Table of Contents

[Specific Aim 1 3](#_Toc15465519)

[Rationale and Background 3](#_Toc15465520)

[Murine Placental Development and Physiology 3](#_Toc15465521)

[Cortisol/Corticosterone Levels in Pregnancy 3](#_Toc15465522)

[Placental HSD11B2 Activity 4](#_Toc15465523)

[Fetal HPA Axis Development 4](#_Toc15465524)

[Glucocorticoid Treatments in Pregnancy 5](#_Toc15465525)

[Effects of Glucocorticoid Exposure on Placenta and Fetus 5](#_Toc15465526)

[Fetal and Placental Development 5](#_Toc15465527)

[Placental Protein Expression 5](#_Toc15465528)

[Placental Glucose and Amino Acid Transporters 6](#_Toc15465529)

[Effect of In Utero Glucocorticoid Exposure on Offspring 6](#_Toc15465530)

[Experimental Design 7](#_Toc15465531)

[Figure 1: Diagram representing the experimental design and respective timeline 8](#_Toc15465532)

[Methods 9](#_Toc15465533)

[Dexamethasone Exposure 9](#_Toc15465534)

[Food Intake 9](#_Toc15465535)

[Body Composition 9](#_Toc15465536)

[Sacrifice and Tissue and Blood Collection 9](#_Toc15465537)

[Real time qPCR 10](#_Toc15465538)

[Western Blotting 10](#_Toc15465539)

[Histology 10](#_Toc15465540)

[Expected Results 11](#_Toc15465541)

[Aim 1.1: How does maternal GC exposure affect placental, fetal IUGR, and offspring survival? 11](#_Toc15465542)

[Aim 1.2: How does maternal GC exposure affect placental endocrine function (specific hormones: lactogen,IGF2 , GDF15…) look at qPCR mRNA expression – will not use ELISA yet since ELISA is expensive and we may not see a difference in qPCR/mRNA expression initially 11](#_Toc15465543)

[Aim 1.3: Is placental mTORC1 signaling altered after maternal GC exposure?  Western blot for 4EBP, S6, PS6, AKT 11](#_Toc15465544)

[Aim 1.4: How does maternal GC exposure affect the expression of placental nutrient transporters?  11](#_Toc15465545)

[Aim 1.5: Is offspring metabolic health survival, wt, mri, if they survive after Dex exposure during gestation only (no 1 week preconception) 11](#_Toc15465546)

[Aim 1.6: Does a placental GR-KO model rescue the placental and fetal effects of GC exposure? 11](#_Toc15465547)

[Potential Pitfalls and alternate Approaches (Aims 1.1-1.6) 12](#_Toc15465548)

# Specific Aim 1

**Determining the effects of chronic stress on placental** **transport of nutrients and endocrine function.**

The placenta is the direct and only site of communication between mother and fetus during *in utero* development (Brett *et al.*, 2014). The placenta is the rate-limiting step for fetal nutrient and gas acquisition (Brett *et al.*, 2014). Additionally, the placenta plays an important endocrine role to promote fetal growth and nutrient supply (Malassine *et al.*, 2003). The placenta is highly regulated to ensure adequate growth of the fetus in normal pregnancies (Napso *et al.*, 2018). In cases of maternal glucocorticoid-induced stress, placental nutrient transport and endocrine function are compromised leading to potentially suboptimal fetal growth (Kipmen-Korgun *et al.*, 2012; Waffarn & Davis, 2012). In Denmark, 20% of women reported use of corticosteroids from 4 weeks prior to delivery until delivery between 1996-2008 (Hviid & Mølgaard-Nielsen, 2011). The mechanisms by which maternal corticosteroids influence fetal health and placental function are understudied (Kemp *et al.*, 2015). Some side effects like reduced birthweight, offspring hypertension, mental illness and higher HPA axis activity remain controversial (Duthie & Reynolds, 2013; Reynolds, 2013; Braun *et al.*, 2013; Moisiadis & Matthews, 2014). My hypothesis is that corticosteroid treatments prior to conception and/or during conception cause altered placental transport and hormonal function in a time-dependent manner by which an early and prolonged exposure during pregnancy has more prominent side effects on the placenta and fetus. To test this hypothesis, we will examine a) how maternal dexamethasone effects on placental development and function, b) how maternal dexamethasone affects fetal and offspring development and health, and c) the role of placental glucocorticoid receptor in mitigating the effects of maternal dexamethasone exposure.

# Rationale and Background

## Murine Placental Development and Physiology

The definitive structure of the mouse placenta is determined at midgestation (Malassine *et al.*, 2003). The placenta encompasses two sides, an arc-shaped surface facing the maternal side and another flat surface facing the fetal side (Georgiades *et al.*, 2002). The mouse placenta has three distinct compartments, a decidual maternal zone which is the outermost compartment, a fetal-derived junctional zone that mediates placental endocrine function, and a fetal-derived labyrinthine zone that comprises the majority of the placenta and is the main site for nutrient and gas exchange (Woods *et al.*, 2018). Three exchange barriers exist moving inwards from the decidua to the fetal compartment including two syncytiotrophoblast layers (in the labyrinthine layer) and one fetal endothelial cell layer (Georgiades *et al.*, 2002). The two syncytiotrophoblast barriers comprise the microvillous membrane facing the maternal circulation and the basal membrane facing fetal circulation (Brett *et al.*, 2014).

At midgestation, placental invasion of the maternal uterine cavity occurs to allow maternal blood flow into the placental cavity (Malassine *et al.*, 2003; Woods *et al.*, 2018). This invasion permits direct nutrient uptake from the maternal circulation to the fetus through the placenta. Prior to this invasion, the embryo acquires nutrients from the yolk sac, the initial placental structure that absorbs nutrients from maternal circulation (Malassine *et al.*, 2003; Woods *et al.*, 2018).

## Cortisol/Corticosterone Levels in Pregnancy

During human pregnancy, mean cortisol rises gradually as pregnancy progresses (Carr *et al.*, 1981). Mean cortisol levels increase during the first, second and third trimester by 1.6, 2.4 and 2.9 folds, respectively (Jung *et al.*, 2011). The increased cortisol levels may be explained by placental secretions of estrogen stimulating maternal cortisol production and mitigating maternal negative feedback (Lindsay & Nieman, 2005; Duthie & Reynolds, 2013) and/or by placental production of corticotropin-releasing hormone (CRH) into the maternal circulation in mid- and late gestation (Duthie & Reynolds, 2013). Maternal cortisol promotes placental CRH production, which in turn promotes maternal HPA axis activity thus acting as a feed-forward positive mechanism.

However, in mouse pregnancy, corticosterone levels do not increase as much as humans near term although there are still increases. In pregnant control mice, corticosterone levels were not significantly different at E11 and E17 despite slightly higher levels at E17 (Jafari *et al.*, 2017). Other studies showed an increase in corticosterone levels at E19 compared to E16 in control unstressed mice (Vaughan *et al.*, 2012). Unstressed pregnant mice had higher corticosterone levels with peak levels at E16 being 60 times higher than non-pregnant mice (Barlow *et al.*, 1973). The levels then dropped after E16 until delivery at E19 (Barlow *et al.*, 1973). The decrease in plasma corticosterone is concordant with reduced activity of placental hydroxysteroid 11-beta dehydrogenase (HSD11B2; discussed in section below), thus allowing more passage of corticosterone to the fetus. Despite the natural increase in maternal circulating cortisol levels, the placenta is efficient at inactivating cortisol by HSD11B2 activity allowing only 10-20% of maternal cortisol to cross to the fetus (Gitau *et al.*, 1998; Ellman *et al.*, 2008).

## Placental HSD11B2 Activity

The two genes in the placenta modulating the flux of the stress hormone to the fetus are HSD11B2 (Benediktsson *et al.*, 1997) and HSD11B1 (Chapman *et al.*, 2013). HSD11B2 is a gene that inactivates cortisol or corticosterone into inactive form cortisone or 11-dehydrocorticosteroone in humans and mice, respectively (Chapman *et al.*, 2013). HSD11B1 is a gene responsible for activating cortisone and 11-dehydrocorticosterone to active form cortisol or corticosterone in humans and mice, respectively (Chapman *et al.*, 2013).

Despite the natural increase in maternal circulating cortisol levels, the placenta is efficient at inactivating cortisol by hydroxysteroid 11-beta dehydrogenase 2 (Hsd11B2) activity allowing only 10-20% of maternal cortisol to cross to the fetus in humans and mice (Montano *et al.*, 1993; Gitau *et al.*, 1998; Ellman *et al.*, 2008; Duthie & Reynolds, 2013). In humans, excessive levels of cortisol can reach the fetus bypassing placental inactivation (Duthie & Reynolds, 2013). Placental HSD11B2 activity in human placenta increases with pregnancy progression and with the natural increases of maternal cortisol, but placental HSD11B2 activity decreases in the last few weeks of the third trimester to allow more cortisol to pass through the placenta to the fetus (Austin & Leader, 2000; Davis & Sandman, 2010). This ensures that the fetus receives adequate cortisol levels to promote lung maturation and ensure preparation for the stressful period of delivery (Austin & Leader, 2000).

In mice, placental HSD11B2 seems controversial. Mouse HSD11B2 gene was highly expressed in the labyrinthine zone at E14.5 then decreased by E16.5 thus allowing a higher corticosterone flux to the fetus later in gestation and near term (Brown *et al.*, 1996; Malassine *et al.*, 2003). Supporting this finding, placental HSD11B2 levels were increasing gradually in unstressed mouse placenta until E13.5, then decreased until E15.5 (Wieczorek *et al.*, 2019). Conversely, HSD11B1, gene responsible for activating 11-dehydrocorticosterone to its active form corticosterone, expression increased from E13.5 until the end of gestation thus allowing more active corticosterone to be delivered to the fetus (Wieczorek *et al.*, 2019). Another study showed unaltered placental HSD11B2 and HSD11B1 expression in unstressed pregnant mice at E16 and E19 (Vaughan *et al.*, 2012).

## Fetal HPA Axis Development

The human fetal hypothalamic-pituitary axis activity is detected as early as 8-12 weeks of gestation (Ng, 2000) and is fully developed in the second trimester of pregnancy (Moisiadis & Matthews, 2014). In early pregnancy, fetal cortisol is thought to primarily be attained from maternal cortisol, as the fetus is believed to sufficiently produce cortisol at 22 weeks of gestation (Buss *et al.*, 2012). Given the critical developmental window by which fetal organs and HPA axis are developing, it is possible that increased maternal cortisol levels in early pregnancy compared to late pregnancy may have more deleterious effects on fetal development (Barker, 2007; Braun *et al.*, 2013). In mice, the offspring HPA develops postnatal in two phases. On postnatal day (PND) 1 through 12, the mouse HPA is considered hypo-responsive, and after PND 12 the HPA system matures (Schmidt *et al.*, 2003).

## Glucocorticoid Treatments in Pregnancy

In addition to the naturally increasing cortisol levels in pregnancy, glucocorticoid treatments are further prescribed during pregnancy for multiple reasons. A single course of synthetic corticosteroid treatment is prescribed to women who are at risk of delivering premature babies. The treatment is proven to increase offspring chances of survival post-delivery (Doyle *et al.*, 2000; Baisden *et al.*, 2007). Glucocorticoid treatments are prescribed as they enhance fetal growth, specifically fetal lung maturation to prevent respiratory distress syndrome (RDS), and aid in overall embryogenesis to prevent perinatal death due to hemorrhages, heart failure and other underlying causes associated with preterm birth (Lunghi *et al.*, 2010; Singh *et al.*, 2012). Specifically, betamethasone, dexamethasone, prednisolone, corticosteroids, or cortisol are prescribed to women who have acute asthma or asthma, hyperemesis gravidarum, depression, stress, or are at risk of delivering preterm babies (Singh *et al.*, 2012). The use of corticosteroids is widespread. In a Danish cohort study encompassing all births in Denmark from 1996-2008, about 20% of women reported use of corticosteroids from 4 weeks prior to delivery until delivery (Hviid & Mølgaard-Nielsen, 2011). In an American cohort study including 152,531 pregnancies between 1996-2000, 3.5% of pregnant women who had a documented diagnosis associated with preterm birth used corticosteroids, while 1.7% of pregnant women who did not have a documented diagnosis used corticosteroid (Andrade *et al.*, 2004). Despite the placenta’s function to protect the fetus from excess maternal corticosteroid, synthetic corticosteroids used in preterm treatments can readily cross the placenta bypassing inactivation by HSD11B2(Cuffe *et al.*, 2011; Singh *et al.*, 2012).

## Effects of Glucocorticoid Exposure on Placenta and Fetus

### Fetal and Placental Development

Pregnant rats treated with dexamethasone at E13 until E20 showed reduced placental and fetal weights, reduced placental labyrinth layer at E20 (Ain *et al.*, 2005). Despite the evident placental and fetal growth restriction, dexamethasone did not affect litter size (Ain *et al.*, 2005). Rats exposed to triamcinolone once at E16 had reduced placental and fetal weights (Hahn *et al.*, 1999). Mice exposed to a sound stressor on E10.5, E12.5, and E14.5 showed reduced fetal body weight and had growth restriction that was more evident in female fetuses (Wieczorek *et al.*, 2019). Pregnant mice exposed to dexamethasone on E15, E16, and E17 had reduced placental and fetal weights and trophoblast hypertrophy in the junctional and labyrinth zones (Baisden *et al.*, 2007).

### Placental Protein Expression

Pregnant rats treated with dexamethasone at E13 until E20 had reduced *Igf2* gene expression of IGFII, a growth factor that modulates placental and fetal growth (Constância *et al.*, 2002), and reduced expression of the active form of AKT, upstream positive mTORC1 regulator, in the junctional area at E20 (Ain *et al.*, 2005). Conversely, pregnant mice exposed to dexamethasone on E15, E16, and E17 showed unaltered placental IGFII expression (Baisden *et al.*, 2007).

mTORC1 is a crucial nutrient sensor that plays a role in integrating maternal and fetal signals to ensure adequate nutrient transport to the fetus through the placenta (Wen *et al.*, 2005; Roos *et al.*, 2007; Mparmpakas *et al.*, 2012; Jansson & Powell, 2013). Fewer studies have assessed the relationship between maternal glucocorticoid exposure and placental mTORC1 activity in rodents or humans, but its activity is reduced in intrauterine growth restriction (Roos *et al.*, 2007). Mice exposed to corticosterone at E14-E19 had reduced mTORC1 activity at E19 evident by the reduced p4E-BP1 and pS6K expression, downstream targets of mTORC1, and increased REDD1 expression which is an inhibitor of mTORC1 signaling (Vaughan *et al.*, 2015). Mice exposed to corticosterone at E11-E16 had reduced pAKT levels but unchanged total AKT levels and unchanged REDD1 expression, suggesting a minimal effect on mTORC1 function at E16 (Vaughan *et al.*, 2015). Additionally, dexamethasone exposure reduces rat placental amino acid transport, suggesting a negative effect of dexamethasone on the nutrient sensing pathway of mTORC1 (Audette *et al.*, 2011; Jansson & Powell, 2013).

GDF15 is produced in the placenta, and changes are associated with a variety of complications including miscarriage, nausea and hypertension (Tong *et al.*, 2004; Chen *et al.*, 2016; Petry *et al.*, 2018). There are no studies assessing placental GDF15 activity in response to glucocorticoid or psychological stress exposures. Placental GDF15 levels are positively correlated with maternal and fetal levels, suggesting that the placenta is the primary source of this hormone during pregnancy (Sugulle *et al.*, 2009).

Placental glucocorticoid receptor (*Nr3c1*) was unchanged with corticosterone exposure in mice at E11-E16 or at E14-E19 (Vaughan *et al.*, 2012, 2015). However, mice treated with corticosterone at E12.5-E15, *Nr3c1* placental expression increased at E14.5 in male placentas (Cuffe *et al.*, 2012). Placentas collected at E17.5, after the exposure also showed a male-specific placental increase in *Nr3c1* expression (Cuffe *et al.*, 2012).

Glucocorticoid receptor unchanged with stress in vaughan paper, GR changed and increased in stressed animals especially female placentas.

### Placental Glucose and Amino Acid Transporters

Mice exposed to corticosterone at E11-E16 had increased placental GLUT1 and GLUT2 expression at E16, while mice exposed at E16-E19 showed reduced fetal radioactive glucose acquisition (Vaughan *et al.*, 2015). Triamcinolone exposure in pregnant rats at E16 reduced placental glucose transport via decreasing GLUT1 expression (Hahn *et al.*, 1999). Overall, stress in rodents seems to cause reductions in placental glucose transporter expression (Braun *et al.*, 2013). Midgestation administration of dexamethasone in mice at E13.5 and E14.5 caused reduced placental System A amino acid transporter expression near term at E18.5 (Audette *et al.*, 2011).

## Effect of In Utero Glucocorticoid Exposure on Offspring

Studies investigating the effect of antenatal glucocorticoid treatment on fetal hypothalamic-pituitary-adrenal axis show blunted offspring HPA activity (Waffarn & Davis, 2012). Women with higher corticotropin-releasing hormone at midgestation, were 7.5 fold more likely to deliver preterm (Inder *et al.*, 2001). Despite popular use of corticosteroids, offspring side effects have been understudied and largely unknown. Some studies have shown increased blood pressure in children, increased risk of preeclampsia, impaired mental development in infants, increased infant cortisol, reduced fetal weight, and other symptoms associated with timing, dosage and type of corticosteroid treatment during pregnancy (Singh *et al.*, 2012). The effects of corticosteroid use further manifest in childhood where maternal third trimester cortisol levels were shown to influence childhood adiposity (Entringer *et al.*, 2016). In mice, studies have shown reduced placental weights after a short period preterm exposure to dexamethasone and potential fetal growth restriction (Cuffe *et al.*, 2011). The mechanisms by which maternal corticosteroids influence fetal health and placental function remain understudied with conflicting results (Kemp *et al.*, 2015). The effects of prenatal glucocorticoid exposure remain controversial, and the exact mechanisms by which they are manifested remain poorly understood (Bandoli *et al.*, 2017). Add offspring outcomes summarized by Duthie et al 2013

Reduced cognitive function in humans as seen in LeWinn

Third trimester maternal corti associated with Lower offspring IQ at 7 years of age. Infant negative reactivity and mental and motor delays seen with high maternal cort levels (refs 8 and 9) (paper by LeWinn K 2009)

# Experimental Design

To determine how glucocorticoid exposure affects placental function, we will obtain n=X females and males /per group 8 week-old C57BL/6 virgin mice from Jackson laboratory. Mice will be given two weeks to acclimatize with *ad libitum* access to normal chow diet and water. After acclimatization, mice will then be single-housed and randomized into one of the following groups, to assess placental morphology (at E14.5) and effects on offspring (at delivery). Pending these results other groups may be evaluated as well:

Cohort A of groups treated one week prior to conception:

1. Water*E-1-14.5:* control group on water one week prior to conception and until midgestation at embryonic day 14.5
2. Dex*E-1-14.5*: experimental group exposed to Dexamethasone in drinking water a week prior to conception and until midgestation at embryonic day 14.5
3. Water*E-1-21.5*: control group on water one week prior to conception and until delivery
4. Dex*E-1-21.5*: experimental group exposed to Dexamethasone in drinking water a week prior to conception and until delivery

Cohort B of groups treated at conception:

1. Water*E0.5-14.5*: control group on water starting at conception and until midgestation at embryonic day 14.5
2. Dex*E0.5-14.5*: experimental group exposed to Dexamethasone in drinking water starting at conception and until midgestation at embryonic day 14.5
3. Water*E0.5-21.*5: control group on water starting at conception and until delivery
4. Dex*E0.5-21.5*: experimental group exposed to Dexamethasone in drinking water starting at conception and until delivery

All groups will have *ad libitum* access to normal chow diet and water or Dexamethasone depending on treatment arm. Experimental groups will receive 1mg/kg/day dexamethasone in their drinking water with *ad libitum* access. For groups of Cohort A (receiving Dex or water a week prior to conception), female mice will be mated with age-matched male mice after one week of treatment. A copulatory plug will be checked daily to identify E0.5 day. For groups of Cohort B (receiving Dex or Water at conception), mice will be mated with age-matched males immediately after acclimatization while having *ad libitum* access to water. We will check for the presence of copulatory plugs daily to determine treatment initiation. Once a copulatory plug is identified, mice will be placed on Dexamethasone or Water based on their assigned group.

For Water and Dex groups that will complete their pregnancy and deliver their pups, they will have *ad libitum* access to normal chow diet and water during lactation. Males will be removed from the cage after a copulatory plug is detected to minimize male exposure to treatment and to better detect potential miscarriages. Dams from all groups will undergo body mass assessment three times weekly using magnetic resonance to assess body composition. Water and food intake will be recorded weekly. For groups that will be sacrificed prior to delivery (E14.5), placental and fetal extractions will occur midgestation at E14.5. Briefly, the dams will be anesthetized using a vaporizer during the placental and fetal extraction. Litter size will be determined per dam and will account for potential resorbed placentas. Placental and fetal weights will be collected. Placentas will be snap frozen in liquid nitrogen while some will be embedded in paraffin for histology. Molecular studies on placental samples will be conducted to determine protein expression.

For the groups that will deliver their pups at E21.5, survival and birth rates will be noted. Pups will be sexed and culled to 2 at PND2.5. The offspring will be weighed at PND0.5, PND7.5, 14.5, 16.5, and at 21.5. Pups will be weaned based on sex and treatment group. The weaned pups will have *ad libitum* access to normal chow diet and water. Their water and food intake will be assessed weekly. They will further undergo body composition analysis by echoMRI at weaning and weekly thereafter. To assess offspring HPA axis activity, retro-orbital bleeds will be done to collect blood followed by sacrifice and tissue collection of fat pads 3 days later. Offspring fat pads (gWAT and iWAT) will be collected and weighed to determine adiposity.

To determine if the effects of dexamethasone exposure on the placenta and the fetus can be rescued using a placenta-specific glucocorticoid receptor (GR) knockout (KO) model. To isolate placental from fetal and maternal glucocorticoid signaling, our knockout model will ablate GR conditionally in the placenta, to my knowledge the first time such a model has been generated. To generate the GR-KO, we will use the Cre-loxP recombination technology. We will leverage the fact that placental tissue is primarily fetal derived, so the genotype of the offspring will dictate the genotype of most of the placenta. First, female mice with homozygously flanked exon 2 of *Nr3c1* will be crossed with a male having placental driver *Cyp19a1-CreTg/+* (Wieczorek *et al.*, 2019). This cross will be generated as depicted in Figure 1. Briefly, the parental strains for this experiment will be male *Nr3c1* +/+;*Cyp19a1-Cre* Tg/+crossed *with* female *Nr3c1* fl/fl ; *Cyp19a1-Cre* +/+. This cross will yield a combination of knockout *Tsc1* fl/fl;*Cyp19a1-CreTg/+* , conditionally heterozygous *Nr3c1* fl/+;*Cyp19a1-CreTg/+* , and wild-type *Nr3c1* fl/fl ; *Cyp19a1-Cre* +/+ (no Cre transgene) at an expected ratio of 1:2:5 with the knockout and wild-type animals only being used for further breeding. The offspring generated from the next generation will all have the floxed allele with the Cre (KO) or without (WT). The offspring generated from the last breed will either be WT with intact placentas or knockout with placental KO and a phenotypically WT embryo.

The dams with GR-KO will be treated with Dexamethasone similar to the previous groups in cohorts A and B to determine placental, embryonic and offspring function and growth.

### Figure 1: Diagram representing the experimental design and respective timeline

Using the above-mentioned model,we will measure placental expression of HSD11B2 mRNA using RT-qPCR. Placental cell lines described above will also be used to quantify HSD11B2 expression using western blotting and RT-qPCR to measure mRNA expression. HSD11B2 activity will be measured by determining the conversion of cortisol to cortisone as mentioned by Zhu et al. (Zhu *et al.*, 2016). We will further determine if there is a correlation between maternal, fetal and placental cortisol levels. In normal pregnancies, fetal cortisol levels are supposed to be less than the maternal serum cortisol, as the placenta efficiently inactivates 80-90% of the maternal cortisol (Duthie & Reynolds, 2013). Increased fetal levels in response to increased maternal cortisol following dexamethasone treatment will highlight the placenta’s failed ability to adequately inactivate the surplus of maternal cortisol and will further corroborate our previous results that may indicate altered placental HSD11B2 function and expression. We will further assess the exact flux of maternal cortisol by using radiolabeled corticosterone as per Li et al. (Li *et al.*, 2015) and further quantify placental and fetal expressions using the HSD11B2 assay kit.

**Aim 2.3: Is placental endocrine function altered with increased maternal cortisol levels?** As previously mentioned, multiple doses of dexamethasone treatment reduced placental and fetal weights (Hahn *et al.*, 1999; Baisden *et al.*, 2007), and small-for-gestational-age babies show reduced PGH levels (Männik *et al.*, 2010). As our preliminary data suggests that the pups of dexamethasone-treated dams are inviable, placental growth hormone and fetal GH secretions can be altered in a dose-dependent manner. We will conduct the previously mentioned studies in Aim 1.2 but using the cortisol exposure.

# Methods

## Dexamethasone Exposure

Water-soluble dexamethasone (Sigma) will be prepared at a concentration of 53 mg/L, which our previous work shown results in a dose of approximately 1 mg/kg/day in non-pregnant mice.

If the dam is single housed or with nursing pups:

(the new added total water/dexamethasone- the last measurement’s water/dexamethasone) / # of days between measurements

If more than one adult mouse is in the cage (when the male is breeding in the same cage), food intake will be calculated as follows:

(the new added total water/dexamethasone - the last measurement’s water/dexamethasone) \* #of days between measurements / sum of days spent by each mouse in that cage between measurements

## Food Intake

Food will be weighed when the treatment starts and throughout the experiment. The weight of the dam’s food will be recorded three times weekly every Monday, Wednesday, and Friday. Food will also be weighed at delivery for the dam. Food will be topped off to ~400g weekly every Friday. Food intake will be calculated as:

If the dam is single housed or with nursing pups:

(the new added total food weight - the last measurement’s food weight) / # of days between measurements

If more than one adult mouse is in the cage (when the male is breeding in the same cage, or when weaned offspring are housed together), food intake will be calculated as follows:

(the new added total food weight - the last measurement’s food weight) \* #of days between measurements / sum of days spent by each mouse in that cage between measurements

## Body Composition

Mice will be weighed by using dynamic weighing to capture accurate weight using a digital scale. The weight will be recorded along with the mouse ear tag number. The mouse will be gently placed in the MRI tube with the plunger slightly compressing along the mouse body to ensure it cannot move during the measurement. Fat, lean, free water and total water mass (g) will be recorded for each animal.

## Sacrifice and Tissue and Blood Collection

Dams of groups E14.5 and E17.5 will be sacrificed on the respective dates based on their treatment group. Dams will be anesthetized using an isoflurane vaporizer. Toe punches will be performed to ensure that the mouse is under anesthesia. A midline incision of the skin from the rectum to the diaphragm will be made while the mouse is still alive and anesthetized using the vaporizer. The uterine horn will be exposed and placental and fetal excision will begin in order along the uterine horn starting from the side (closer to the ovaries). The amniotic sac for each pup will be ruptured using fine scissors. The placenta will be detached from the maternal tissue and the umbilical cord then weighed and immediately snap frozen or cryopreserved and in paraffin for future molecular and histological studies. Fetuses will be weighed after removal from the amniotic sac then they will be immediately sacrificed by decapitation using surgical scissors. After the complete extraction of tissue, dams will be euthanized while under anesthesia by cardiac exsanguination.

Offspring of dams that will be allowed to deliver and nurse (groups of E21.5) will be dissected at 6 weeks of age. Offspring will be first anesthetized using isoflurane drop jar. Retro-orbital bleeds will be performed while the offspring are under anesthesia using capillaries injected into the peritoneal area of the eye. Blood will be collected in 1.5ml tubes then stored in ice. Blood will then be centrifuged for 20 minutes at 5000rpm to collect the plasma. Plasma will be stored at -80C for later corticosteroid analysis. Immediately after the retro-orbital bleed, offspring will be sacrificed using isoflurane drop jar. Cervical dislocation will be performed as a secondary measure to confirm euthanasia. We will dissect the offspring fat pads by a midline incision of the skin from the rectum to the diaphragm, extract inguinal and gonadal white adipose tissue. Inguinal white adipose tissue (iWAT) will be collected from the mouse right side first by pulling the peritoneum away from the skin. Inguinal fat will be carefully extracted, weighed then snap frozen in liquid nitrogen for further molecular studies. Right gonadal white fat tissue (gWAT) will be collected next by scraping the fat along the gonads (ovaries or testis), weighed, and then snap frozen in liquid nitrogen in 2ml tubes. The fat pads will be stored at a temperature of -80C.

## Real time qPCR

Using the placental tissues collected from the dams, we will assess RNA expression of macronutrient transporters and endocrine hormones. RNA samples will be prepared from the mouse tissues using the PureLink RNA Mini Kit. Briefly, tissues will be cut to ~50mg samples that will be homogenized and treated to collect the RNA. The RNA will be quantified using a nanodrop. Later, first strand cDNA will be synthesized from the purified RNA samples using High Capacity cDNA Reverse Transcription Kit. The cDNA samples will be diluted and added to the clear 384 well plate in triplicates. A Primer/SYBR Green mix will be prepared for each primer. Briefly, we will use sequence-specific primers to amplify the genes ACC1, SREBP1c, ACLY and FASN using primer pairs (forward and reverse). This will allow us to assess the overall endocrine and transport function of the placentas of Dex- and Water-treated dams. Real-time PCR was performed for *Sry* (sex-determining region Y, Mm00441712\_s1).

## Western Blotting

Using the placentas collected at E14.5 and E17.5, mTORC1 activity will be assessed. Briefly, a portion of the sample will be boiled and loaded into different wells with a ladder control. Proteins will transfer to nitrocellulose overnight. The matrix will be stained for total protein using Revert total protein and scanned by LiCOR to normalize against total protein. Samples will be incubated with the primary then the secondary antibodies. Briefly, antibodies against total and phosphorylated mTORC1 targets (S6K, 4EBP1, S6) and regulators (Akt, IRS and TSC2) will be used.

## Histology

Placentas collected from control and experimental at E14.5 and E17.5 will be embedded in paraffin and stained at the Rogel Cancer Center’s Tissue and Molecular Pathology. Slides will be blindly assessed for decidual, junctional and labyrinthine thickness. CD68+ cells will be assessed to determine

# Expected Results

## **Aim 1.1:**How does maternal GC exposure affect placental, fetal IUGR, and offspring survival?

## **Aim 1.2:** How does maternal GC exposure affect placental endocrine function (specific hormones: lactogen,IGF2 , GDF15…) look at qPCR mRNA expression – will not use ELISA yet since ELISA is expensive and we may not see a difference in qPCR/mRNA expression initially

Maternal GDF15 levels were lower in mothers who had intrauterine growth restriction

The experiments conducted above will determine the placental efficiency at inactivating maternal cortisol and will shed light on potential side effects of the treatment dose and timing. We expect that placental expression of HSD11B2 will be upregulated in a time-dependent manner by which an earlier and more prolonged dexamethasone exposure will manifest this placental change in gene expression. Despite the increased placental expression of HSD11B2, we expect the placenta to fail to overcompensate for the excess maternal cortisol thus allowing the increased passage of cortisol to the fetus. We expect the fetal cortisol levels to increase and correlate with maternal levels. We expect that fetal weight will be reduced and thus placental growth hormone secretion will be decreased in a corticosteroid dose-dependent manner.

## **Aim 1.3:** Is placental mTORC1 signaling altered after maternal GC exposure?  Western blot for 4EBP, S6, PS6, AKT

## **Aim 1.4:** How does maternal GC exposure affect the expression of placental nutrient transporters?

The experiments conducted in this aim will examine the effect of dexamethasone treatment on placental transport and transporters. We predict that placental glucose transporters (see Table 1) will have increased expression (Kipmen-Korgun *et al.*, 2012) suggesting that glucose flux across the placenta to the fetus will be increased . We further expect increased placental lipid transporters and flux but reduced amino acid transporters and flux. We predict maternal corticosteroid treatment will alter placental glucose, lipid, and amino acid transporters and nutrient flux and will influence fetal and placental viability in a time-dependent manner. Earlier exposure at E5.5 and E12.5 will have the most prominent effect on placental transport and may lead to reduced nutrient flux overall, while later exposure at E17.5 will only increase glucose and lipid transporters and flux.

## **Aim 1.5:** Is offspring metabolic health survival, wt, mri, if they survive after Dex exposure during gestation only (no 1 week preconception)

## **Aim 1.6:**Does a placental GR-KO model rescue the placental and fetal effects of GC exposure?

Based on the results of Aims 1.1-1.5 we will have identified critical glucocorticoid-induced changes in placental gene expression, signaling , placental and fetal size and offspring health. Those models however do not separate effects of dexamethasone on the mother from those on the placenta. To separate these we will use placental GR knockouts and repeat these studies. We expect that placental-derived glucocorticoid actions will be blocked by GR knockout in the placenta, but maternal glucocorticoid actions will be retained.

# Potential Pitfalls and alternate Approaches (Aims 1.1-1.6)

It is possible that mice in groups E-1Water or Dex will not conceive immediately at mating thus causing Dex exposure to be more than a week. We will have to eliminate all dams that will be exposed to Dex fro more than a week and the half prior to conception, thus we may need more mice ordered. It is also possible for both groups that the mice may have spontaneous abortions and resorptions due to induced stress. Thus we will need to try this experiment with more mice.

If both treatments prove to reduce

**Potential Pitfalls and Alternate Approaches:** It might be that we are unable todetermine *in vitro* uptake since cortisol and dexamethasone treatments may alter placental cell differentiation and function, in which case we will resort to looking at different cell lines that may be less influenced in regards to differentiation by cortisol and dexamethasone. Another potential problem that may arise is fetal resorption with early dexamethasone exposures. Our pilot study has shown that some dams on dexamethasone who were pregnant did not deliver pups which highlights the possibility of fetal death. In this case, we will have to resort to testing later dexamethasone exposures at 1-6 days prior to conception, instead of starting one full week prior to conception, to determine which treatment leads to the highest birth rate and least fetal resorption rate. We may also need to limit our exposure time to better mimic the human one-course treatment of corticosteroids and to ensure the viability of the pups.

**Potential Pitfalls and Alternate Approaches:**If our hypothesis is correct and fetal cortisol is upregulated, then the change in fetal and placental weights may be due to the cortisol and not the nutrient acquisition. Using results from Aim 2.1, we need to distinguish the effects of fetal nutrient uptake and fetal cortisol uptake to prevent misinterpreting our findings. Although both effects synergistically will yield the final phenotype, we need to be diligent about interpreting our data keeping the full results in mind.Corticosteroid treatment may cause perinatal death and thus placental collection to determine placental function may be difficult. If this is the case, we will collect the placentas at an earlier time point prior to E18.5 when perinatal death may have already occurred. Finally, quantification of radiolabeled corticosterone is uncommon, and so we may need to develop our own protocol using the available references.

Our preliminary data show that dams on dexamethasone (1 mg/kg/day) one week prior to conception and throughout pregnancy are fertile but their offspring are inviable and die either at postnatal day one or prior to delivery suggesting that the placental transport of nutrient, placental endocrine function, or both were impaired or that the fetuses were small for gestational age and thus inviable. Dams on dexamethasone do not gain as much weight during pregnancy compared to the control dams on water, and they mainly lose lean mass consistent with other studies on chronic dexamethasone treatment, while maintaining a constant fat mass. This further directs our future investigation to determine the underlying mechanisms altering placental function and leading to perinatal fetal death during dexamethasone-treated pregnancy compared to a normal pregnancy. This aim will help determine the effect of corticosteroids on placental nutrient transport and placental hormone-secreting capacity in a stress-induced environment.

Ain R, Canham LN & Soares MJ (2005). Dexamethasone-induced intrauterine growth restriction impacts the placental prolactin family, insulin-like growth factor-II and the Akt signaling pathway. *J Endocrinol* **185,** 253–263.

Andrade SE, Gurwitz JH, Davis RL, Chan KA, Finkelstein JA, Fortman K, Mcphillips H, Raebel MA, Roblin D, Smith DH, Yood MU, Morse AN & Platt R (2004). Prescription drug use in pregnancy. *Am J Obstet Gynecol* **191,** 398–407.

Audette MC, Challis JRG, Jones RL, Sibley CP & Matthews SG (2011). Antenatal Dexamethasone Treatment in Midgestation Reduces System A-Mediated Transport in the Late-Gestation Murine Placenta. ; DOI: 10.1210/en.2011-0104.

Austin MP & Leader L (2000). Maternal stress and obstetric and infant outcomes: epidemiological findings and neuroendocrine mechanisms. *Aust N Z J Obstet Gynaecol* **40,** 331–337.

Baisden B, Sonne S, Joshi RM, Ganapathy V & Shekhawat PS (2007). Antenatal dexamethasone treatment leads to changes in gene expression in a murine late placenta. *Placenta* **28,** 1082–1090.

Bandoli G, Palmsten K, Forbess Smith CJ & Chambers CD (2017). A Review of Systemic Corticosteroid Use in Pregnancy and the Risk of Select Pregnancy and Birth Outcomes. *Rheum Dis Clin North Am* **43,** 489–502.

Barker DJP (2007). The origins of the developmental origins theory. *J Intern Med* **261,** 412–417.

Barlow SM, Morrison PJ & Sullivan FM (1973). Plasma corticosterone levels during pregnancy in the mouse. *Br J Pharmacol* **48,** 346P.

Bayliss RIS, Browne JCM, Round B & Steinbeck AW (1955). PLASMA-17-HYDROXYCORTICOSTEROIDS IN PREGNANCY. *Lancet* **265,** 62–64.

Benediktsson R, Calder AA, Edwards CR & Seckl JR (1997). Placental 11 beta-hydroxysteroid dehydrogenase: a key regulator of fetal glucocorticoid exposure. *Clin Endocrinol (Oxf)* **46,** 161–166.

Braun T, Challis JR, Newnham JP & Sloboda DM (2013). Early-Life Glucocorticoid Exposure: The Hypothalamic-Pituitary-Adrenal Axis, Placental Function, and Long-term Disease Risk. *Endocr Rev* **34,** 885–916.

Brett K, Ferraro Z, Yockell-Lelievre J, Gruslin A & Adamo K (2014). Maternal–Fetal Nutrient Transport in Pregnancy Pathologies: The Role of the Placenta. *Int J Mol Sci* **15,** 16153–16185.

Brown RW, Diaz R, Robson AC, Kotelevtsev Y V, Mullins JJ, Kaufman MH & Seckl JR (1996). The ontogeny of 11 beta-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor gene expression reveal intricate control of glucocorticoid action in development. *Endocrinology* **137,** 794–797.

Buss C, Davis EP, Shahbaba B, Pruessner JC, Head K & Sandman CA (2012). Maternal cortisol over the course of pregnancy and subsequent child amygdala and hippocampus volumes and affective problems. *Proc Natl Acad Sci U S A* **109,** E1312-9.

Carr BR, Parker CR, Madden JD, MacDonald PC & Porter JC (1981). Maternal plasma adrenocorticotropin and cortisol relationships throughout human pregnancy. *Am J Obstet Gynecol* **139,** 416–422.

Chapman K, Holmes M & Seckl J (2013). 11β-hydroxysteroid dehydrogenases: intracellular gate-keepers of tissue glucocorticoid action. *Physiol Rev* **93,** 1139–1206.

Chen Q, Wang Y, Zhao M, Hyett J, da Silva Costa F & Nie G (2016). Serum levels of GDF15 are reduced in preeclampsia and the reduction is more profound in late-onset than early-onset cases. *Cytokine* **83,** 226–230.

Cuffe JSM, Dickinson H, Simmons DG & Moritz KM (2011). Sex specific changes in placental growth and MAPK following short term maternal dexamethasone exposure in the mouse. *Placenta* **32,** 981–989.

Davis EP & Sandman CA (2010). The timing of prenatal exposure to maternal cortisol and psychosocial stress is associated with human infant cognitive development. *Child Dev* **81,** 131–148.

Doyle LW, Ford GW, Rickards AL, Kelly EA, Davis NM, Callanan C & Olinsky A (2000). Antenatal corticosteroids and outcome at 14 years of age in children with birth weight less than 1501 grams. *Pediatrics* **106,** E2.

Duthie L & Reynolds RM (2013). Changes in the maternal hypothalamic-pituitary-adrenal axis in pregnancy and postpartum: influences on maternal and fetal outcomes. *Neuroendocrinology* **98,** 106–115.

Ellman LM, Schetter CD, Hobel CJ, Chicz-Demet A, Glynn LM & Sandman CA (2008). Timing of fetal exposure to stress hormones: effects on newborn physical and neuromuscular maturation. *Dev Psychobiol* **50,** 232–241.

Entringer S, Buss C, Rasmussen JM, Lindsay K, Gillen DL, Cooper DM & Wadhwa PD (2016). Maternal cortisol during pregnancy and infant adiposity: a prospective investigation. *J Clin Endocrinol Metab* **102,** jc.2016-3025.

Georgiades P, Ferguson-Smith AC & Burton GJ (2002). Comparative Developmental Anatomy of the Murine and Human Definitive Placentae. *Placenta* **23,** 3–19.

Gitau R, Cameron A, Fisk NM & Glover V (1998). Fetal exposure to maternal cortisol. *Lancet* **352,** 707–708.

Hahn T, Barth S, Graf R, Engelmann M, Beslagic D, Reul JMHM, Holsboer F, Dohr G & Desoye G (1999). Placental Glucose Transporter Expression Is Regulated by Glucocorticoids 1. *J Clin Endocrinol Metab* **84,** 1445–1452.

Hviid A & Mølgaard-Nielsen D (2011). Corticosteroid use during pregnancy and risk of orofacial clefts. *CMAJ* **183,** 796–804.

Inder WJ, Prickett TCR, Ellis MJ, Hull L, Reid R, Benny PS, Livesey JH & Donald RA (2001). The Utility of Plasma CRH as a Predictor of Preterm Delivery. *J Clin Endocrinol Metab* **86,** 5706–5710.

Jafari Z, Mehla J, Afrashteh N, Kolb BE & Mohajerani MH (2017). Corticosterone response to gestational stress and postpartum memory function in mice ed. Pawluski J. *PLoS One* **12,** e0180306.

Jansson T & Powell TL (2013). Role of placental nutrient sensing in developmental programming. *Clin Obstet Gynecol* **56,** 591–601.

Jung C, Ho JT, Torpy DJ, Rogers A, Doogue M, Lewis JG, Czajko RJ & Inder WJ (2011). A Longitudinal Study of Plasma and Urinary Cortisol in Pregnancy and Postpartum. *J Clin Endocrinol Metab* **96,** 1533–1540.

Kemp MW, Newnham JP, Challis JG, Jobe AH & Stock SJ (2015). The clinical use of corticosteroids in pregnancy. *Hum Reprod Update* **22,** dmv047.

Kipmen-Korgun D, Ozmen A, Unek G, Simsek M, Demir R & Korgun ET (2012). Triamcinolone up-regulates GLUT 1 and GLUT 3 expression in cultured human placental endothelial cells. *Cell Biochem Funct* **30,** 47–53.

Li L, Wu X, Guan H, Mao B, Wang H, Yuan X, Chu Y, Sun J & Ge R-S (2015). Zearalenone Inhibits Rat and Human 11 *β* -Hydroxysteroid Dehydrogenase Type 2. *Biomed Res Int* **2015,** 1–7.

Lindsay JR & Nieman LK (2005). The Hypothalamic-Pituitary-Adrenal Axis in Pregnancy: Challenges in Disease Detection and Treatment. *Endocr Rev* **26,** 775–799.

Lunghi L, Pavan B, Biondi C, Paolillo R, Valerio A, Vesce F & Patella A (2010). Use of Glucocorticoids in Pregnancy. *Curr Pharm Des* **16,** 3616–3637.

Malassine A, Frendo J-L & Evain-Brion D (2003). A comparison of placental development and endocrine functions between the human and mouse model. *Hum Reprod Update* **9,** 531–539.

Männik J, Vaas P, Rull K, Teesalu P, Rebane T & Laan M (2010). Differential expression profile of growth hormone/chorionic somatomammotropin genes in placenta of small- and large-for-gestational-age newborns. *J Clin Endocrinol Metab* **95,** 2433–2442.

Moisiadis VG & Matthews SG (2014). Glucocorticoids and fetal programming part 1: outcomes. *Nat Rev Endocrinol* **10,** 391–402.

Montano MM, Wang M-H & vom Saal FS (1993). Sex differences in plasma corticosterone in mouse fetuses are mediated by differential placental transport from the mother and eliminated by maternal adrenalectomy or stress. *Reproduction* **99,** 283–290.

Mparmpakas D, Zachariades E, Goumenou A, Gidron Y & Karteris E (2012). Placental DEPTOR as a stress sensor during pregnancy. *Clin Sci (Lond)* **122,** 349–359.

Napso T, Yong HEJ, Lopez-Tello J & Sferruzzi-Perri AN (2018). The Role of Placental Hormones in Mediating Maternal Adaptations to Support Pregnancy and Lactation. *Front Physiol* **9,** 1091.

Ng PC (2000). The fetal and neonatal hypothalamic-pituitary-adrenal axis. *Arch Dis Child Fetal Neonatal Ed* **82,** F250-4.

Petry CJ, Ong KK, Burling KA, Barker P, Goodburn SF, Perry JRB, Acerini CL, Hughes IA, Painter RC, Afink GB, Dunger DB & O’Rahilly S (2018). Associations of vomiting and antiemetic use in pregnancy with levels of circulating GDF15 early in the second trimester: A nested case-control study. *Wellcome open Res* **3,** 123.

Reynolds RM (2013). Glucocorticoid excess and the developmental origins of disease: Two decades of testing the hypothesis – 2012 Curt Richter Award Winner. *Psychoneuroendocrinology* **38,** 1–11.

Roos S, Jansson N, Palmberg I, Säljö K, Powell TL & Jansson T (2007). Mammalian target of rapamycin in the human placenta regulates leucine transport and is down-regulated in restricted fetal growth. *J Physiol* **582,** 449–459.

Schmidt M, Enthoven L, van der Mark M, Levine S, de Kloet ER & Oitzl MS (2003). The postnatal development of the hypothalamic–pituitary–adrenal axis in the mouse. *Int J Dev Neurosci* **21,** 125–132.

Singh RR, Cuffe JS & Moritz KM (2012). Short- and long-term effects of exposure to natural and synthetic glucocorticoids during development. *Clin Exp Pharmacol Physiol* **39,** 979–989.

Sugulle M, Dechend R, Herse F, Weedon-Fekjaer MS, Johnsen GM, Brosnihan KB, Anton L, Luft FC, Wollert KC, Kempf T & Staff AC (2009). Circulating and placental growth-differentiation factor 15 in preeclampsia and in pregnancy complicated by diabetes mellitus. *Hypertens (Dallas, Tex 1979)* **54,** 106–112.

Tong S, Marjono B, Brown DA, Mulvey S, Breit SN, Manuelpillai U & Wallace EM (2004). Serum concentrations of macrophage inhibitory cytokine 1 (MIC 1) as a predictor of miscarriage. *Lancet* **363,** 129–130.

Vaughan OR, Sferruzzi-Perri AN & Fowden AL (2012). Maternal corticosterone regulates nutrient allocation to fetal growth in mice. *J Physiol* **590,** 5529–5540.

Waffarn F & Davis EP (2012). Effects of antenatal corticosteroids on the hypothalamic-pituitary-adrenocortical axis of the fetus and newborn: experimental findings and clinical considerations. *Am J Obstet Gynecol* **207,** 446–454.

Wen HY, Abbasi S, Kellems RE & Xia Y (2005). mTOR: A placental growth signaling sensor. *Placenta* **26,** S63–S69.

Wieczorek A, Perani C V., Nixon M, Constancia M, Sandovici I, Zazara DE, Leone G, Zhang M-Z, Arck PC & Solano ME (2019). Sex-specific regulation of stress-induced fetal glucocorticoid surge by the mouse placenta. *Am J Physiol Metab* **317,** E109–E120.

Woods L, Perez-Garcia V & Hemberger M (2018). Regulation of Placental Development and Its Impact on Fetal Growth-New Insights From Mouse Models. *Front Endocrinol (Lausanne)* **9,** 570.

Zhu H, Zou C, Fan X, Xiong W, Tang L, Wu X & Tang C (2016). Up-regulation of 11β-Hydroxysteroid Dehydrogenase Type 2 Expression by Hedgehog Ligand Contributes to the Conversion of Cortisol Into Cortisone. *Endocrinology* **157,** 3529–3539.