thyroid hormone synthesis has been observed as early as day 29 of gestation (dGA; Kratzsch & Pulzer, 2008), but there are other conflicting reports that state thyroglobulin is not observed in the thyroid gland until 70 dGA. By 50 dGA, the thyroid gland has migrated to the anterior portion of the neck where it will remain for the rest of gestation and post-natal life. It is around

this time that the cells in the developing thyroid gland start to mature. Canaliculus that evolve from the endoplasmic reticulum migrate towards the apex of the cell where they will begin to discharge their contents outside of the cell and fuse with other canaliculi to create a larger space for colloid accumulation. Around 70 dGA, an increase in iodine concentrations in the colloid is observed (Polk & Fisher, 2011). This increase along with the increasing amounts of thyroglobulin result in the synthesis and accumulation of thyroid hormones (Polk & Fisher, 2011).

Thyroid Hormones:

T_4 and T_3 are amino acid derived hormones that are produced in the follicular cells of the thyroid gland (Barrett, 2012). These hormones are involved in numerous systems in the body but are mainly known for the role they play in the regulation of one's basal metabolic rate (BMR). Excess TH increases basal metabolic rate, whereas insufficient amounts of TH reduce the basal metabolic rate (Barrett, 2012). Thyroid hormones are necessary to regulate lipid metabolism, carbohydrate metabolism and protein metabolism, therefore changes to these pathways will lead to an abnormal BMR (Barrett, 2012). It has also been reported that THs are necessary for normal fetal growth and brain development (Korevaar et al., 2017). Thyroid hormone deficiency due to an iodine deficiency can lead to a condition called cretinism. This condition is characterized by impairments in both mental and physical development such as mental retardation and delayed motor skill development (Barrett, 2012). Thyroid hormones are also necessary for normal female reproductive function as well. Hypothyroidism has been linked to delayed onset of puberty, anovulation, ovarian cysts, menstrual irregularity, and infertility (Silva et al., 2018). These examples of systems affected by dysregulation of thyroid hormones clearly illustrate the

importance of maintaining regulated levels of thyroid hormone for normal overall physiological function.

TH secretion is regulated and stimulated by a pathway called the hypothalamic-pituitary-thyroid axis. Thyrotropin releasing hormone (TRH) is released from the arcuate nucleus into the median eminence of the hypothalamus. TRH then binds to receptors in the anterior pituitary which stimulates the release of thyroid stimulating hormone (TSH) into circulation where it will bind to receptors on the thyroid follicular cells. This binding activates the mechanism for thyroid hormone production in the thyroid gland. In short, TSH stimulates sodium iodine cotransporter activity which allows for the accumulation of iodine in the follicular cells. This iodine will leave the follicular cells along with thyroglobulin and enter the follicular lumen where it will be oxidized. Once oxidized, iodination of thyroglobulin occurs. Conjugation of the iodinated tyrosine leads to the production of T_4 and T_3 linked to thyroglobulin. Processing of this molecule occurs in the follicle cells of the thyroid gland and eventually T_4 and T_3 are secreted into circulation (Barrett, 2012).

T_4 is produced in much greater abundance than T_3 (Rousset et al., 2015), but T_3 is known to be more biologically active due to its higher affinity for thyroid hormone receptors (THR) (Barrett, 2012). Thyroid hormones elicit most of their actions by binding to a thyroid hormone receptor in the nucleus (Adu-Gyamfi et al., 2020). This receptor is comprised of two subunits (THR: Retinoid X Receptor) dimerized together on a stretch of DNA called the thyroid response element (TRE). When TH is not present this receptor is in an inactive state due to the binding of corepressor molecules. Once TH binds to this complex a conformational shift occurs that displaces corepressors and allows for the binding of activators, leading to the THR's activation

(Adu-Gyamfi et al., 2020). When active, thyroid hormone receptors act as a transcription factor, leading to the transcription of the gene that it sits on (Anyetei-Anum et al., 2019). Thyroid hormones can also elicit a response through pathways that do not involve transcription and translation. They may act on receptors in plasma membrane, cytoplasm or mitochondria to initiate pathways that increase cellular oxygen consumption or activate ion channels, second messengers and protein kinases to initiate a cellular response (Barrett, 2012; Anyetei-Anum et al., 2019; Kalyanaraman et al., 2014).

Thyroid hormones produced by a human fetus's own thyroid gland are not present in fetal circulation until about week 14 of gestation (Barrett, 2012). Thus, the fetus relies on maternal thyroid hormones transported across the placenta during the first trimester (de Escobar et al., 2004). Thyroid hormones are released from the thyroid gland and enter maternal circulation bound to a thyroid carrier protein. When TH-carrier protein complexes approach the placental interface, TH is released and transported into and out of the placenta via transport proteins such as large amino-acid transporters (LATs), organic anion transporting polypeptides (OATP1C1, OATP4A1; Mendoza & Hollenberg, 2017), or monocarboxylate transporters (MCT8, MCT10; Abe et al., 2012; Bernal et al., 2017). These transporters allow the transport of other molecules into and out of the placenta as well.

While thyroid hormone transport throughout gestation is not a novel topic of study, and hypothyroidism is known to be associated with asymmetric intrauterine growth restriction (Harris et al., 2017), recent data in ovine models of IUGR pregnancies have increased our interest in this system. In 2021, Steinhauser et al. observed that there was a decrease of plasma T_4 in maternal nutrient restricted models of IUGR near term compared to control animal

pregnancies in samples taken from the fetal heart, with no change in T_3 levels. Interestingly, they observed that maternal thyroid hormone levels were impacted as well. They reported that maternal T_4 and T_3 levels were reduced in pregnancies effected by an IUGR phenotype compared to those that were not (Steinhauser et al. 2021).

Thyroid Hormone Action in the Placenta:

The placenta is commonly thought of only as a barrier which nutrients pass through. However, it is important to remember that it is a metabolically active organ that produces substrates necessary for fetal growth, development, and maternal adaptation to pregnancy (Constância et al., 2005). While thyroid hormones do travel across the placenta to enter fetal circulation, these molecules also have a physiological role in the placenta itself, specifically involving trophoblast proliferation, differentiation, and invasion (Barber et al., 2005; Handwerger, 2010; Maruo et al., 1991; Silva et al., 2014; Cartwright et al., 1999).

Regarding thyroid hormones roll in human trophoblast proliferation, different studies have produced conflicting reports. When JEG-3 choriocarcinoma cells were treated with T_3, rate of cell proliferation increased, whereas when extra villous-like cell line SGHPL-4 cells were treated with T_3, rate of cell proliferation decreased. The difference in thyroid hormone responsiveness observed was thought to be due to differential deiodinase responsiveness or just simply because these are two different cell types (Barber et al., 2005). Thyroid hormones may also be aiding in the process of human cytotrophoblast (CTB) to syncitiotrophoblast (STB) differentiation. One of the functions of syncitiotrophoblast cells is producing and secreting hormones (Handwerger, 2010). It has been observed that when first trimester placental tissues are exposed to optimal levels of T_3 or T_4, there is an increase in secretion of placental derived

hormones such as placental lactogen estradiol-17 beta, progesterone, and human chorionic gonadotropin Interestingly however, when thyroid hormones were added in concentrations that were less than or greater than those established as optimal to the placental tissue, overall hormone secretion was impaired (Maruo et al., 1991). There are also multiple studies illustrating that TH is involved in extravilllous trophoblast (EVT) invasiveness. In 2014, Siliva et al. observed that in hypothyroid rat pregnancies, EVT migration is impaired (Silva et al., 2014). They deduced that this was due to a reduction in the expression of molecules that are known to mediate invasiveness of EVTs such as MMP2, MMP9, placental leptin and NOS2. MMP molecules aid in the degradation of endometrial extracellular matrix while NOS2 helps EVTs invasive ability, thus when expression is reduced, invasive ability is decreased (Cartwright et al., 1999).

Due to the influence that thyroid hormones have on trophoblast cells, it is plausible to hypothesize that thyroid hormone dysregulation may play a role in pregnancy complications related to shallow invasion of EVTs such as preeclampsia, multiple miscarriages or impaired nutrient transfer leading to IUGR. Dysregulation of thyroid hormone levels during gestation could be related to impaired transport of maternal TH to the fetus via a disruption in one or multiple thyroid hormone carrier proteins. It could also be due to a change in the deiodinase enzyme expression in the placenta which under normal circumstances, tightly regulate the concentrations of thyroid hormones through the process of activating and inactivating THs from maternal circulation (Köhrle, 1999; Adu-Gyamfi et al., 2020).

Deiodinase Enzymes:

Deiodinases are selenium dependent enzymes found throughout the body, including the placenta. These enzymes act to regulate the activation and inactivation of thyroid hormones. It does so through either the removal of iodine at the phenolic ring or the tyrosyl ring (Gereben et al., 2008). There are three known types of deiodinase enzymes, characterized by their function and location in the body: deiodinase 1 (DIO1), deiodinase 2 (DIO2) and deiodinase 3 (DIO3). DIO1 is present in the thyroid gland, liver, and kidneys but not the placenta therefore for the scope of this paper it will not be discussed further (Chan et al., 2003). In the human placenta, DIO2 is expressed strongly in the CTB and weakly in the STB. It is mostly expressed in the membrane of the endoplasmic reticulum. The opposite is true for DIO3. It is highly expressed in STB and weakly expressed in CTB and is mostly expressed in the plasma membrane of these cells (Gutiérrez-Vega et al., 2020).

Deiodinase Activity in the Placenta:

As previously mentioned, DIO3 is expressed primarily in STB and DIO2 is expressed primarily in the CTB (Gutiérrez-Vega et al., 2020). DIO3's function is to convert T_4 to reverse triiodothyronine (rT_3), or T_3 to diiodothyronine (T_2) which are both inactive forms of TH. DIO2 activates the biologically active form of TH by converting T_4 to T_3. It also converts T_3 to T_2 (Figure 1; adapted from Adu-Gyamfi et al., 2020).

While it might seem counter intuitive to inactivate thyroid hormones from the maternal circulation, DIO3 evolved as a mechanism to ensure that if maternal circulation has excess TH, the large concentration of THs will not enter fetal circulation. The opposite is true for DIO2. If DIO2 is dysregulated not enough active thyroid hormone can be transported to the fetus (Adu-Gyamfi et al., 2020). This system is more important in early gestation when the fetus is

dependent on a regulated level of maternal thyroid hormones before it can produce its own endogenous supply. Once the fetus can produce their own thyroid hormones, TH transport capacity changes, and expression of these enzymes decreases (Chan et al., 2003).

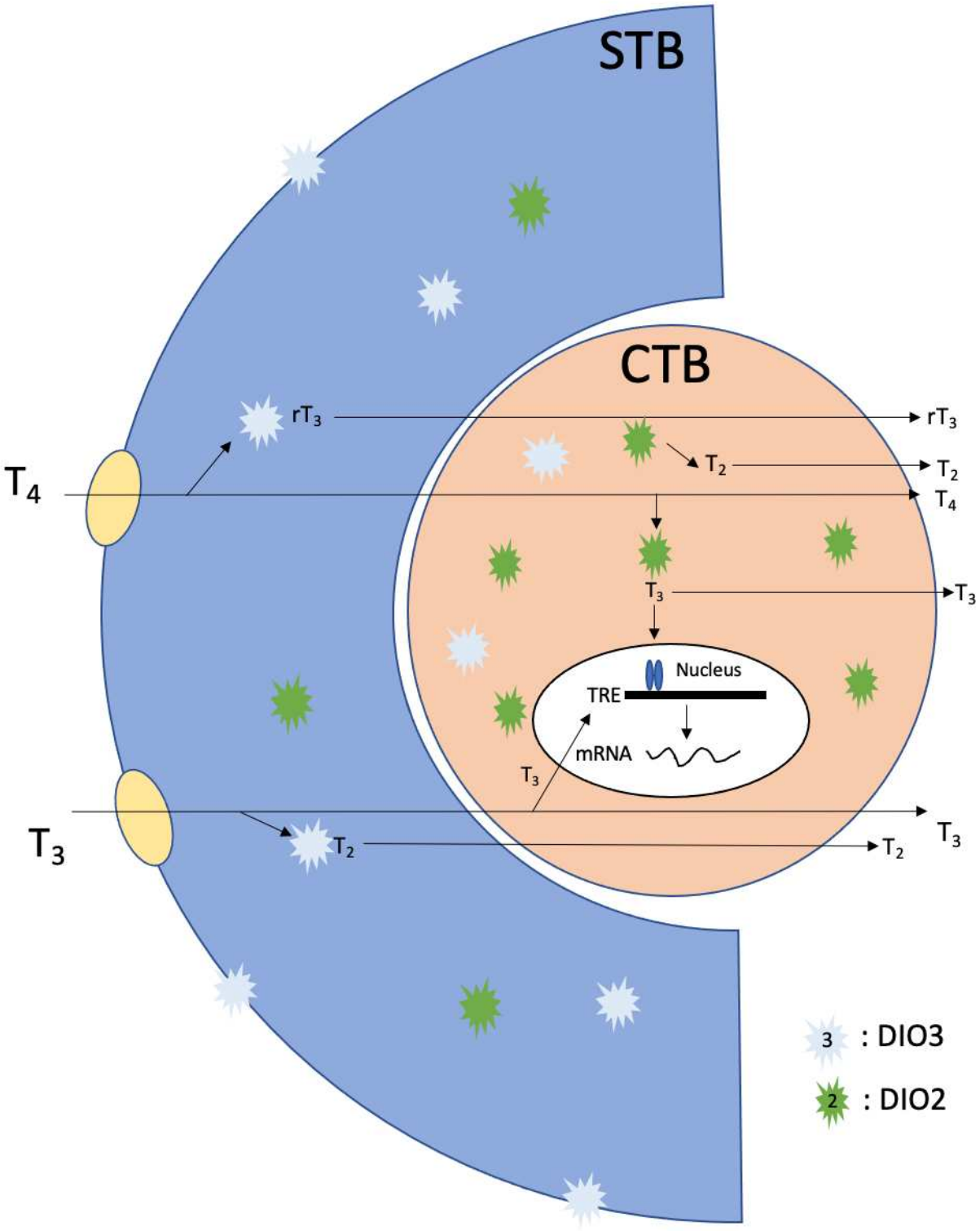


Figure 1: Deiodinase location and activity in human placenta. Figure adapted from Adu-Gyamfi et al. 2020 Biology of Reproduction 102 (1), 8-17

Steinhauser et al. (2021) investigated the difference in gene expression of DIO2 and DIO3 in sheep placentomes in a nutrient restricted model of IUGR pregnancy compared to control pregnancies. They observed that expression of DIO2 in placentomes at 135 dGA did not significantly change between maternal nutrient restricted and control animals, but DIO3 gene expression in placentomes did increase in the IUGR pregnancies near term (Steinhauser et al. 2021). While this is interesting data, it only gives insight into full placentome gene expression, therefore does not allow for any conclusions to be drawn about spatial gene expression between DIO2 and DIO3 in sheep pregnancies. This data also does not account for any physiological changes that occurred due to animal stress such as temporary fasting or anesthesia during the time of tissue collection.

Even though in humans TH first passes through the STB on its way to fetal circulation and is exposed to DIO3, it has been observed that maternal T_4 passes through the placenta and enters fetal circulation without being deactivated (Sampson et al., 2000), suggesting that there may be another mechanism for T_4 transport across the placenta that bypasses DIO3 activity with the help of a thyroid hormone carrier protein.

Thyroid Hormone Carrier Proteins:

In human circulation there are three main thyroid carrier proteins: thyroxine binding globulin (TBG), transthyretin (TTR) and albumin (Pappa et al., 2015). While different carrier proteins have different affinities for T_4 compared to T_3 (Rutchanna et al., 2022), thyroxine binding globulin (TBG) has the highest affinity for T_4. Transthyretin (TTR) has the next highest affinity for T_4 in circulation. Albumin has the lowest binding affinity for T_4 out of the three main carrier proteins (Schreiber, 2002). These carrier proteins bind free thyroid hormones after release

from the thyroid gland and shuttle them throughout circulation. Once TH-carrier protein complexes reach cell surfaces, for the sake of this paper the placental interface, TH is released from the carrier protein and enters the cells through the membrane transporters mentioned earlier (OATPs, LATs, MCTs; Barrett, 2012).

The free hormone hypothesis states that hormones can only carry out their function when they are in a free state and unbound to any other molecules. This hypothesis includes the process of hormone entry into a cell (Mendel, 1989). Because of this, it has been thought that once a thyroid carrier protein drops off TH at the placental interface, its job is complete, and it will find another free thyroid hormone molecule to bind to and carry throughout circulation. Recently however, it has been postulated that although TTR does act like other carrier proteins by dropping TH off at a cell membrane, it may also act as a protective shuttle system. It has been hypothesized that T_4 binding to TTR creates a complex that can travel into and across the placenta where T_4 is secreted into fetal circulation to be used by the developing fetus (Landers et al. 2009). While this transport is necessary, it is important to remember that TH acts in the placenta itself to stimulate cellular processes necessary for placental function as previously mentioned. This TTR-T_4 complex could also be acting as a protective mechanism to ensure that thyroid hormones are active in the placenta itself. This would be a unique function compared to other thyroid carrier proteins such as TBG and albumin

Transthyretin:

Transthyretin (TTR), formerly known as prealbumin, is s 56 kilodalton homotetrameric secreted protein consisting of 4 identical 14 kilodalton subunits which are oriented around a central channel (Gonzalez, 1971; Kanda, 1974). While transthyretin is commonly thought of as

only a thyroid carrier protein, it is also responsible for transporting vitamin A throughout circulation. For TTR to bind vitamin A, it must form a stable complex with retinol binding protein (RBP; Raz, 1969).

Regarding thyroid hormone transport, TTR transports both T_3 and T_4, but it preferentially binds to and transports T_4 (Mondal et al., 2016). TH binding occurs in the channel that sits in-between the four TTR monomers. There are two binding sites for two thyroid hormone molecules to bind, but because the binding affinity of the second site is greatly reduced once the first site is occupied, usually only one TH molecule binds (Refetoff, 2015). Transthyretin is synthesized in the liver, choroid plexus, retinal pigment, pancreas, neurons, placenta, and intestines in humans (Schreiber et al., 1976; Dickson et al., 1985; McKinnon et al., 2005; Cavallaro et al., 1990; Ong et al., 1994; Loughna et al., 1995; Kato et al., 1985). It is responsible for about 10-15% of total thyroxine transport in the body and all of the thyroid hormone transport in the cerebrospinal fluid (Schreiber, 2002; Landers et al., 2013). Clinically, TTR is most widely known for the TTR amyloidosis pathology. This is a condition in which TTR proteins unfold, aggregate, and misfold oligomers and monomers causing the formation of insoluble amyloid fibrils leading to a disturbance in many different systems in the body, most notably in the heart (Kapoor et al., 2019). While there are conflicting results in literature reviews hypothesizing that TTR misfolding may be occurring in the placenta, Fruscalzo et al. was able to show that this was not occurring in vivo (Fruscalzo et al., 2012).

In 2005, Mckinnon et al. obtained human placentas from consenting mothers who underwent an uncomplicated cesarean section at term and discovered that the placenta itself was synthesizing TTR in trophoblast cells. Not only was TTR found to be produced in the placenta, but it also was observed in in vitro systems that TTR is secreted out of JEG-3 cells into both

basal media and apical media when Transwell permeable supports were used. More interestingly, placental cells were able to internalize TTR on the apical side of cells (Landers et al., 2009). While this has been observed in hepatocytes this was the first time it was observed in trophoblast cells (Divino & Schussler, 1990; Landers et al., 2009). It was also noted that when T_4 and T_3 were added to the cell culture environment, the rate of TTR internalization increased (Landers et al., 2009). Later it was confirmed that TTR protein is secreted into both fetal and maternal circulation in human placental perfusion models (Mortimer et al., 2012).

Impairment of Nutrient Transfer in a Ruminant Placenta:

The transfer of nutrients from maternal circulation to fetal circulation throughout gestation ensures that the developing fetus receives the necessary materials from mom for proper growth and development. While the placenta is this exchange site, it is also a functioning organ that produces its own nutrients that are necessary for fetal growth and development (Costa, 2016). Impairments placentation, fetal villi development, placental perfusion, and placental endocrine function can lead to placental insufficiency (PI), which is characterized as a progressive deterioration in placental functioning leading to fetal hypoxemia. This impairment in functioning stimulates a downregulation of fetal metabolic demands to preserve the nutrients that are already present and accessible (Wardinger & Ambati 2022). A typical phenotype of PI IUGR is a decrease in placental size and function which can result in impaired fetal growth leading to intrauterine growth restricted (IUGR) pregnancies (Gagnon, 2003). PI IUGR accounts for about 60% of all fetal growth restricted pregnancies and thus is an important topic of research (Ghidini, 1996).

Intrauterine Growth Restriction:

Intrauterine growth restriction (IUGR), sometimes referred to in the literature as fetal growth restriction (FGR), is a pregnancy disorder that effects about 6% of pregnancies worldwide and is a leading cause of fetal death (Gagnon, 2003). It is defined as the failure of the fetus to reach its growth potential in utero (ACOR, 2019). To diagnose IUGR, serial ultrasounds are performed to measure fetal weight, asymmetric growth, any decreases in amniotic fluid volume and abnormal Doppler waveforms in the umbilical artery, ductus venosus and middle cerebral artery (Crocker, 2011). IUGR has been shown to increase the risk of developing metabolic diseases in adult life such as diabetes, heart disease and stroke (Barker, 1990). The etiology of IUGR is difficult to study because it is not caused by just one perturbation. These pregnancies can be caused by genetic changes, environmental changes, or changes occurring directly in the placenta which lead to placental insufficiency (Crocker, 2011).

Because placental insufficiency accounts for the majority of IUGR cases, this will be the category of IUGR that will be discussed from this point on (Audette & Kingdom 2018). There are two different growth patterns observed in IUGR pregnancies. The first is symmetrical where the fetus does not reach its growth potential, but both the head and trunk of the body grow together symmetrically (Anthony et al., 2003). The other growth pattern is asymmetrical growth which is commonly caused by PI-IUGR. (Anthony et al., 2003). Asymmetrical growth means that fetal head circumference is maintained while other abdominal organs do not reach their growth potential. This is called brain sparing, or the adaptive phenomenon that allows the brain to acquire enough substrates for normal growth and development at the expense of the liver. To achieve this, vasodilation of both the ductus venosus and arteries to the brain occurs to keep nutrient rich and oxygenated blood flowing to the brain (Benítez-Marín et al., 2021). While this

is a great compensatory mechanism for fetal brain development, it does result in a decrease in blood flow to abdominal organs leading to decreased growth and development of those organs, especially the liver (Cahill et al., 2019). Phenotypically these fetuses appear to have much larger heads in relation to the trunk of their body therefore making their growth appear asymmetrical (Anthony et al., 2003).

Animal Models of IUGR:

Due to ethical and practical roadblocks, in depth human IUGR studies are not performed in vivo throughout gestation making it necessary for animal models to be developed and used. The sheep has been determined to be an excellent model to study maternal-fetal interactions due to the ability to catheterize maternal and fetal vessels during gestation (Barry Anthony, 2008). In addition to this, compared to other animal models such as a mouse, the body size of a pregnant ewe is more comparable to that of a pregnant human. Regarding placental structure and function, when comparing human discoid placentas and ruminant cotyledonary placentas, the branching structures at the site of interdigitation are very similar in both species (Meschia et al., 1965). Because of these similarities in villi structures at maternal/fetal nutrient exchange sites, sheep models have been established as an appropriate animal model to study human pregnancy disorders such as IUGR (Barry & Anthony, 2008). Many different strategies have been used to produce IUGR pregnancies of varying severities including nutrient manipulation, injection of glucocorticoids, utero-placental embolization, carunclectomy, and exposure to increased temperatures during pregnancy (Anthony et al., 2003).

To produce nutrient manipulated models of IUGR pregnancies in sheep, ewes can either be underfed or overfed (Anthony et al., 2003). In maternal nutrient restricted models, the severity

of the IUGR phenotype observed depends on when nutrient restriction occurs. When nutrient restriction occurs early to mid-gestation there is no reduction in fetal or placental weights (Steyn et al., 2001). If ewes are nutrient restricted from mid to late gestation, there is a more consistent production of reduced fetal body weights with no reduction in placental weights. Late gestation nutrient restriction tends to produce mixed results as well, but these fetuses do have reduced umbilical glucose uptake (Marconi et al. 1996). Inconsistent production of IUGR pregnancies makes this model relatively unreliable for studying the etiology of placental insufficiency induced IUGR.

IUGR pregnancies can be induced when a pregnant ewe is overfed as well. When pregnant ewes are over nourished, fetal weight and placental weight has been observed to be reduced (Wallace et al., 1999b). It is postulated that this is due to the maintenance of maternal growth becoming priority over nutrient transfer to the fetus (Anthony et al., 2003). While changes caused by over nutrition early in gestation do appear to be reversible (Wallace et al., 1999b), overnutrition continued over gestation can result in fetal hypoxia and hypoglycemia due to a reduction in umbilical vein blood flow (Wallace et al., 2002).

Another strategy for producing IUGR pregnancies is the administration of glucocorticoids to pregnant ewes (Seckl, 2001). In a normal pregnancy, a fetus responds to a lack of nutrients by increasing the levels of circulating cortisol (Barker & Osmond, 1998). It has been observed that when glucocorticoids are administered to pregnant ewes in late gestation, fetal and post-natal growth is reduced (Jobe et al., 1998, Moss et al., 2001; Long et al., 2012). While these reductions in size have been observed, it is speculated that this is due to a direct effect on the fetus, therefore making it an inadequate model for studying placental insufficiency driven IUGR (Anthony et al., 2003).

Utero-placental embolization is an IUGR producing strategy that purposefully slows down blood flow from maternal circulation to fetal circulation either through infusion of microspheres, vessel occlusion or ligation of a single uterine artery in late gestation (Anthony et al., 2003). These strategies decrease uterine artery and umbilical artery blood flow, thus decreasing nutrient and oxygen transfer across the placenta (Lang et al., 2000). It has been observed that by using this model, fetal wight and placental weights can be reduced, and fetal hypoxemia and hypoglycemia occur (Clapp et al., 1981; Louey et al., 2000). While this model mimics important changes that occur in utero-placental blood flow due to IUGR, the reason for these changes is accounted for and therefore is not an ideal model to study underlying factors that are causing placental insufficiency in the first place. (Anthony et al., 2003).

Carunclectomies can be performed to make IUGR pregnancies as well. This procedure is the surgical removal of uterine caruncles before pregnancy (Alexander, 1964). As previously described in this review, the caruncles are the highly vascularized sites in the endometrium where nutrient exchange will occur from maternal circulation to fetal circulation (Stegman, 1974). When the number of these sites are reduced, cotyledon numbers are reduced and the surface area for placental exchange is also reduced. While this seems like a guaranteed way to induce IUGR, not all pregnancies produced with this strategy result in IUGR. When IUGR phenotypes are observed however, it appears to show a similar phenotype to those seen in human IUGR such as reduced placental weights, reduced fetal weights, fetal hypoxemia, fetal hypoglycemia, fetal hypoinsulinemia, reduced uterine and umbilical blood flows and reduced utero-placental oxygen consumption (Darby et al., 2020; Anthony et al., 2003).

A model that is commonly used to make IUGR pregnancies in sheep is the maternal hyperthermia model. The production of IUGR pregnancies is accomplished by exposing sheep to

high temperatures throughout gestation leading to heat stress in pregnant ewes (Alexander and Williams, 1971). This model shows some of the largest decreases in fetal weight and placenta weight compared to other models (Anthony et al., 2003). Along with reduced weights, fetal growth is asymmetric like most IUGR cases caused by placental insufficiency. In this model fetuses are hypoxic and hypoglycemic. Uterine and umbilical blood flows are also reduced. Interestingly, the hyperthermic model has been shown to alter angiogenic growth factors and their receptor leading to changes in placental vasculature which mimics changes seen in placental insufficiency IUGR pregnancies (Regnault et al., 2002a,b).

While the studies mentioned above have provided important insights into IUGR pregnancies, there was not a sheep model that explored a gene specific role in placental insufficiency leading to IUGR. Because of this, a new technique for creating IUGR pregnancies was established using lentiviral mediated RNA interference (RNAi) to knock down chorionic somatomammotropin (CSH) in the trophectoderm of day 9 blastocysts (Baker et al., 2016). When this strategy was implemented, it led to reductions in umbilical blood flow, uterine blood flow, uterine weight, fetal IGF-1 production, fetal insulin production, and fetal weight near term. These pregnancies impacted by IUGR also experienced global reductions in the uptakes of oxygen, glucose, and amino acids (Tanner et al., 2021). These results mimic impaired transfer of materials from maternal systems to fetal systems that would be observed in IUGR pregnancies caused by placental insufficiency thus making it a great model to study PI IUGR.

In addition to using RNAi to reduce the amount of CSH in the placenta, Tanner et al. (2021) used chronic catheterization to collect blood samples in real time, thus the samples were collected in an environment that mimics steady state physiology (Tanner el al., 2021). Catheters can be placed in the following vessels: the fetal descending aorta to represent umbilical artery

blood, umbilical vein, maternal femoral artery to represent uterine artery blood, and uterine vein (Figure 2).

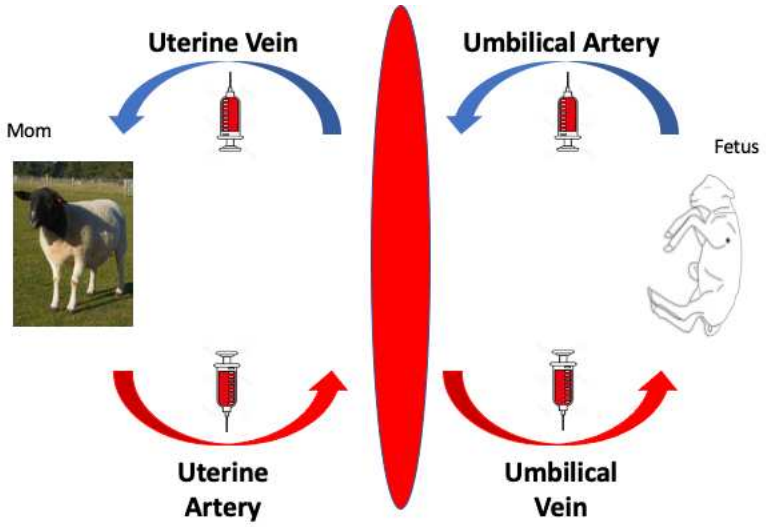


Figure 2: Schematic representation of the maternal and fetal circulation, identifying catheter placement for sampling maternal and fetal blood under steady-state non-anesthetized/non-stressed conditions at130 dGA.

When catheters are surgically placed in these vessels, blood can be collected under non-stressed steady states in vivo to observe the physiology of IUGR pregnancies without having to account for physiological changes due to increased animal stress including fasting prior to surgery and euthanasia drug administration (Barry & Anthony, 2008; Tanner et al., 2021).

RNA Interference (RNAi):

RNAi is a helpful technique that allows one to determine the function of a specific gene or protein in a system by knocking protein of that gene of interest. The observed difference between the control population and the knocked down population allows one to start to elucidate the function that the gene of interest has on overall physiological function under normal circumstances. This strategy can also be utilized to create a desired phenotype, for example

knocking down CSH in the placenta to produce an IUGR pregnancy (Baker et al., 2016; Tanner et al., 2021).

Lentiviral mediated RNAi mimics the natural phenomenon of short interfering RNA (siRNA) gene regulation by using an engineered short hairpin RNA (shRNA) that acts like endogenous micro RNA would and targets specific endogenous mRNA for either degradation or translational repression (Igarashi, 2012). Lentiviral vectors are constructed by inserting a gene of interest targeting sequence into a plasmid. Because the lentivirus cannot replicate in the host cell, these viral particles infect only the cells that they come into contact with. Once the lentivirus integrates its viral genome into the host cells genome, normal transcription and translation occurs leading to the production of short-hairpin RNA (shRNA) in the nucleus. Endogenous dicers target this shRNA and process it into siRNA. The siRNA binds to endogenous RNA Induced Silencing Complexes (RISC), which bind to and degrade the endogenous mRNA of the gene if interest. Moving forward, the host cells are no longer able to translate the mRNA into protein resulting in a functional knockdown of the protein of interest (Anthony et al., 2010; Purcell et al., 2009; Tanner et al., 2022).

History of Trophectoderm Specific RNAi:

While RNAi has been used in numerus studies, it wasn't until 2007 that RNAi was used to perform trophectoderm specific gene knockdowns (Georgiadas et al. 2007). This study showed that when engineered lentiviral particles were introduced to hatched mouse blastocysts, they were able to create a functional knock down of their gene of interest only in the trophectoderm layer of the developing embryo while the inner cell mass remained unaffected.

In 2009, Purcell et al. published the first study where lentiviral mediated RNAi was used to perform a trophectoderm specific knockdown in sheep. The goal of this study was to determine proline rich 15 (PRR15) mRNA expression timing and localization during conceptus development and observe the effects that this functional knockdown has on conceptus development. This paper established a general procedure for the development of lentiviral mediated RNAi pregnancies in sheep. To develop the pregnancies used in this study, sheep were naturally mated and on day 8 of gestation total hysterectomies were performed, uterine horns were flushed, and blastocysts were collected. Blastocysts were cultured in lentivirus containing oil drops for 6 hours where they were then transferred back into a recipient ewe. They observed that by using this technique they were able to eliminate PRR15 gene expression in the conceptus at day 15 (Purcell et al., 2009). With some modifications, this process has been used to target chorionic somatomammotropin (CSH), Lin28A/B, and SLC2A3 in the sheep placenta. (Baker et al., 2016; Tanner et al., 2021; Ali et al., 2020; Lynch et al., 2022).

Chorionic Somatomammotropin:

Chorionic somatomammotropin (CSH), also referred to as placental lactogen (PL), is a placental derived peptide hormone produced and secreted from the binucleate cells of the fetal sheep trophectoderm epithelium and the syncitiotrophoblast cells of the human trophectoderm epithelium (Ali et al., 2020, Gootwine, 2004). This hormone is secreted into both fetal and maternal circulation during gestation, leading to placental driven changes to both maternal and fetal systems (Tanner et al., 2021). A study from the late 70s showed that low levels of circulating CSH during pregnancy were associated with fetuses that exhibited decreased fetal weight, decreased placental weight, and an increased rate of fetal death (Spellacy et al., 1975).

While there is still work that needs to be done to deduce CSH's function during gestation, previous studies have shown that when CSH is knocked down in the ovine placenta, both IUGR pregnancies and non IUGR pregnancies are produced (Baker et al., 2016; Tanner et. al., 2021). Tanner et al. published work in 2021 that explored the physiological ramifications of an RNAi induced CSH knockdown and observed that when this procedure is performed, normal gestation is disturbed leading to reduced fetal and uterine weight at term. Umbilical and uterine blood flows (mL/min) were reduced, leading to reduced umbilical nutrient uptake, reduced uterine nutrient uptakes, and reduced uteroplacental glucose utilization (Tanner et al., 2021). It has been shown that when CSH was knocked down and IUGR pregnancies were produced, an 80% reduction in fetal glycogen storage occurred (Ali et al., 2020). Fetal insulin production also is impacted by reduced levels of placental CSH. Pancreatic islet cells in vitro showed a decrease in insulin production (Martin & Friesen1969), Tanner et al. also observed that when CSH in the placenta is reduced in the ovine placenta in vivo, there tended to be a reduction in fetal insulin production (Tanner et al. 2021). CSH may also influence nutrient transfer to the fetus and alter placental utilization of oxidative substrates leading to a decrease in nutrient availability for fetal uptake (Tanner et al. 2022). Further studies will be necessary to deduce the systemic effect of a placental CSH knockdown during and after pregnancy.

Livestock Industry Impact:

In addition to the clinical relevance these studies have for human research, ruminant studies also benefit the livestock industry. This industry has been greatly impacted by low levels of reproductive efficiency. Because successful reproduction leads to profitability in this industry, decreased fertility means decreased profitability. Dairy cow reproduction in particular has been

impacted due to trait selection for increased milk production being linked to a decline in fertility (Walsh et al., 2011). It is estimated that infertility costs the dairy industry up to $484 million annually (Bellows et al., 2002). Currently, pregnancy success rates are low. In 2019 a survey of sixteen different dairy herds deemed to show excellent reproductive performance was performed where it was found that there was pregnancy success rate of only 32% (Willmore & Davis, 2019). Sheep are also experiencing issues in fertility with a reported embryonic loss rate estimated to be between 12-26% (Diskin & Morris, 2008).

Because of these dismal statistics, it is imperative reproduction in ruminants such as sheep and cattle be investigated further in hopes of increasing fertility and rates of neonatal survival.

Research Objective:

In recent literature, there is a lack of research relating to the details of thyroid hormone transport in ovine pregnancies. There is very little data available about the connection between dysregulated thyroid hormone levels and intrauterine growth restricted pregnancies as well. In 2021, Steinhauser et al. published a study which caught our attention due to their use of an IUGR sheep model to study physiological changes that occur at the level of the placenta, a topic that we are interested in. They created a nutrient restricted model of IUGR and investigated the effect that this phenotype had on both maternal and fetal plasma thyroid hormone levels, while also looking deeper into the expression of genes involved in thyroid hormone transport such as membrane transporters and deiodinase enzymes.

While this paper was one of the first to study thyroid hormone regulation in the sheep throughout gestation, there were certain issues in the methodology that do not allow for an

accurate representation of nutrient transfer and placental physiology. The blood samples that were collected in this study, may not illustrate what is occurring around the placenta because they do not specify where they took blood from on the maternal side, we must assume they took a jugular sample as it is common practice when collecting blood from a sheep. If this is the case, data reported from this study could be an inaccurate depiction of what is occurring at the placental interface. The proximity of the blood sample to the thyroid gland may lead to results which are higher than what they would be by the time they travel through circulation and arrive at the placenta. Fetal blood samples were collected from the heart during necropsy, which also does not give an accurate representation of thyroid hormone levels transferred across the placenta to fetal circulation. This paper also does not explore potential changes thyroid hormone carrier protein levels which we think is necessary to understand the complete process of thyroid hormone transport.

Our lab is interested in investigating the impact of CSH RNAi IUGR on nutrient exchange. In this CSH RNAi model of IUGR pregnancy, it has been observed that a reduction in CSH negatively impacts the transport of many substances from maternal circulation to fetal circulation including glucose, oxygen, and amino acids (Tanner et al., 2021). Therefore, we deemed it important to conduct similar studies to Steinhauser's and investigate the impact that this reduction in CSH in the placenta has on thyroid hormone transport across the placenta near term. Because there is limited information on this topic, our research was more exploratory in nature.

Lentiviral mediated RNA interference was used disrupt translation of CSH leading to a functional knock down in the trophectoderm of day 9 blastocysts. This strategy allowed for a placental specific knockdown of this placental peptide hormone while leaving the inner cell mass

to develop unaffected. The infected blastocysts were transferred into recipient ewes where the pregnancies were carried out until 126 dGA. At that time point, pregnant ewes were transported at 115 dGA to University of Colorado Anschutz Medical Center's Perinatal Research Center where catheters were placed into both maternal and fetal circulation. These catheters as previously mentioned allow for a better understanding of nutrient transport at the level of the placenta itself in real time under steady state conditions. At day 130, blood samples were taken from uterine vein, uterine artery, umbilical vein, and umbilical artery catheters and processed into serums. Necropsy was also performed, and full placentomes, maternal livers and fetal livers were collected from all pregnancies. Placentomes were separated into maternal caruncles and fetal cotyledons. All collected samples were used for the experiments described below.

Competitive enzyme-linked immunosorbent assays (ELISA) were used to determine the levels of total thyroxine T_4 in all four sample locations (uterine artery, uterine vein, umbilical artery, and umbilical vein) in both control and CSH knockdown IUGR pregnancies. Unfortunately, we did not have enough serum samples to run a total T_3 ELISA.

To determine the effect of a CSH knockdown IUGR pregnancy on the thyroid carrier protein transthyretin, polyclonal antibodies were used to determine the relative abundance of TTR protein in uterine artery, uterine vein, umbilical artery, umbilical vein, maternal liver and fetal liver samples for both knock down and pregnancies. Those abundances were used for uptake calculations.

Deiodinase 2 and Deiodinase 3 gene expression was also investigated in both caruncles and cotyledons in control vs IUGR pregnancies. We wanted to determine if the CSH knockdown model of IUGR pregnancies caused any changes to the finetuned thyroid hormone activation/inactivation regulatory system which could explain the differences in thyroid hormone

levels we observed. To do this cotyledon and caruncle RNA was isolated and quantitative real-time PCR (qRT-PCR) was performed.

These experiments allowed us to determine that CSH RNAi pregnancies produced through lentiviral mediated CSH RNAi in a developing ovine placenta does in fact do show impaired thyroid hormone transport and regulation in late gestation. Further studies need to be conducted to further deduce the mechanistic impacts of CSH RNAi on thyroid hormone transport.

Chapter II: The Effect of Placental CSH RNAi on Thyroid Hormone Transport and Regulation In Vivo

Introduction:

Maternally supplied thyroid hormones are necessary for normal fetal growth and development. Thyroid hormones are also important regulators of placental specific growth and function during pregnancy and are therefore necessary for the development of both the placenta and the fetus (Adu-Gyamfi et al., 2020; Korevaar et al., 2017; Thorpe-Beeston et al., 1991; Klein et al., 1997; Kilby et al., 1998). When the placenta does not function to its full capacity, it cannot transfer adequate nutrients to the developing fetus. This phenomenon is termed placental insufficiency, which may lead to intrauterine growth restricted (IUGR) pregnancies (Anthony, 2003). IUGR is the second leading cause of perinatal morbidity and affects about 6% of pregnancies worldwide (Gagnon et al., 2003). In a previous study, IUGR pregnancies had impaired glucose, oxygen, and amino acid transport to the fetus throughout gestation, and increased risk for adult-onset metabolic diseases when compared to control pregnancies (Tanner et al., 2021; Barker, 1990). Maternal nutrient restricted models of IUGR have also been associated with impaired thyroid hormone transport in ruminant pregnancies during mid and late gestation. It was previously hypothesized that this perturbation is due to the disruption in expression of various genes and proteins associated with thyroid hormone transport therefore leading to reductions in thyroid hormones in maternal and fetal circulation (Steinhauser et al., 2021).

The transport of thyroid hormones from maternal circulation to fetal circulation requires the cooperation of multiple biological processes. Thyroid hormones are produced in the maternal thyroid gland, where they are secreted into maternal circulation and transported throughout

maternal circulation via thyroid hormone carriers (Barrett, 2012). At the placental interface, thyroid hormones pass through cell membranes via membrane transporters to enter the placental unit (Mendoza and Hollenberg 2017; Abe et al., 2012; Bernal et al., 2015). Once thyroid hormones pass into the placenta, they encounter deiodinase enzymes. These enzymes either activate (Deiodinase 2; DIO2) or inactivate (Deiodinase 3; DIO3) thyroid hormones to ensure that a highly regulated amount of thyroid hormone is passed along to the fetus (Chan et al., 2003). Inside the placenta, thyroid hormones can either be transported across the placenta to fetal circulation or be used in the placenta itself to aid in gene transcription and translation (Adu-Gyamfi et al., 2020). Dysregulation in any one of the mechanisms can lead to insufficient thyroid hormone transport to fetus or the placenta during gestation. While this dysregulation may be particularly detrimental before mid-gestation when the fetal thyroid gland develops and starts to produce its own thyroid hormone, it may also impact nutrient transport during late gestation (Barrett, 2012).

Transthyretin (TTR) is a 56 kilodalton homotetrameric secreted protein that is one of three main thyroid carrier proteins in circulation that aids in bringing thyroid hormone to the placental interface (Pappa et al., 2015). TTR is mainly synthesized in the liver but is also produced in other parts of the body including the placenta (McKinnon et al., 2005; Gonzalez et al., 1971; Kanda, 1974) and is known to function as a carrier protein for TH and retinol throughout circulation (Raz, 1969). While TTR binds to both T_3 and T_4, it preferentially binds to T_4 (Mondal et al., 2016). In contrast to other thyroid carrier proteins, it has been observed that TTR may do more than just drop TH off at the placental interface (Landers et al., 2009). TTR can be internalized back into trophoblast cells via endocytosis. The rate of internalization increases when T_4 is present and bound to TTR, leading to the hypothesis that TTR-T_4

complexes are endocytosed together into the placenta potentially to create a protective shuttle mechanism for T_4 (Mortimer et al., 2012). If thyroid hormones avoid inactivation, increased concentrations of active thyroid hormones could be reaching fetal circulation.

Deiodinase enzymes regulate the activation and inactivation of thyroid hormones throughout the body (Gereben et al., 2008). While there are three different deiodinase enzymes, only DIO2 and DIO3 are present in the placenta. In humans, these enzymes are present in differing concentrations in various cell types of the placenta. DIO3 enzymes are more abundant in the syncitiotrophoblast layer (STB) and are responsible for the inactivation of T_4 to rT_3. DIO2 enzymes are more abundant in the cytotrophoblast (CTB) layer of the placenta and are responsible mostly for activating T_4 to a more biologically active T_3 (Gutiérrez-Vega et al., 2020). Recently, deiodinase mRNA expression in ovine placentome tissues was investigated in maternal nutrient restriction models of IUGR pregnancies (Steinhauser et al., 2021). DIO3 gene expression in full placentomes was increased in nutrient restricted IUGR fetuses compared to control pregnancies at term (Steinhauser et al., 2021). DIO2 and DIO3 regulation ensures that an appropriate amount of biologically active thyroid hormones from maternal circulation will enter fetal circulation. As mentioned previously, it has recently been postulated that TTR may be acting as a protective mechanism, but we believe this mechanism evolved to protect TH from DIO3 regulation in the placenta specifically.

Due to our labs interest in the physiological changes that occur to nutrient exchange systems due to placental insufficiency driven IUGR, and previous reports of perturbations in thyroid hormone transport in maternal nutrient restricted models of IUGR, we were interested in investigating thyroid hormone transport in CSH RNAi models of IUGR pregnancy. It was our hypothesis that a reduction in placental CSH negatively impacts thyroid hormone transport from

maternal circulation to fetal circulation due to perturbations in both thyroid hormone carrier protein activity and placental deiodinase enzyme expression. To investigate this, we measured thyroid hormone concentration, transthyretin protein concentration and uptakes, and deiodinase 2 and 3 enzyme gene expression in various samples from CSH RNAi induced IUGR pregnancies with the objective of better understanding thyroid hormone transport during late gestation specifically in CSH RNAi models of IUGR pregnancies.

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) as described in Tanner et al. 2021.

Lentiviral Vector Construction:

Lentiviral infection was used to functionally knock down Chorionic Somatomammotropin (CSH) the trophectoderm of day 9 blastocysts. Virus generation and titration were performed as previously reported in Baker et al. 2016. Briefly, non-targeting sequence (NTS) and CSH targeting short hairpin RNA sequences used in this procedure are provided in Table 1.

Table 1. NTS and CSH-targeting short-hairpin RNA (shRNA) sequences (Baker et al., 2016).

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

Blastocyst Collection and Transfer:

Dorper breed composition ewes were used in all experiments. All ewes were provided access to hay, trace mineral and water in alignment with the National Research Council requirements. They were housed in pens at Colorado State University Animal Reproduction and Biotechnology Laboratory. The protocol for animal care, estrus synchronization, breeding, embryo collection, and embryo transfer were described in Baker et al. 2016. In short, dorper ewes were introduced to vasectomized rams, to determine standing estrus in all ewes. Blastocyst donor and recipient ewes were given two intramuscular injections of PGF-2α four hours apart. (10 mg/dose, Lutalyse; Pfizer, New York, NY). 48 hours after synchronization, donor ewes were allowed to breed with intact rams. Nine days after breeding, complete hysterectomies were performed on donor ewes, and late-stage blastocysts were flushed from uterine horns using DMEM-F-12 (1:1) medium supplemented with 0.25% BSA. Flushed blastocysts were washed in HEPES- buffered chemically defined medium (HCDM-2) for late-stage embryos. Blastocysts were either infected with CSH or NTS control lentivirus. Fully expanded and hatched blastocysts were incubated in 100 ul drops containing 100,000 transducing units of concentrated lentivirus, 50 ng of Polybrene (5ng/ul; Sigma-Aldrich, St. Louis, MO) and CDM-2, overlaid with mineral oil. Blastocysts were cultured in 5% CO_2- 5% O_2- 90% N_2 at 37° C for 5 hours before being surgically transferred into recipient ewes. Blastocyst were washed in HEPES-buffered chemically defined medium (HCDM-2) immediately before transfer. A single infected blastocyst was transferred into the uterine horn ipsilateral to the corpus luteum of recipient ewes that had been synchronized with donor ewes. These recipient ewes showed standing estrous within 24 hours of the donor ewes. Recipient ewes were fasted for 16 hours prior to transfer and sedated as previously described in Baker et al. 2016. After surgical transfer of the blastocyst, recipient ewes

were given an ab libitum diet of alfalfa hay and water and monitored for 72 hours. Each recipient ewe was monitored for return to standing estrus and pregnancy was confirmed at 50 dGA by ultrasonography.

Tissue and Blood Collection:

Experiments were performed on tissues and blood samples collected from two different cohorts of animals. The difference between the two cohorts was the method of blood collection, which will be described further below. The cohort of samples collected from Baker et al. 2016 pregnancies and fetal data were previously described in Baker et al. 2016 Am J Physiol Regul Interg Comp Physiol 310: R837-R846. The cohort of samples collected from Tanner et al. 2021pregnancies and fetal data were previously described in Tanner et al. 2021 Int J Mol Sci. 22, 8150.

Baker et al Samples:

At 135 dGA, terminal surgeries were performed to collect uterine artery, uterine vein, umbilical artery and umbilical vein blood and tissue samples. This procedure was described in detail in Baker et al. 2016. In summary, pregnant ewes were food and water restricted 16 hours prior to surgery. Ewes were anesthetized, and blood samples were collected. Blood samples were left to coagulate and spun down to collect serum only. Serum samples were frozen and stored in −80 ◦C for future analysis.

Once blood sample collection was complete, both the ewe and fetus were euthanized, and necropsy was performed. Fetal characteristics were recorded. Placentomes, fetal livers, and maternal livers were collected, weighed and snap frozen. Tissue samples were snap frozen in liquid N_2 and stored in −80 ◦C.

Tanner et al Samples:

At approximately 115 dGA, pregnant recipient ewes were transported to the University of Colorado Anschutz Medical Campus, Perinatal Research Center (Aurora, CO, USA). At 126 dGA pregnant recipient ewes underwent surgical placement of fetal and maternal catheters to determine blood flow and nutrient flux as previously described in Tanner et al 2021. Ewes had access to ad libitum alfalfa pellets. Catheters were placed in the fetal descending aorta (to represent umbilical artery blood), umbilical vein, maternal femoral artery (to represent uterine artery blood), and uterine vein.

At day 130 dGA samples were collected from the uterine artery, uterine vein, umbilical vein, and umbilical artery of each pregnant ewe. Uterine and umbilical blood flow were determined using the steady state $^{3}H_2O$ transplacental diffusion technique as previously described in Tanner (2021). Briefly, samples were collected from uterine artery, uterine vein, umbilical artery, and umbilical vein catheters simultaneously. After the initial draw, a 3 mL bolus of $^{3}H_2O$ was infused into the fetal umbilical artery until isotopic steady state was reached through continuous infusion for 90 minutes. Samples were taken from all catheters simultaneously for analysis and quantification of $^{3}H_2O$ content and used to determine umbilical and uterine blood flow (Table 2).

Table 2. Calculations for umbilical blood flow and uterine blood flow (Tanner et al., 2021)

Measurement	Calculation
Umbilical Blood Flow (mL/min)	$(R_{inf} - R_{acc(f)})/ ([^{3}H_2O]_{\alpha(WB)} - [^{3}H_2O]_{\gamma(WB)})$
Uterine Blood Flow (mL/min)	$(R_{inf} - R_{acc(m)})/ ([^{3}H_2O]_{V(WB)} - [^{3}H_2O]_{A(WB)})$

R_{inf}: $^{3}H_2O$ Infusion Rate Racc: Transplacental Diffusion Constant (Meschia et al., 1965)

Collected blood from baseline samples were allowed to clot at room temperature and spun down. Supernatant was collected and snap frozen. Serum samples were stored in −80 ◦C for future analysis.

After sample collection, ewes and fetuses were euthanized and tissues were harvested as described in Tanner er al. 2021. To summarize, placentomes were dissected from each placenta and separated into cotyledonary and caruncle components then snap frozen in liquid N_2 and sored at -80 C. Fetal weights and organ weights were recorded and tissue samples were snap frozen in liquid N_2 and stored in −80 ◦C.

Maternal and Fetal Thyroid Hormone Concentration Analysis:

Total T_4 in serum was measured using a human T_4 ELISA (Cat # IB 19108, IBL America, Minneapolis, MN, USA) according to the manufacturer's instructions. Total T_3 in serum was measured using a human T_3 ELISA (Cat#IB19107; IBL America) according to manufacturer's instructions. Both assays were validated for linearity using sheep plasma prior to use.

Western Immunoblotting Analysis:

For this procedure serum samples (uterine artery, uterine vein, umbilical artery, and umbilical vein) from both cohorts and maternal and fetal liver samples from both cohorts were used to assess relative transthyretin protein levels using western immunoblot analysis in CSH RNAi IUGR pregnancies compared to NTS RNAi control pregnancies. For analysis, protein was electrophoresed through a 4-15% Tris-glycine Stain-free gels (Bio-Rad Laboratories Inc, Hercules, CA) and transferred via a Trans-Blot- Turbo semi-dry transfer system (Bio-Rad Laboratories Inc.) to a .2- uM pore nitrocellulose membrane. For normalization, total protein per lane was visualized after transfer using the ChemiDoc XRS+ chemiluminescence system (Bio-Rad Laboratories Inc.) To detect ovine transthyretin, a polyclonal antibody generated in a 1:1000

dilution (in 5% Non-Fat Dry Milk / 1X Tris-Bis Solution+ 1% Tween) of rabbit-Transthyretin (product no. PA5-27220 Thermofisher Scientific Waltham, MA) for 20 hours at 4◦C. After the membrane was washed 3 times for 15 minutes each, it was transferred to a 1:20,000 dilution (in 5% Non-Fat Dry Milk / 1X Tris-Bis Solution+ 1% Tween) of anti-rabbit IgG conjugated to horseradish peroxidase-conjugated secondary antibody (product no. ab97051 Abcam, Cambridge, UK). Membranes were developed using a SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermofisher Scientific) and imaged using the Chemi DOC XRS+ chemiluminescence system (BioRad Laboratories Inc.). To correct for technical error between western blot immunoblots, a common sample was included in each western immunoblot. Densitometry measurements were then taken using Image Lab Software (BioRad Laboratories Inc.)

TTR Uptake Calculations:

Uterine uptakes, umbilical uptakes and uteroplacental utilization were calculated in samples from Tanner et al. tissues using the calculations provided in Table 3. The normalized volume of TTR calculated by western blot analysis was used for these calculations. Because these values were relative to total protein in serum samples, the uptake calculations show the change in uptake between CSH RNAi and NTS RNAi pregnancies rather than absolute values.

TTR uterine uptake is the measurement of TTR that was taken up by the placenta from maternal circulation. Uterine uptake was calculated by subtracting uterine vein TTR from uterine artery TTR and multiplying that value by uterine blood flow value previously calculated. Umbilical uptake is the measurement of TTR that is taken into fetal circulation from the placenta. To calculate this, umbilical artery TTR is subtracted from umbilical vein TTR, and that

value is multiplied by umbilical blood flow. Uteroplacental utilization, or the amount of TTR that was metabolized in the placenta itself was calculated by subtracting umbilical uptakes from uterine uptakes.

Table 3. Calculations for uterine uptake, umbilical uptake, and nutrient utilization (Tanner et al., 2021)

Measurement	Calculation
Uterine Transthyretin Uptake (UtTTRU; mmol/min)	$UBF \times ([TTR]_{A}(WB) - [TTR]_{V}(WB))$
Umbilical Transthyretin Uptake (UTTRU; mmol/min)	$UBF \times ([TTR]_{\gamma}(WB) - [TTR]_{\alpha}(WB))$
Uteroplacental Transthyretin Utilization (mmol/min)	UtTTRU – UTTRU

RNA Isolation:

Total RNA was extracted from frozen cotyledons and caruncles using the RNeasy Mini Kit (Ref# 74104 Qiagen, Hilden, Germany) according to manufacturer's specifications. RNA concentration was quantified using BioTek Synergy 2 Microplate Reader and the Gen5 version 3.09 Microplate Reader and Imager software (BioTek, Winooski, VT) For quality assurance, the 260 to 280 nm absorbance ratio was measured with a 2.0-2.3 requirement for all samples. Isolated RNA samples were stored at -80°C.

cDNA synthesis:

cDNA was synthesized from 2 μg of total cellular RNA using iScript Reverse Transcriptase Supermix for qRT-PCR (Bio-Rad Laboratories Inc.) according to the manufacturer's protocol. cDNA was used immediately after synthesis for qRT-PCR procedure.

qRT-PCR:

Quantitative real-time PCR (qRT-PCR) was performed using the CFX 384 Touch Real-Time PCR detection System (BioRad Laboratories Inc.) All primer sets for qRT-PCR were designed using the Custom Oligo software (Thermofisher Scientific) to amplify an intron spanning product. Primer sequences and PCR conditions are summarized in Table 4. To generate a standard curve, a PCR product for each gene of interest and housekeeping genes were generated using cDNA from 130 dGA fetal cotyledons as a template and cloned into the StrataClone vector (Agilent Technologies, Santa Clara, CA). Each PCR product was sequenced to verify the incorporation and amplification of the correct cDNA. Standard curves were generated using the PCR products that were amplified from each mRNA from $1x10^{2}$ to $1x\ 10^{-5}$ and used to measure amplification efficiency. The starting quantity was normalized by dividing the starting quantity of mRNA of interest by the starting mRNA quantity of ovine Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) for caruncular mRNA and ribosomal protein S15 (RPS15) for cotyledonary mRNA.

Table 4. Primers and product sizes for cDNA used in qRT-PCR

cDNA	Forward Primer (5'-3')	Reverse Primer (5'-3')	Annealing Temp °C	BP length
DIO2	GACCTCAGAAGGAATGCGCT	CGCTGTTTTCTCCTGGGCAT	62	157
DIO3	TAGACTTCCTGTGCATCCGC	GTTGTCATCGGACACGCAGA	62	134
oGAPDH	GGGCAGCCCAGAACATCAT	CCAGTGAGCTTCCCGTTCAG	63	88
oRPS15	ATCATTCTGCCCGAGATGGTG	TGCTTTACGGGCTTGTAGGTG	64	134

DIO2, Deiodinase 2; DIO3, Deiodinase 3; oGAPDH, ovine Glyceraldehyde-3-Phosphate Dehydrogenase; oRPS15, ribosomal protein S15

Statistical Analysis:

Data were analyzed by Student's t-test in GraphPad Prism (8.3.1)/ Excel. 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). Each pregnancy/fetus was its own independent unit. The data figures are presented as bar graphs, with the capped vertical lines representing the SEM. There were no treatment by fetal sex interactions, therefore the data are presented as the main effect of treatment only.

Results:

Baker (et al., 2016) Tissue Results:

Tissue and serum from the following pregnancies were previously described in Baker et al. 2016

Total Triiodothyronine and Thyroxine ELISA:

ELISA assays were performed using serum from uterine artery, uterine vein, umbilical artery, and umbilical vein samples at 135 dGA to determine if there is a disruption in amount of total triiodothyronine (T_3) or total thyroxine (T_4) in CSH RNAi pregnancies compared to NTS RNAi pregnancies near the placental unit. Uterine artery serum T_3 concentrations tended to be increased by 36.7% ($P \leq 0.1$; Figure 3a) in CSH RNAi pregnancies compared to NTS RNAi pregnancies. There were no differences in T_3 concentrations between CSH RNAi pregnancies and NTS RNAi control pregnancies in uterine vein, umbilical artery, or umbilical vein serum.

There was a 29% reduction ($P \leq 0.05$; Figure 4b) in total T_4 in uterine vein serum from CSH RNAi samples compared to NTS RNAi samples. There was no change in T_4 levels between CSH RNAi pregnancies and NTS RNAi pregnancies in uterine artery, umbilical artery, or umbilical vein serum.

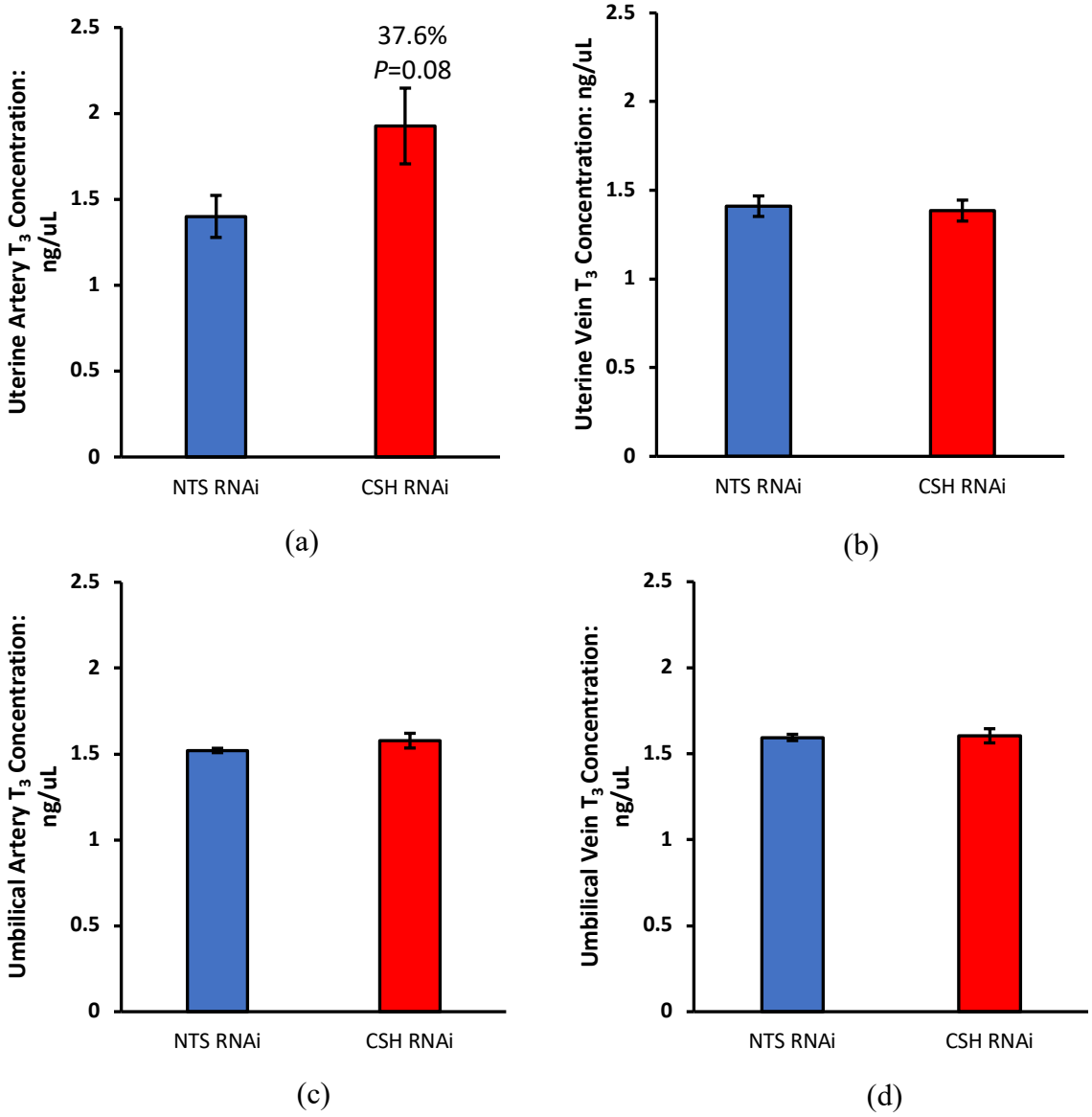


Figure 3 Total Triiodothyronine ELISA (**a**) Uterine artery, ng/uL (**b**) uterine vein, ng/uL (**c**) umbilical vein ng/uL and (**d**) umbilical artery, ng/uL total T_3 as assessed by ELISA at130 dGA: Data are shown as means ± SEM for all pregnancies in each treatment group. NTS, non-targeting sequence, n=8; CSH, chorionic somatomammotropin, n=8; RNAi, RNA interference

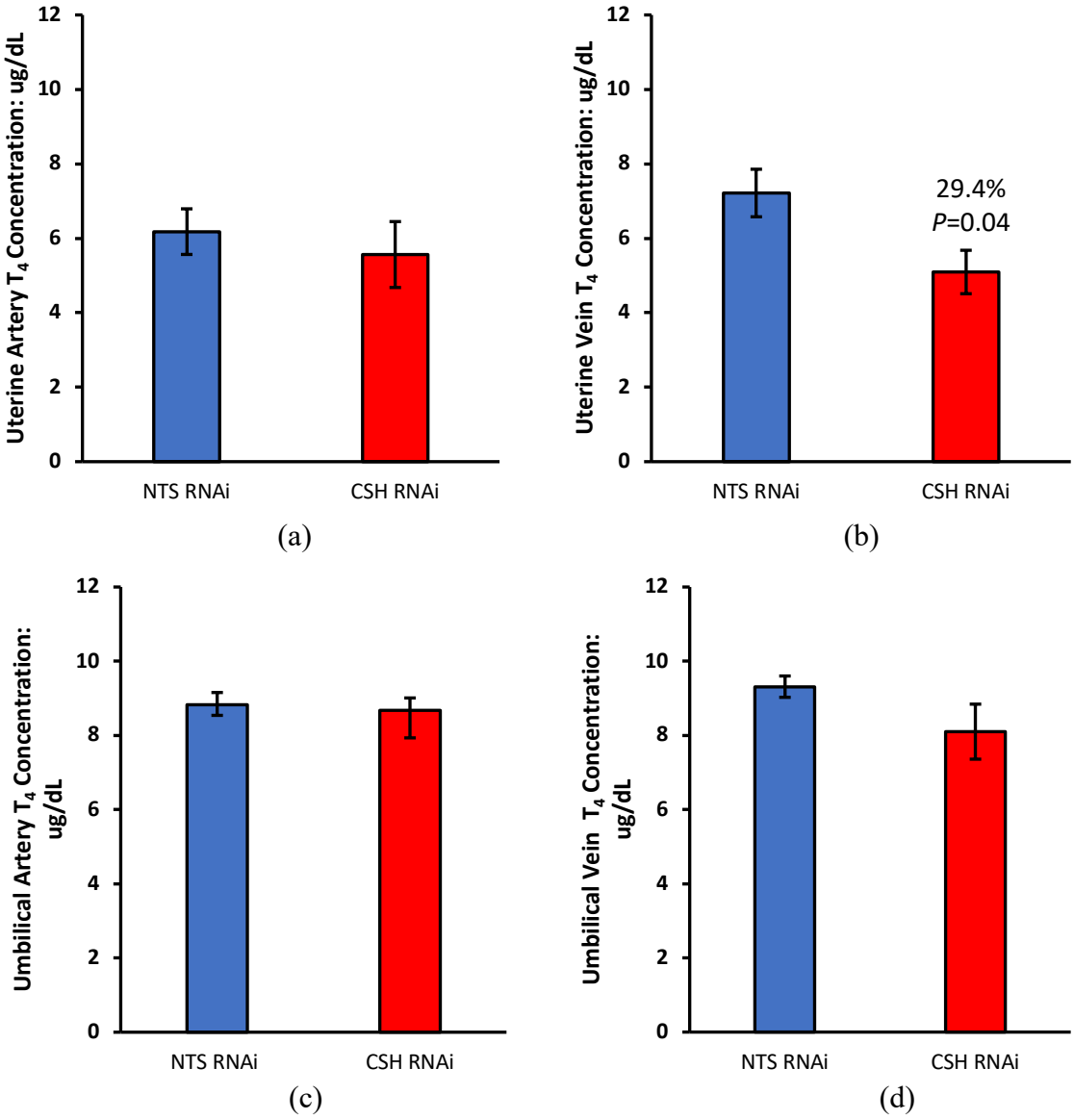


Figure 4 Total Thyroxine ELISA (**a**) Uterine artery, ug/dL (**b**) uterine vein, ug/dL (**c**) umbilical vein ug/dL and (**d**) umbilical artery, ug/dL total T_4 as assessed by ELISA at130 dGA: Data are shown as means ± SEM for all pregnancies in each treatment group. NTS, non-targeting sequence, n=7; CSH, chorionic somatomammotropin, n=6; RNAi, RNA interference

Serum Transthyretin Protein Concentrations:

Western blot analysis was performed to measure the change in protein expression of transthyretin in NTS RNAi pregnancies compared to CSH RNAi pregnancies. Serum from uterine veins, uterine arteries, umbilical veins, and umbilical arteries at 135 dGa were analyzed

for total protein concentration using BCA analysis, and 80ug of total protein were used for each sample. CSH RNAi resulted in a 71% reduction in TTR concentration ($P \leq 0.05$; Figure 5) as compared to NTS RNAi infected pregnancies in uterine artery samples. There was no significant difference in TTR protein concentration in CSH RNAi pregnancies compared to NTS RNAi pregnancies in uterine vein samples (Figure 6). There was no significant difference in TTR protein concentration in CSH RNAi pregnancies compared to NTS RNAi pregnancies in uterine artery samples (Figure 7). CSH RNAi resulted in a 64% reduction in TTR concentration ($P \leq 0.005$; Figure 8) as compared to NTS RNAi infected pregnancies in umbilical artery samples.

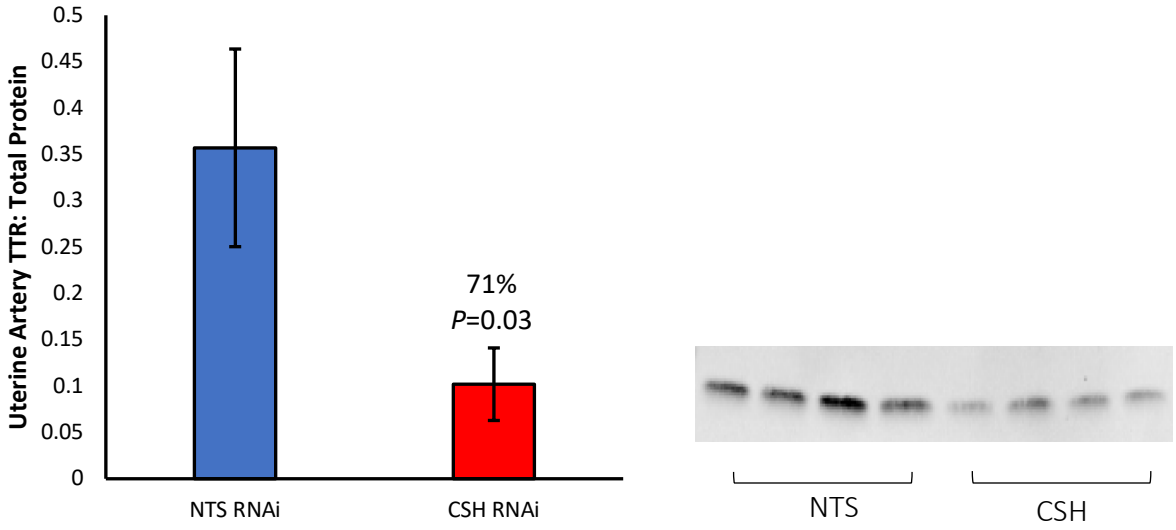


Figure 5: Uterine artery serum TTR protein concentration as measured by Western blot. Data are shown as means ± SEM for all pregnancies in each treatment group. NTS, non-targeting sequence, n=7; CSH, chorionic somatomammotropin, n=8; RNAi, RNA interference.

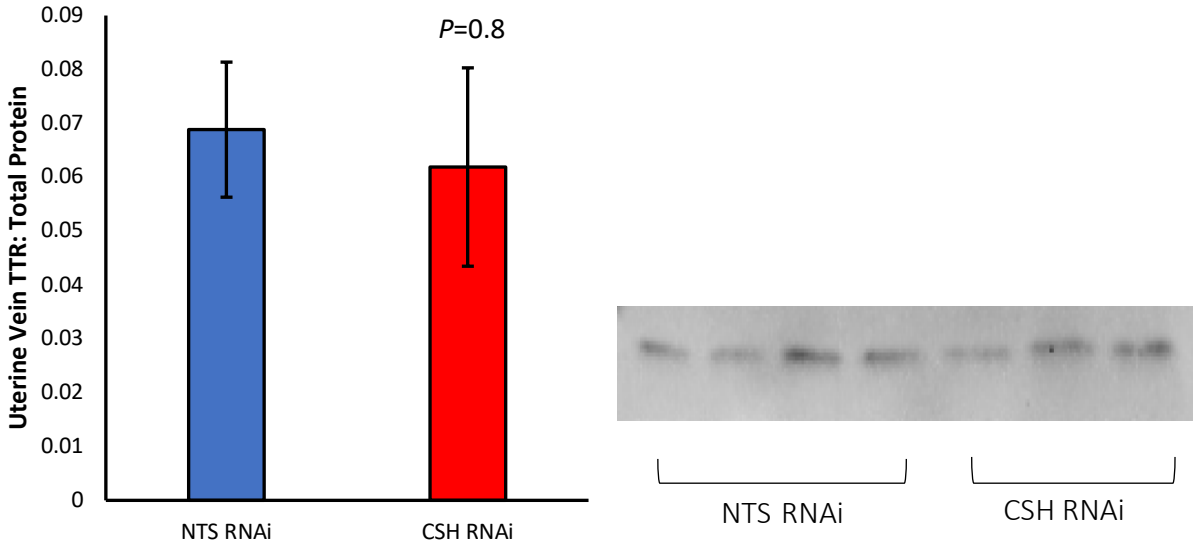


Figure 6: Uterine vein serum TTR protein concentration as measured by Western blot. Data are shown as means ± SEM for all pregnancies in each treatment group. NTS, non-targeting sequence, n=7; CSH, chorionic somatomammotropin, n=6; RNAi, RNA interference.

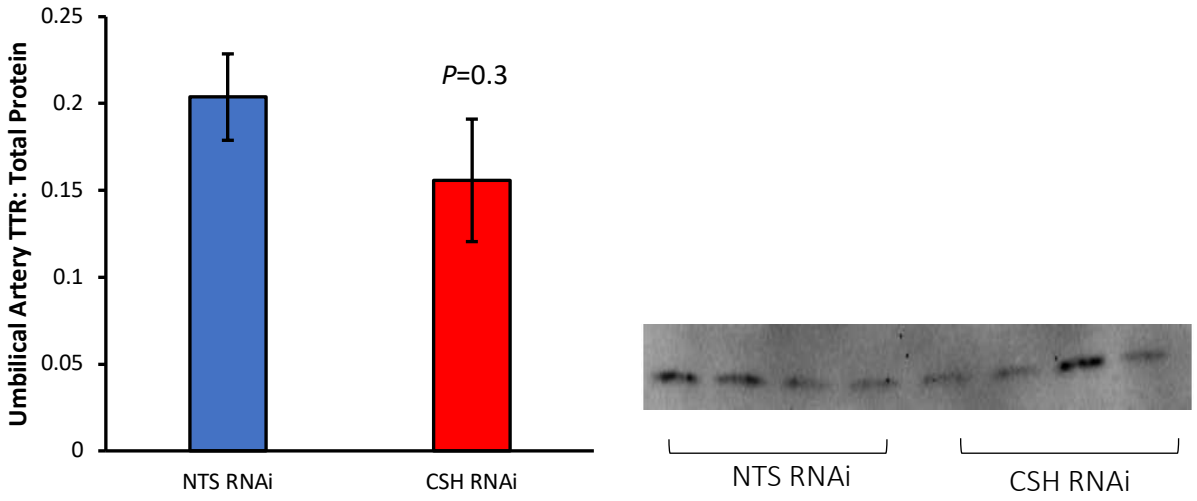


Figure 7: Umbilical Artery serum TTR protein concentration as measured by Western blot. Data are shown as means ± SEM for all pregnancies in each treatment group. NTS, non-targeting sequence, n=8; CSH, chorionic somatomammotropin, n=7; RNAi, RNA interference.

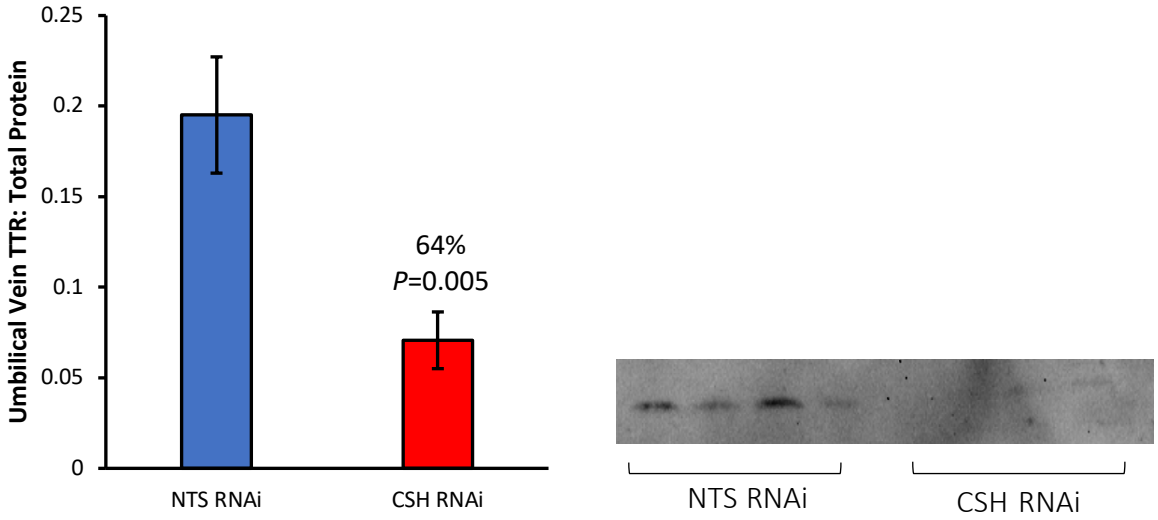


Figure 8: Umbilical vein serum TTR protein concentration as measured by Western blot. Data are shown as means ± SEM for all pregnancies in each treatment group. NTS, non-targeting sequence, n=8; CSH, chorionic somatomammotropin, n=7; RNAi, RNA interference.

Liver Transthyretin Protein Concentrations:

Western blot analysis was performed to measure the change in protein expression of transthyretin in in NTS RNAi maternal liver and fetal liver compared to CSH RNAi maternal liver and fetal liver. Tissues from maternal and fetal livers at 135 dGa were analyzed for total protein concentration using BCA analysis, and 40ug of total protein were used for each sample. CSH RNAi resulted in no change of maternal liver TTR protein concentration when compared to NTS RNAi control pregnancies. CSH RNAi resulted in a 29.58% reduction in TTR concentration ($P \leq 0.05$; Figure 10) as compared to NTS RNAi infected pregnancies in fetal liver samples

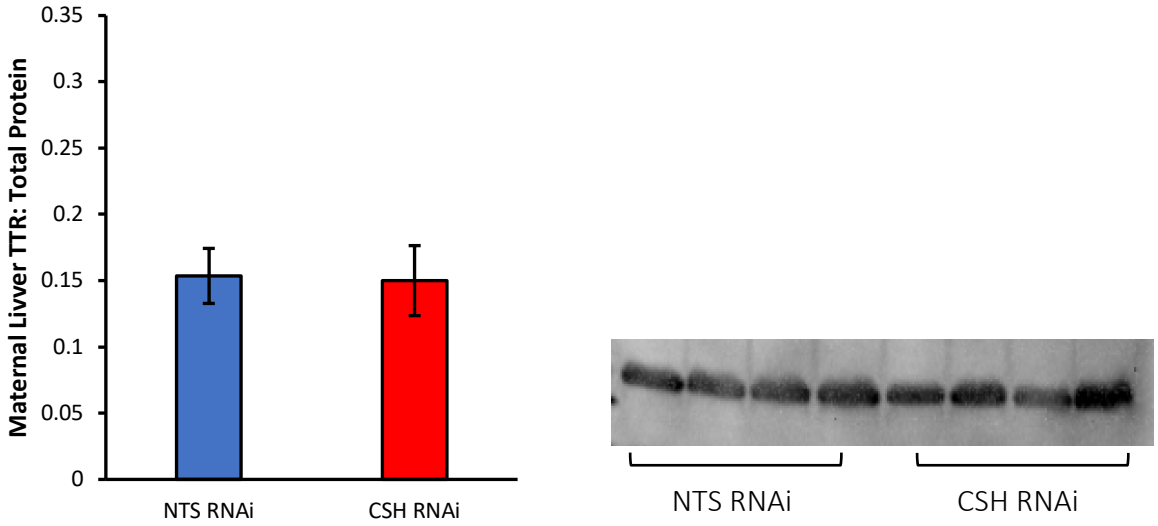


Figure 9: Maternal Liver TTR protein concentration as measured by Western blot. Data are shown as means ± SEM for all pregnancies in each treatment group. NTS, non-targeting sequence, n=8; CSH, chorionic somatomammotropin, n=8; RNAi, RNA interference.

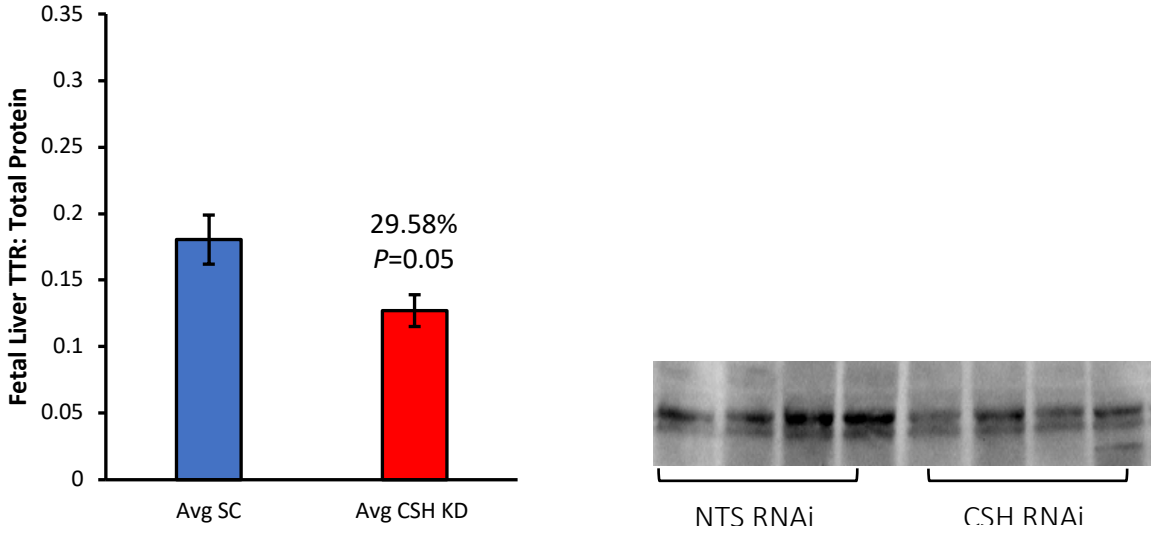


Figure 10: Fetal Liver TTR protein concentration as measured by Western blot. Data are shown as means ± SEM for all pregnancies in each treatment group. NTS, non-targeting sequence, n=4; CSH, chorionic somatomammotropin, n=4; RNAi, RNA interference.

Tanner (et al., 2021) Tissue Results:

Tissue and serum from the following pregnancies were previously described in Tanner et al., 2021.

Relevant Pregnancy Characteristics:

As previously described in Tanner et al. 2021, pregnancy characteristics were measured at 130 dGA. Fetal weight was reduced by 30% ($P \leq 0.05$; Table 5) in CSH RNAi pregnancies compared to NTS RNAi control pregnancies. Placental weight did not differ between CSH RNAi and NTS RNAi pregnancies. Uterine weight was reduced by 43% ($P \leq 0.05$; Table 5) in CSH RNAi pregnancies compared to NTS RNAi pregnancies. To calculate uterine and umbilical blood flow measurements, the $^{3}H_{2}0$ transplacental diffusion technique was used. There was no difference in uterine blood flow between the two samples, but umbilical blood flow was reduced by 40% ($P \leq 0.05$; Table 5) in CSH RNAi pregnancies compared to NTS RNAi pregnancies. Because fetal and placental function is dependent on adequate nutrient and oxygen delivery, the decrease in umbilical blood flow in CSH RNAi pregnancies, led to questions about what substates may be impacted by this perturbation to normal pregnancies, specifically related to thyroid hormone transport.

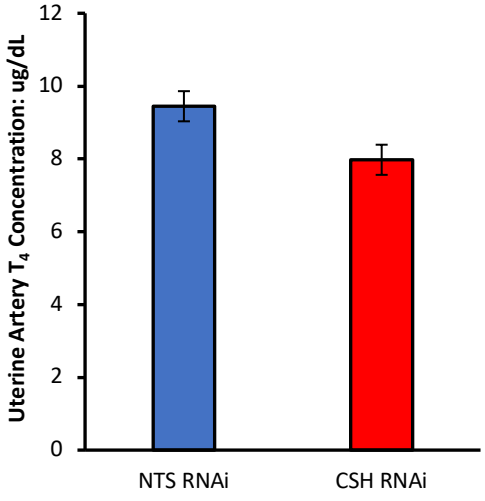
Table 5. Pregnancy characteristics assessed at necropsy (130 dGA).

Measurement	NTS RNAi	CSH RNAi	P-value	% Difference
Fetal Weight (g ± SEM)	3972 ± 241	2781± 402	0.04	30%
Placental Weight (g ± SEM)	463 ± 55	364 ± 80	0.34	21%
Uterine Weight (g ± SEM)	882 ± 136	499 ± 26	0.03	43%
Uterine Blood Flow (mL/min± SEM)	2034 ± 461	1182 ± 301	0.17	42%
Umbilical Blood Flow (mL/min± SEM)	738 ± 67	442 ± 82	0.03	40%

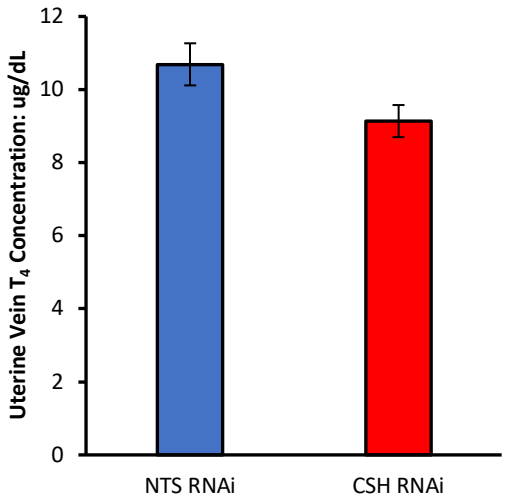
These pregnancies and fetal data were previously described in Tanner et al. 2021Data are shown as mean values ± SEM for all ewes in each treatment group.

Total Thyroxine ELISA:

ELISA assays were performed using serum from uterine artery, uterine vein, umbilical artery, and umbilical vein samples at 130 dGA to determine if there is a disruption in amount of total thyroxine (T_4) in CSH RNAi pregnancies compared to NTS RNAi pregnancies. There were no differences in T_4 levels between CSH RNAi pregnancies and NTS control pregnancies in either uterine artery or uterine vein serum. CSH RNAi resulted in a 16% reduction in T_4 concentration ($P \leq 0.05$; Figure 11c) as compared to NTS RNAi infected pregnancies in umbilical vein samples. CSH RNAi resulted in a 29% reduction in T_4 concentration ($P \leq 0.05$; Figure 11d) as compared to NTS RNAi infected pregnancies in uterine artery samples.



(a)



(b)

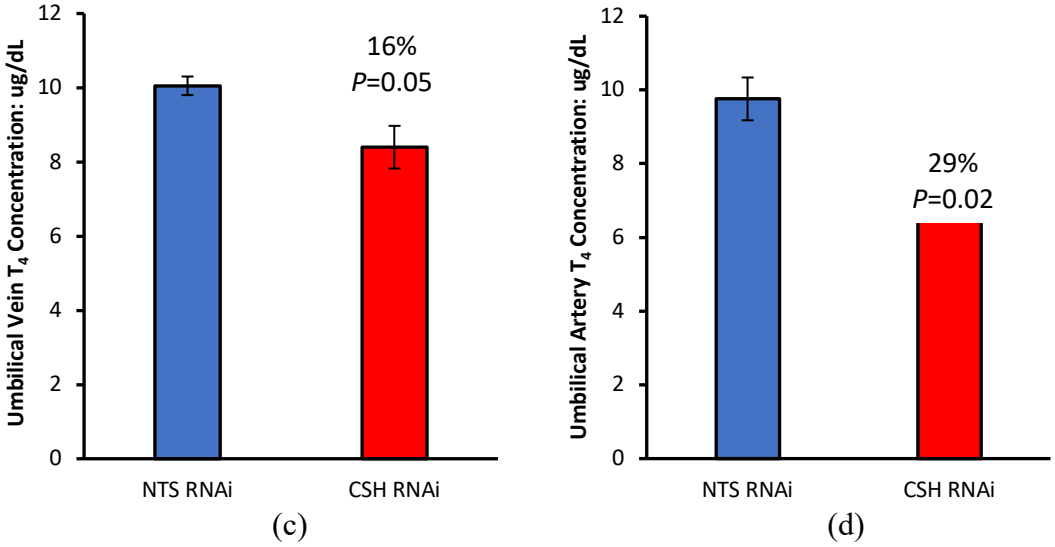


Figure 11 Total Thyroxine ELISA. (a) Uterine artery, ug/dL (b) uterine vein, ug/dL (c) umbilical vein ug/dL and (d) umbilical artery, ug/dL total T_4 as assessed by ELISA at130 dGA: Data are shown as means ± SEM for all pregnancies in each treatment group. NTS, non-targeting sequence, n=4; CSH, chorionic somatomammotropin, n=4; RNAi, RNA interference

Serum Transthyretin Protein Concentrations:

Western blot analysis was performed to measure the change in protein expression of transthyretin in in NTS RNAi pregnancies compared to CSH RNAi pregnancies. Serum from uterine veins, uterine arteries, umbilical veins, and umbilical arteries at 130 dGA were analyzed for total protein concentration using BCA analysis, and 80ug of total protein were used for each sample. CSH RNAi tended to result in a 47% reduction in TTR concentration ($P \leq 0.10$; Figure 12) as compared to NTS RNAi infected pregnancies in uterine artery samples. CSH RNAi resulted in a 64% reduction in TTR protein concentration ($P \leq 0.05$; Figure 13) as compared to NTS RNAi infected pregnancies in uterine vein samples. CSH RNAi resulted in a 47% reduction in TTR concentration ($P \leq 0.01$; Figure 14) as compared to NTS RNAi infected pregnancies in umbilical artery samples. There was no significant difference in TTR protein concentration in CSH RNAi pregnancies compared to NTS RNAi pregnancies in umbilical vein samples (p = .4; Figure 15).

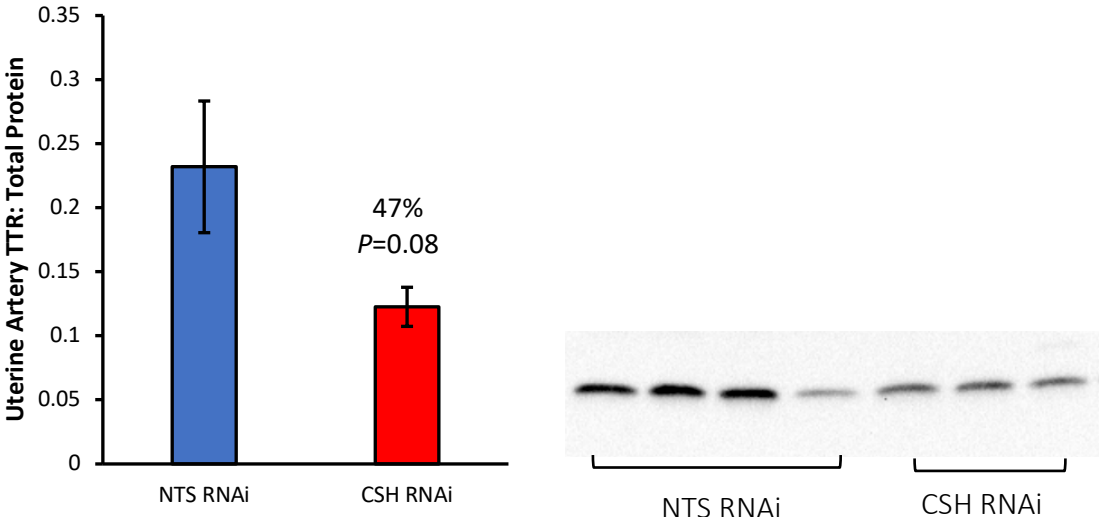


Figure 12: Uterine artery serum TTR protein concentration as measured by Western blot Data are shown as means ± SEM for all pregnancies in each treatment group. NTS, non-targeting sequence, n=4; CSH, chorionic somatomammotropin, n=3; RNAi, RNA interference.

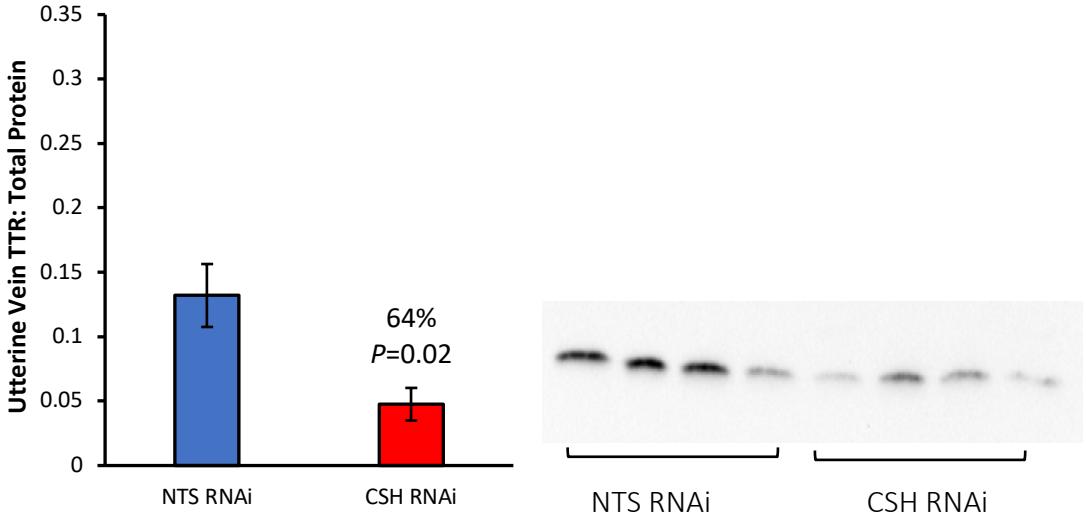


Figure 13: Uterine vein serum TTR protein concentration as measured by Western blot. Data are shown as means ± SEM for all pregnancies in each treatment group. NTS, non-targeting sequence, n=4; CSH, chorionic somatomammotropin, n=3; RNAi, RNA interference.

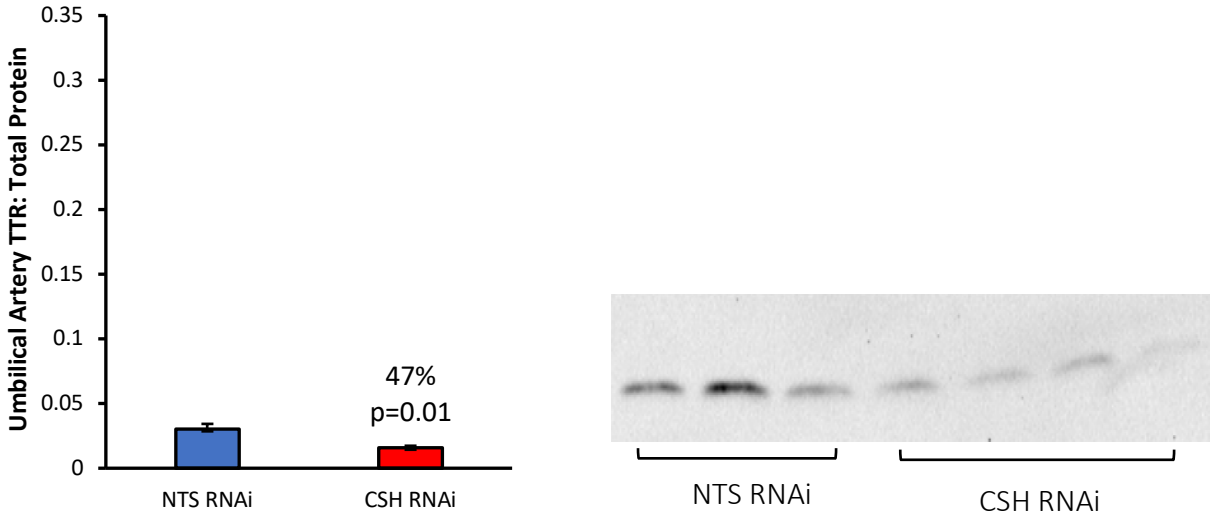


Figure 14: Umbilical artery serum TTR protein concentration as measured by Western blot. Data are shown as means ± SEM for all pregnancies in each treatment group. NTS, non-targeting sequence, n=3; CSH, chorionic somatomammotropin, n=4; RNAi, RNA interference.

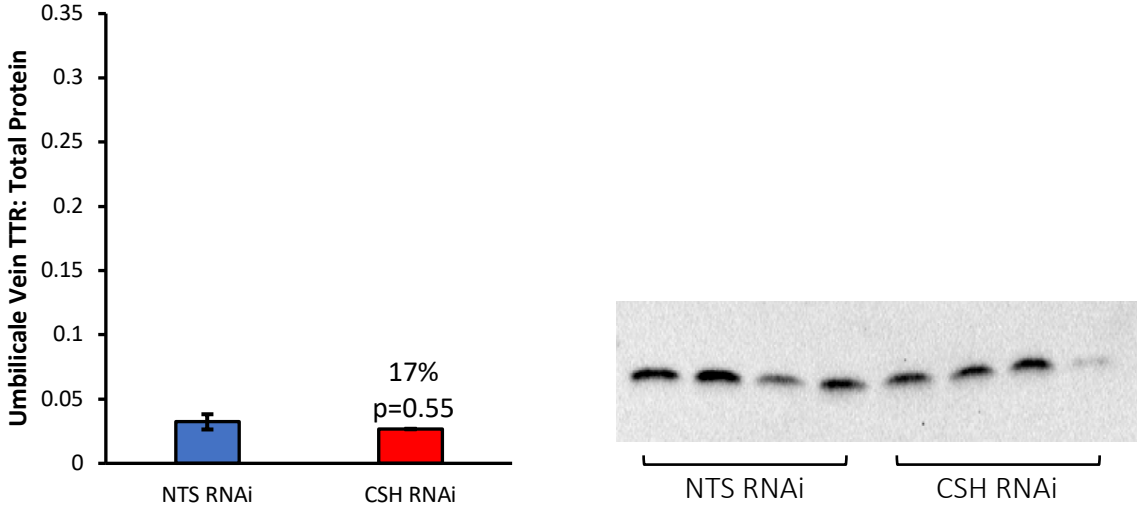


Figure 15: Umbilical vein serum TTR protein concentration as measured by Western blot. Data are shown as means ± SEM for all pregnancies in each treatment group. NTS, non-targeting sequence, n=4; CSH, chorionic somatomammotropin, n=4; RNAi, RNA interference.

Transthyretin Uptake Calculations:

Umbilical uptake, uterine uptake and uteroplacental utilization calculations use collected blood flow data to calculate the amount of Transthyretin that the placenta takes up from maternal circulation, what the placenta itself uses, and how much transthyretin is transferred from the placenta to fetal circulation. The uterine uptake of transthyretin tended to be reduced ($P \leq 0.1$;

Figure 16a) by 45% in CSH RNAi pregnancies, meaning that in CSH RNAi pregnancies, the placenta is taking up 45% less transthyretin than in NTS RNAi pregnancies. Uteroplacental transthyretin utilization however tended to be reduced ($P \leq 0.1$; Figure 16b) by 48% in CSH RNAi pregnancies. The placenta itself is metabolizing 48% less transthyretin in CSH RNAi pregnancies compared to NTS RNAi pregnancies. While less TTR is being taken up from maternal circulation, less is being used at the placenta itself in CSH RNAi pregnancies. Due to negligible umbilical gradients reported, we concluded fetal circulation is not taking up TTR from the placenta near term.

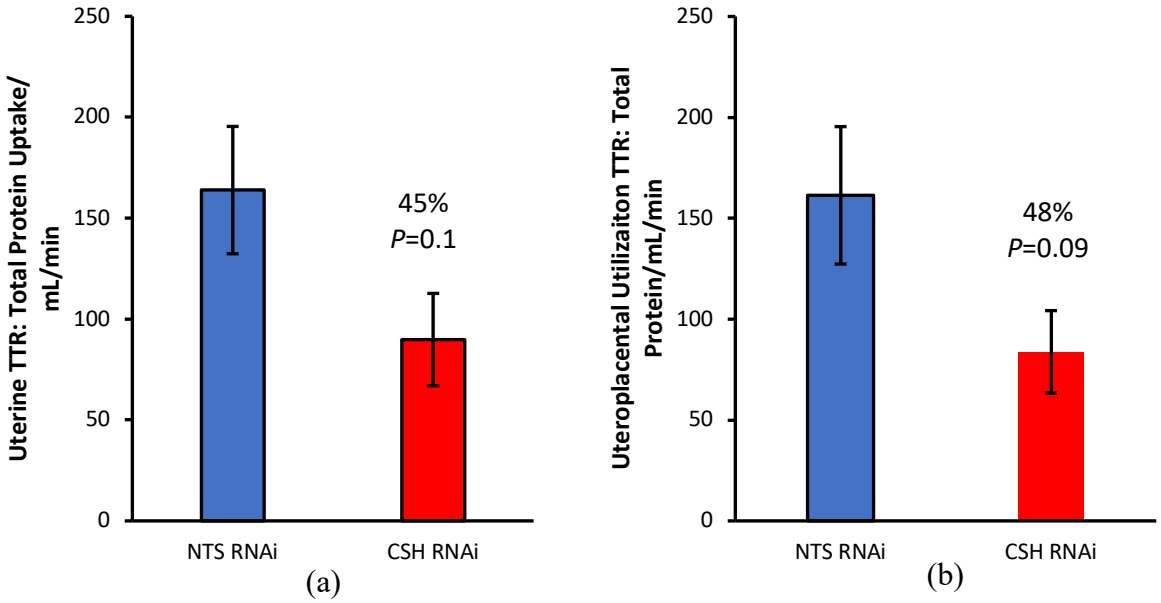


Figure 16. Uterine, and uteroplacental TTR uptakes as assessed by $^{3}H_{2}0$ transplacental diffusion at 130 dGA: (**a**) uterine transthyretin uptakes, mmol/min (**b**) uteroplacental transthyretin utilization, mmol/min. Data are shown as means ± SEM for all pregnancies in each treatment group. NTS, non-targeting sequence, n=4; CSH, chorionic somatomammotropin, n=4; RNAi, RNA interference

Liver Transthyretin Protein Concentrations:

Western blot analysis was performed to measure the change in protein expression of transthyretin in in NTS RNAi maternal liver and fetal liver compared to CSH RNAi maternal

liver and fetal liver. Tissue from maternal and fetal livers at 130 dGA were analyzed for total protein concentration using BCA analysis, and 40ug of total protein were used for each sample. CSH RNAi resulted in no change of maternal liver TTR protein concentration or fetal liver TTR protein concentration when compared to NTS RNAi control pregnancies.

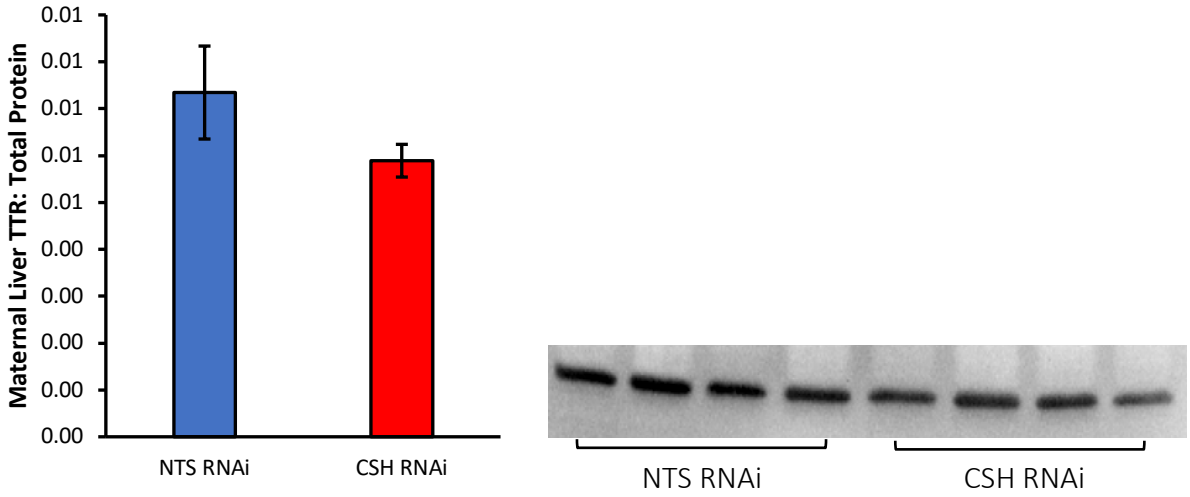


Figure 17: Maternal Liver TTR protein concentration as measured by Western blot. Data are shown as means ± SEM for all pregnancies in each treatment group. NTS, non-targeting sequence, n=4; CSH, chorionic somatomammotropin, n=4; RNAi, RNA interference.

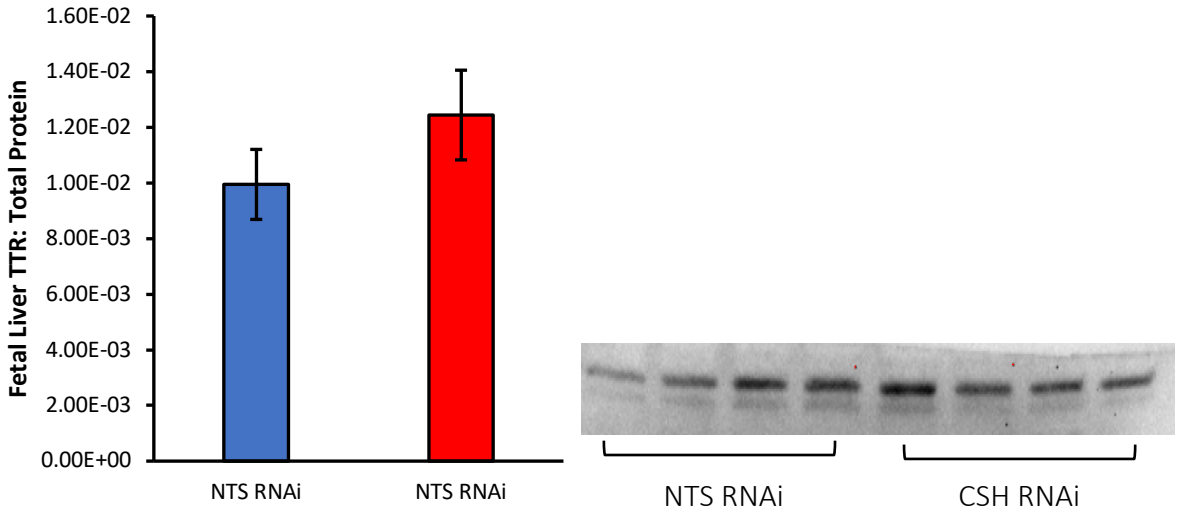


Figure 18: Fetal Liver TTR protein concentration as measured by Western blot. Data are shown as means ± SEM for all pregnancies in each treatment group. NTS, non-targeting sequence, n=4; CSH, chorionic somatomammotropin, n=4; RNAi, RNA interference.

Caruncle DIO2 and DIO3 mRNA Concentrations:

Day 130 caruncle and cotyledon tissues were assessed for differences in DIO2 and DIO3 mRNA concentration between NTS RNAi pregnancies and CSH RNAi pregnancies. There was no significant difference in DIO3 expression in caruncle tissue when comparing NTS RNAi pregnancies to CSH RNAi pregnancies. Caruncle DIO2 mRNA expression decreased by 60% ($P \leq 0.05$; Figure 19b) in CSH RNAi pregnancies compared to NTS pregnancies. There was no difference in DIO2 or DIO3 expression in cotyledon tissue when comparing NTS RNAi pregnancies and CSH RNAi pregnancies (Figure 20).

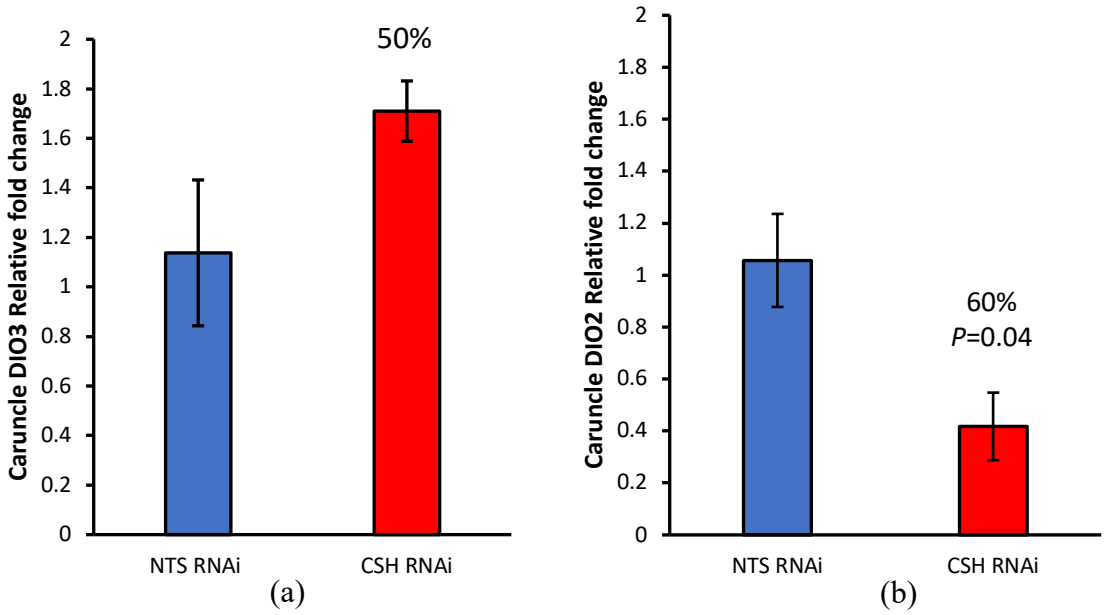


Figure 19: Deiodinase 3 and Deiodinase 2 gene expression in Caruncle tissue (**a**) Caruncle DIO3 fold change (**b**) Caruncle DIO2 fold change mRNA concentration at 130 dGA. Data are shown as means ± SEM for all pregnancies in each treatment group. NTS, non-targeting sequence, n=5 CSH, chorionic somatomammotropin, n=6; RNAi, RNA interference.

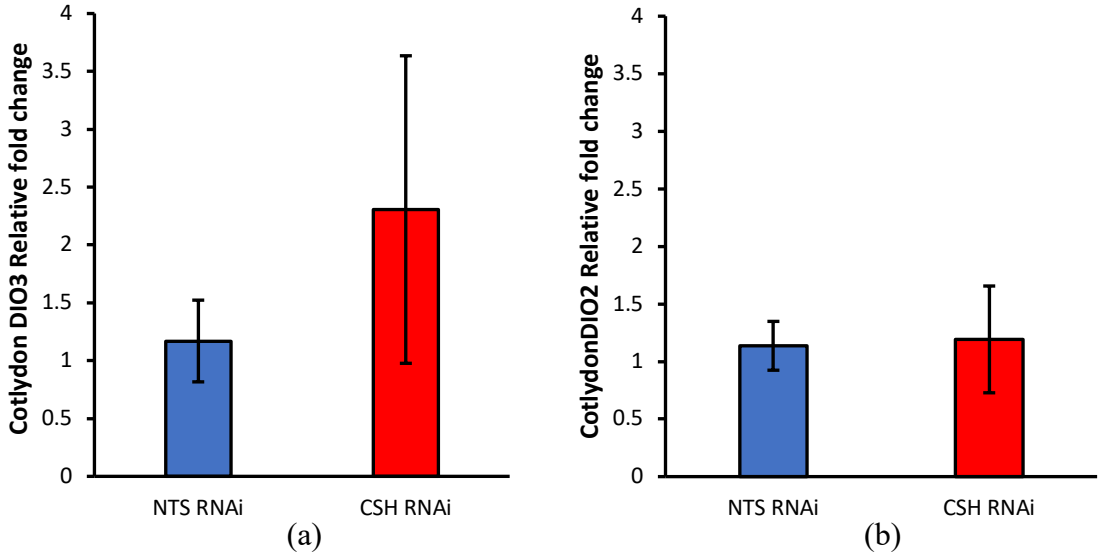


Figure 20: Deiodinase 3 and Deiodinase 2 gene expression in cotyledon tissue (**a**) Cotyledon DIO2 fold change (**b**) Cotyledon DIO2 fold change in mRNA concentration at 130 dGA. Data are shown as means ± SEM for all pregnancies in each treatment group. NTS, non-targeting sequence, n=5 CSH, chorionic somatomammotropin, n=6; RNAi, RNA interference.

Discussion:

Thyroid hormones are necessary for proper fetal growth and fetal brain development (Korevaar et al., 2017). Early in gestation, before the fetus develops its own thyroid gland, maternally derived thyroid hormones cross the placenta and enter fetal circulation in order to supplement the fetus with adequate amounts of thyroid hormone (de Escobar et al., 2004). Steinhauser et al. (2021) reported that in nutrient restricted models of IUGR pregnancy, perturbations were observed in thyroid hormone concentrations in both maternal and fetal circulation. In these IUGR pregnancies there were also observed changes in placental deiodinase 3 (DIO3) mRNA expression, the enzyme responsible for inactivating thyroid hormones (Steinhauser et al., 2021). Unfortunately, besides the data reported in Steinhauser et al. (2021), there is very little data available pertaining to the impact of IUGR on thyroid hormone transport in ovine pregnancies. It has been reported however that perturbations in the transport of other essential substrates from maternal circulation to fetal circulation in ovine IUGR pregnancies

occurs. Tanner et al. (2021) reported that in lentiviral-mediated chorionic somatomammotropin (CSH) RNA interference (RNAi) induced IUGR pregnancies, there was a reduction in uterine and umbilical uptake of oxygen, glucose, and many amino acids when compared to control pregnancies. Because of the observed changes reported with this specific model of IUGR along with the general lack of information surrounding thyroid hormone transport near term in ovine pregnancies, the main objective of our study was to better understand thyroid hormone transport during late gestation specifically in CSH RNAi models of IUGR pregnancies. While two cohorts of samples were used for experiments, Baker et al. (2016) samples and Tanner et al. (2021) samples, we believe that the data from the Baker et al. (2016) cohort does not accurately represent what is occurring near term under steady state non stressed conditions in ovine pregnancies due to the method of sample collection used in that year's study. While this data was important for preliminary studies, the conclusions drawn from our analysis come from data collected from samples in the Tanner et al. (2021) cohort.

CSH RNAi samples had reduced concentrations of T_4 in umbilical vein and umbilical artery serum samples at 135 dGA when compared to control samples, with no changes in either uterine artery or uterine vein samples. Because maternal concentrations of T_4 were not reduced in the CSH RNAi model of IUGR it can be concluded that the reductions in fetal T_4 circulation were not due to dysregulation in maternal production, but perhaps perturbations in processes such as transport across the placenta, dysregulated placental deiodinase enzyme expression, or reduced fetal production of T_4. It has been speculated that in sheep pregnancies specifically, no maternally produced thyroid hormone is transported across the placenta near term (Forhead & Fowden, 2014; Hopkins & Thorburn, 1972). This conclusion was based on thyroid hormone concentrations measured in the serum from umbilical artery blood samples taken from

pregnancies where fetal thyroidectomies were performed (Forhead & Fowden, 2014). This data only demonstrates that a fetus with no thyroid gland has reduced levels of thyroid hormone leaving the fetus and returning to the placenta. Because no thyroid hormone measurements were taken in umbilical vein serum in these studies, one cannot assume that the reduction of thyroid hormone in umbilical artery serum is due to lack of maternal transport but could be due to the fetus utilizing maternally derived thyroid hormone before TH can enter the umbilical artery. To determine if maternally derived TH is transported in fetal thyroidectomized sheep, umbilical vein blood samples would need to be analyzed for the presence of thyroid hormone. It is because of this oversight we believed it was necessary to explore the mechanisms behind thyroid hormone transport to deduce if CSH RNAi negatively impacted this process rather than just assuming zero maternally supplied T_4 was being transported to the fetus at 135 dGA.

Transthyretin (TTR) is one of three main thyroid hormone carrier proteins (Pappa et al., 2015), but the only thyroid hormone carrier protein that can be internalized into the placenta while bound to T_4 (Landers et al. 2009). Due to this unique action, we were interested in observing the impact that CSH RNAi had on TTR protein expression in maternal and fetal circulation to determine if TTR dysregulation was involved in the observed reduced concentration of T_4 in fetal circulation. CSH RNAi pregnancies exhibited reduced TTR protein concentrations in uterine artery, uterine vein, and umbilical artery samples compared to control samples. Because our study is seemingly the first to explore TTR concentrations in these four vessels in ovine IUGR pregnancies, there are very few studies for us to compare our observations. Fruscalzo et al. (2012) studied the impact of IUGR on TTR in human placental tissue however, and reported that in IUGR pregnancies, placental tissue TTR protein concentration increased compared to placental tissue from non IUGR pregnancies (Fruscalzo et

al., 2012). While these results seemingly contrast results from our current study, it must be kept in mind that Fruscalzo et al. (2012) did not measure TTR protein amounts in circulation, only protein present in placental tissue. They also measured TTR in humans rather than sheep. The difference in sample type and species are most likely responsible for the difference in TTR expression pattern reported in Fruscalzo et al. (2012) compared to data reported in this paper.

Due to the high level of TTR production in the liver (Schreiber et al., 1976), we analyzed maternal and fetal liver tissue from both control sheep and CSH RNAi IUGR sheep to see if we observed any differences in TTR protein concentration between the two groups. Our hope was that we would observe a reduced concentration of TTR in CSH KD liver samples which could explain the reduction in TTR that was observed in circulation. Unfortunately, we observed that CSH RNAi had no impact on either maternal or fetal liver tissue TTR protein concentrations. This information only allows us to conclude that liver TTR protein production is not responsible for reduced circulating TTR protein in CSH RNAi IUGR pregnancies. TTR is produced in many other organs including the choroid plexus, retinal pigment, pancreas, neurons, placenta, and intestines in humans (Schreiber et al., 1976; Dickson 1985; McKinnon 2005; Cavallaro et al., 1990; Ong et al., 1994; Loughna et al., 1995; Kato et al., 1985), therefore future studies need to be completed in order to determine if perturbed production of TTR in these tissues could be the cause of the reduction in circulating TTR that our current study reported.

To determine if the transport of TTR from maternal circulation to fetal circulation is impacted by CSH RNAi, uterine and umbilical uptakes were calculated. Previous studies of nutrient transport in CSH RNAi IUGR pregnancies have reported perturbations in uterine uptake of oxygen, glucose, alanine, arginine, asparagine, glutamine, histidine, isoleucine, leucine, lysine, ornithine, phenylalanine, serine, threonine, tyrosine, and valine (Tanner et al., 2021). The

same study also reported reductions in umbilical uptake of oxygen, glucose asparagine, leucine, and tyrosine (Tanner et al., 2021). With a well-documented history of impaired substrate transport in CSH RNAi models of IUGR, we wanted to investigate TTR uterine and umbilical uptake to determine if uptake dysregulation was a potential cause for reduced fetal thyroid hormone concentrations observed in fetal circulation of CSH RNAi induced IUGR pregnancies. Our current study indicated that there tended to be a reduction in uterine uptake of TTR in CSH RNAi pregnancies compared to control pregnancies, with no umbilical uptake of TTR observed in either CSH RNAi or control pregnancies. We can postulate that this reduction in uterine TTR uptake is due to the reduction in TTR observed in uterine artery serum samples mentioned earlier since this is the vessel providing blood to the placenta. Another avenue of thought is that this reduction in uterine uptake could be due to perturbations in membrane transporter expression at the placental interface. Previous studies done in maternal nutrient restricted models of IUGR pregnancy reported that there was no difference in mRNA expression of SLC16A2, SLC16A10, SLC01C1, SLCO4A1, and SLC5A5 leading us to believe that transporters associated with thyroid hormone transport are not negatively impacted by an IUGR phenotype (Steinhauser et al., 2021). TTR-T_4 could be entering the placenta via endocytosis, therefore these transporters mentioned above may not be involved in uptake at all (Landers et al., 2009). Mechanisms of endocytosis should be further investigated to ensure that the reduction of uptake is due to a reduction of concentration of TTR and not with issues relating to entry into the placenta. Umbilical uptake in both NTS RNAi control pregnancies and CSH RNAi pregnancies, was seemingly nonexistent, meaning that according to our data it does not appear that the fetus at 135 dGA is taking up maternal TTR in a concentration that would contribute to normal fetal physiological processes. While we still believe that thyroid hormones are being transported

through the placenta via TTR into fetal circulation, it appears that TTR itself if not being transported into fetal circulation. Therefore, the reduction in TTR that was observed in fetal circulation in CSH RNAi pregnancies was not due to reductions in maternal systems or the placenta, but rather a change that has yet to be elucidated in either the fetus itself or fetal circulation

We believe that TTR-T_4 complexes could be forming to protect thyroid hormones from deiodinase driven inactivation during transport across the placenta. Under normal physiological conditions, the placenta expresses both deiodinase 2 (DIO2) and deiodinase 3 (DIO3) (Chan et al., 2003). DIO2 converts a portion of the maternally supplied T_4 to rT_3 thus inactivating TH, or to T_3 leading to increased volume of biologically active T_3 (Adu-Gyamfi et al., 2020). DIO3 however converts some T_4 to rT_3 and T_3 to T_2 thus inactivating maternally derived thyroid hormone (Adu-Gyamfi et al., 2020; Gutiérrez-Vega et al., 2020). Maintaining a proper balance of both enzymes in the placenta is necessary to ensure that the fetus receives adequate amounts of biologically active TH while also protecting the fetus from situations where there is an excess of thyroid hormones in maternal circulation. When the balance of these enzymes is dysregulated, improper thyroid hormone transport is observed (Adu-Gyamfi et al., 2020). We observed that CSH RNAi leads to increased expression of DIO3 mRNA and decreased expression of DIO2 mRNA in the caruncle when compared to control samples. No differences were observed in cotyledon tissue between CSH RNAi and control pregnancies. Increased expression of DIO3 could indicate that there is more thyroid hormone inactivation occurring, which could potentially lead to the reduced amount of thyroid hormone observed in fetal circulation. It could also explain why TTR-T4 complexes are necessary for thyroid hormone transport across the placenta. Because thyroid hormones in the placenta could be more vulnerable for inactivation, there may

be an increased need for TTR to stay bound to T_4 while in the placenta to aid in protecting thyroid hormones from increased concentrations of DIO3. A decreased expression of DIO2 could indicate that CSH RNAi IUGR leads to reduced thyroid hormone activation in the placenta, thus making it an environment that is not conducive to maintaining proper active thyroid hormone concentration. Deiodinase dysregulation may be contributing to the reductions observed in fetal T_4 or perturbations in TTR observed in CSH RNAi pregnancies. Previous studies done in nutrient restricted models of IUGR pregnancies have reported similar results where DIO3 mRNA expression was increased in IUGR sheep compared to control sheep (Steinhauser et al., 2021). In contrast to our results however, they did not observe a difference in DIO2 expression near term. Steinhauser's study measured mRNA expression in full placentomes near term rather than separating caruncles and cotyledons which could account for the differing results for DIO2 mRNA expression. It has also been reported that IUGR had no impact on DIO2 or DIO3 mRNA expression in human placentas near term (Chan et al., 2003), potentially indicating that human placentas are not impacted in the same way by IUGR that sheep placentas are impacted.

Thyroid hormones are necessary to regulate metabolism, and the placenta is a very metabolically active organ that 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.) Previous studies have reported that in CSH RNAi pregnancies, uteroplacental utilization of glucose, or the amount of glucose the placenta itself is using, is significantly reduced when compared to control pregnancies (Tanner et al., 2021). If deiodinase enzymes are dysregulated in a manner that limits the availability of active thyroid hormones in the placenta like our current study shows, oxidative metabolism may be reduced preserving glucose for transfer to the fetus

rather than being used in the placenta. Further studies are necessary for this hypothesis to be deemed true or false.

Summary/Conclusion:

It was our objective to determine the impact that lentiviral mediated chorionic somatomammotropin RNA interference had on thyroid hormone transport and the mechanisms involved in that transport near term in ovine models of IUGR. We hypothesized that a reduction in placental CSH negatively impacts thyroid hormone transport from maternal circulation to fetal circulation due to perturbations in both thyroid hormone carrier protein activity and placental deiodinase enzyme expression.

CSH RNAi resulted in a 16% reduction ($P \leq 0.10$) in umbilical vein total thyroxine concentrations, and a 29% reduction ($P \leq 0.05$) in umbilical artery total thyroxine concentrations. It also resulted in a 47% reduction ($P \leq 0.10$) in thyroid hormone carrier protein transthyretin in uterine artery serum, a 64% reduction ($P \leq 0.05$) in uterine vein serum TTR, and a 47% reduction ($P \leq 0.05$) in umbilical artery serum TTR. It was observed that CSH RNAi resulted in a 45% reduction ($P \leq 0.10$) in uterine TTR uptake, did not impact umbilical uptake, and resulted in a 48% reduction ($P \leq 0.10$) in uteroplacental utilization to compensate for reduced uterine uptake. CSH RNAi did not impact maternal or fetal liver TTR production. These CSH RNAi pregnancies also resulted in a 60% reduction ($P \leq 0.05$) in deiodinase 2 mRNA expression in caruncle tissue meaning that less activation of thyroid hormone in the placenta is occurring. The perturbations that were observed in CSH RNAi IUGR pregnancies suggest that CSH presence in the placenta is important for proper thyroid hormone activity in the placenta and transport across the placenta near term.

As previously mentioned, this study in sheep is relatively novel, and to understand the full impact that IUGR has on thyroid hormone transport near term, many other experiments need to be performed. To determine why there is a reduction in circulating TTR protein, while observing no reduction in maternally derived liver TTR concentration, it is necessary to collect and analyze a wider range of maternal tissues for TTR expression. Because TTR is one of three main thyroid carrier proteins it would also be interesting to perform lentiviral mediated RNAi targeted to knockdown TTR in the placenta to determine the importance of this carrier protein. It could very well be the case that TBG and albumin can maintain normal thyroid hormone transport in the absence of TTH. To fully deduce what is occurring regarding deiodinase activity, future studies would need to investigate enzymatic activity of both DIO2 and DIO3. MRNA expression tells us nothing about enzyme activity. For example, DIO2 expression could be decreasing, but its enzymatic activity may increase, meaning there is no change in its ability to activate thyroid hormone and therefore having no impact on T_4 levels. We would also like to perform lentiviral mediated RNA interference to knock down the expression of either or both enzymes and to observe the resulting placental and fetal phenotype to better understand deiodinase's role in maintaining proper thyroid hormone transport throughout gestation.

While there are large gaps to fill in terms of understanding the impact of CSH RNAi on the entire process of thyroid hormone transport near term, we believe the results reported here are an important piece of the puzzle in understanding chorionic somatomammotropin's roll in pregnancy.

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