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# Specific Aim 2

**Identify the relationship between glucocorticoid exposure and mammary function.** The specific mechanisms by which glucocorticoid exposure at preconception, during pregnancy, or during lactation affects mammary gland function remain unclear. Available studies have not thoroughly assessed the effects of timed glucocorticoid exposure on the development and function of mammary glands. Many of the studies are further conducted on cow models and much less data is available on rodent models or humans. For humans, there are no contraindications to taking glucocorticoids during lactation or pregnancy for a short period of time as necessary. The evidence regarding potential side effects is lacking and medical consensus states that the treatment benefits outweigh the harms. My hypothesis is that maternal glucocorticoid exposure will impair mammary gland development and reduce milk output and milk macronutrient composition ultimately leading to reduced offspring growth prior to weaning and impaired adult metabolic health. To test this, we will identify how a) dexamethasone exposure affects mammary gland size and development, b) how dexamethasone exposure affects milk output volume and carbohydrate, protein and fat composition, and c) the effect of the exposure on maternal and offspring health via assessing body composition and metabolic and glucose or insulin tolerance.

# Rationale and Background

Glucocorticoids are important for proper mammary gland development (Anderson & Turner, 1956). Primarily, prolactin is the main hormone that promotes the transcriptional activity of STAT5 and mediates mammogenesis- the development of the alveolar duct in preparation for lactation (Feng *et al.*, 1995; Yang & Friedl, 2015). Prolactin is also responsible for milk synthesis, and oxytocin promotes milk ejection by alveolar contractions (Anon, 2009). Dexamethasone was found to work in collaboration with prolactin to coactivate the prolactin/STAT5 and GC/GR pathways that drive milk production in a synchronized manner (Kobayashi *et al.*, 2016). Dexamethasone alone was not found to promote milk synthesis (Kobayashi *et al.*, 2016). In adrenalectomized rats, mammary gland size was reduced, suggesting the importance of glucocorticoids (Anderson & Turner, 1956). Upon injection of prednisone to adrenalectomized-ovariectomized rats, mammary gland development was then normalized to the size in ovariectomized rats (Anderson & Turner, 1956). In hypophysectomized-ovariectomized-adrenalectomized mice, cortisol acetate treatment improved mammary gland ductal branching (NANDI, 1958). Treatment with deoxycorticosterone acetate at lower doses improved ductal branching but caused mammary gland regression at higher doses (NANDI, 1958). Despite the need of both prolactin and glucocorticoids for normal development of mammary glands, the effects of glucocorticoids on lactation remain conflicting and scarce. Dexamethasone administration in lactating rats after a short and prolonged period of pup separation showed inhibition of suckling-induced prolactin release that later normalized (BARTHA *et al.*, 1991). This indicates a potential direct inhibitory effect of cortisol on pituitary prolactin production. In concordance with this, adrenalectomized and dexamethasone-treated male rodents had reductions in prolactin levels (BARTHA *et al.*, 1991). As a drop in glucocorticoid level is necessary to promote involution, exogenous glucocorticoid exposure after suckling cessation has been shown to prevent mammary gland involution and was shown to preserve alveolar structure and increase alveolar size in mice (Feng *et al.*, 1995; Li *et al.*, 1997). Lactating PND6 rats that received dexamethasone 2 hours before nursing had impaired suckling-response when pups were returned to the cage after a 12-hour separation (Vilela & Giusti-Paiva, 2011). In the dexamethasone group, oxytocin and prolactin levels were lower than the control groups that received saline. Furthermore, pups of dexamethasone-treated rats gained less weight after nursing for 15 minutes, indicating that milk volume was impaired (Vilela & Giusti-Paiva, 2011). In a case study, a lactating woman who received local corticosteroid injection reported cessation of milk production 30 hours post injection with a spontaneous resumption of lactation within another day (Babwah *et al.*, 2013). Furthermore, in preterm deliveries, maternal betamethasone treatment had a time-dependent effect on milk volume but not composition (Henderson *et al.*, 2007). Women who delivered within 0-2 days after the treatment had increased milk volume compared to women who delivered 3-9 days post-treatment. This indicates an immediate postpartum effect of glucocorticoids on mammary gland function.

Glucocorticoids can also have an impact on milk composition. Glucocorticoids reduce protein synthesis by inhibiting mTORC1 (Wang *et al.*, 2006; Wolff *et al.*, 2014)*.* Dexamethasone exposure in cows reduced milk output to its lowest after one day of treatment, then gradually increased afterward (Shamay *et al.*, 2000). Lactose levels in milk were unaltered, while milk protein and fat composition increased reaching a maximum after one day of treatment then gradually decreased to normal with a prolonged reduced concentration of whey protein (Shamay *et al.*, 2000). Conversely, adrenocorticotropin injection in lactating cows reduced milk yield and protein yield after injection (Varner & Johnson, 1983). Lactose is thought to be the main regulator of milk output (Kronfeld & Hartmann, 1973). Hence, the reduced milk macronutrient yield was suggested to be due to mammary gland’s reduced ability to utilize glucose for lactose synthesis after glucocorticoid treatment (Varner & Johnson, 1983).

During lactation, maternal glucocorticoids can cross to the offspring through milk by passive diffusion due to their lipid-like profile (Hollanders *et al.*, 2017). Cortisone is reportedly higher in milk than cortisol, and this is thought to be due to HSD11B2 activity in the mammary epithelial cells (Smith *et al.*, 1996; Hollanders *et al.*, 2017). Following the maternal diurnal rhythm, maternal plasma glucocorticoid levels are highest in the early morning at 7am, and milk cortisol concentrations were found to be associated with maternal plasma levels (Patacchioli *et al.*, 1992; van der Voorn *et al.*, 2016).

Currently, there are no contraindications to using glucocorticoids during lactation for asthma, allergies, irritable bowel syndrome and other symptoms. Nevertheless, lactating women are advised to breastfeed after 4 hours of treatment to minimize transfer of glucocorticoids to the newborn via milk[[1]](#footnote-1). Evidence regarding the effects of glucocorticoids on mammary gland development in preparation for lactogenesis II and during lactation remains very scarce (Anon, 2006).

It appears that the effects of glucocorticoids may be more essential at low doses during early pregnancy when the alveolar ducts are still developing with a negative effect at high doses. At midgestation, alveolar development seems to be almost complete as their capacity to produce milk seemed intact despite glucocorticoid treatment (Henderson *et al.*, 2009). Glucocorticoid exposure near preterm delivery had time-dependent effects on lactogenesis II initiation (Henderson *et al.*, 2007). Glucocorticoid exposure at weaning seemed to prolong lactogenesis and inhibit mammary gland apoptosis (Feng *et al.*, 1995). The mechanisms by which glucocorticoids mediate their effect on mammary gland development remain poorly understood. Furthermore, milk output and macronutrient composition after glucocorticoid exposure during pregnancy and/or lactation is insufficient in rodents and humans.

Regardless of popular maternal use of corticosteroids, their long-term effects on the offspring remain largely unknown. Children of mothers who used glucocorticoids during pregnancy had an altered stress response and altered neurodevelopment (Alexander *et al.*, 2012; Asztalos *et al.*, 2014). 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). Animal studies show that offspring of glucocorticoid-treated mothers are at higher risk for developing adult-onset diseases with hyperinsulinemia, hyperglycemia, increased blood pressure and impaired kidney function (Singh *et al.*, 2012). 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). In lactating rats, maternal dexamethasone exposure at a dose of 100ug/kg/day showed altered offspring lipid profile at adulthood with increased liver cholesterol, low-density lipoproteins, and triglycerides (Jeje & Raji, 2015). Liver high-density lipoprotein levels were reduced. The effect of this exposure on the offspring kidneys at 12 weeks of age, showed signs of necrosis and increased oxidative stress (Jeje *et al.*, 2016). There is a paucity of research on the mechanisms by which maternal corticosteroids influence offspring health.

**Aim 2.1:** Is mammary gland development altered after maternal GC exposure during gestation and/or lactation?

**Aim 2.2:** How does maternal time-dependent GC exposure affect milk output and macronutrient composition?

**Aim 2.3:** Is maternal and offspring metabolic health altered after maternal GC exposure during gestation and/or lactation?

# Experimental Design

To assess the effects of glucocorticoids on milk production and milk volume, we will obtain NUMBER (n=X for each of the four groups) 8-week old virgin C57BL6/J female and male mice from The Jackson Laboratory. Mice will be given two weeks to acclimatize with *ad libitum* access to normal chow diet and water. After acclimatization, dams will be assigned to one group of the following: control PND0.5-16.5, experimental PND0.5-16.5, control PND0.5-21.5, or experimental PND0.5-21.5 (See Figure 1). After being assigned a group, the dams will be mated with age-matched males and *ad libitum* normal chow diet and water. Male breeders from both cohorts will be removed from the cage after 18 days of mating to avoid the occurrence of a second pregnancy, which may bias our results due to changes in the hormonal milieu.In all groups, the dams will undergo body mass assessment three times weekly throughout the experiment and immediately postpartum using magnetic resonance to assess body composition. We will measure dam food and water intake weekly. We will check for litters on a daily basis after 2.5 weeks of mating. The number of pups born will be recorded to determine maternal fertility and pup viability. After delivery (PND0.5), the dams will either receive water (controls) or dexamethasone (experimental) at a dose of 1mg/kg/day. Control and experimental groups of PND0.5-16.5 will be receiving water or dexamethasone throughout lactation and until PND16.5, where the dams and the pups will be sacrificed and maternal mammary glands will be weighed and collected for cryosectioning and molecular studies. Control and experimental groups of PND0.5-21.5 will be allowed to complete nursing fully then the dams will be sacrificed and the pups will be weaned.

Pups will be culled to four animals (2 females and 2 males) per litter at PND2.5. The offspring will be weighed at PND0.5, PND7.5, 14.5, 16.5 (for PND0.5-16.5 groups only), and at 21.5 (for PND0.5-21.5 groups only). The pups of groups PND0.5-21.5 will be weaned into new cages 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 reported weekly. They will further undergo body composition analysis by echoMRI at weaning and weekly thereafter. To determine glucose homeostasis, a glucose tolerance test (GTT) will be done at 6 weeks of age followed by sacrifice and tissue collection of fat pads 3 days later. Their fat pads (gWAT and iWAT) will be collected for molecular studies to assess mTORC1 protein expression via Western blotting.

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



# Methods

## Dexamethasone Exposure

To prepare the dexamethasone water, 0.053g of dexamethasone will be weighed using a metric scale. Dexamethasone will be added to 1L of distilled water and shaken well for proper mixing. Dexamethasone water will be measured using a graduated cylinder. Water and dexamethasone intake will be measured as:

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 using a digital balance scale when the breeding cages are set up for mating. 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

## MRI

Mice will be weighed by using dynamic weighing to capture accurate weight using a digital balance 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. Body fat, lean, free water and total water mass (g) will be recorded for each animal.

## Sacrifice and Tissue Collection

All animals will be sacrificed using anesthetic gas inhalation (5% isoflurane drop jar). Cervical dislocation will be done as a secondary method to confirm euthanasia. The mice will be pinned on a dissection board in a supine position. For dams from control and experimental groups PND0.5-16.5, we will dissect the dams by a midline incision of the skin from the rectum to the diaphragm , extract thoracic, abdominal and inguinal mammary glands. The peritoneum will be pulled apart from the skin. The lower glands will be excised carefully then weighed using a metric scale. A portion of the upper and lower glands will be embedded in paraffin for histology, while the rest will be collected in 2ml tubes and snap frozen in liquid nitrogen then alter stored at -80C for possible future molecular studies. Offspring of dams from control and experimental groups PND0.5-21.5 will be sacrificed similarly at 6 weeks of age. For the offspring, fat pad collection will be done. 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.

## Determining Milk Output Volume

At PND10.5, we will determine milk output volume for the control and experimental groups PND0.5-16.5. To determine milk volume, we will use the weigh-suckle-weigh technique (Boston *et al.*, 2001).Briefly, we will weigh the dam then determine the aggregate weight of the pups. The dam and pups will then be separated for two hours. During the two-hour separation, the pups will be placed in a new cage and will be kept warm using a heating pad. In the meantime, the dam will remain in its initial cage with *ad libitum* access to normal chow diet and water or dexamethasone-water based on its assigned group. After the two-hour separation period, the dam will be weighed again and the aggregate weight of the pups will be measured. The pups will then be returned to the dam’s cage and will be allowed to nurse for one hour. At the end of the nursing timepoint, the dam will be weighed and the aggregate weight of the pups will be determined. After the one-hour nursing period, milk volume will be determined as the weight change of the pups after nursing and after the 2-hour separation. The difference in the dam’s weight after nursing and after the 2-hour separation will help further ascertain the dam’s milk supply.

## Determining Milk Composition

On PND16.5, we will collect milk samples (~0.5ml) from the nursing dams in groups control and experimental PND0.5-16.5. Briefly, we will separate the dam and pups for 2 hours. The pups will be weighed and will undergo body composition assessment using echoMRI. Afterwards, the pups will be sacrificed using isoflurane and a secondary measure of cervical dislocation.. We will anesthetize the dam after two hours of separation by intraperitoneal injection of Ketamine (0.1275g/kg body weight). We will then perform an intraperitoneal injection of oxytocin into the forelimb (2U/dam) to induce milk production. The dam’s nipples will be manually squeezed to promote milk letdown, and the milk will be collected into a 1.5 ml tube via suction. After milking is complete, the dam will immediately be sacrificed using isoflurane and a secondary measure of cervical dislocation. We will then dissect the dam by a midline incision of the skin, extract thoracic, abdominal and inguinal mammary glands. The lower mammary gland pads will be weighed. A small section of the lower mammary glands will be saved for paraffin embedding for histology while the rest will be snap frozen in liquid nitrogen and cryopreserved to later determine mTORC1 expression as previously discussed via Western blotting. Milk protein composition will be analyzed using milk gels and diluted milk samples (by a factor of 4, 1:3 ratio).

## Determination of Milk Protein

Milk samples collected from control and experimental groups of PND0.5-16.5 will be assessed for protein content. Milk will be diluted to a factor of 4 (1:3 in PBS+EDTA). Skim milk will be collected after centrifuging. Samples will be heated to ~95C and loading cocktail will be added onto the plastic plate with the gel along with a ladder. Coomasiee Destaining Solution will be added to the gel and fixed. On the next day, the destaining solution will be added every 30 minutes to the gel until the solution runs clear. The gel will be placed in 5% acetic acid until imaging. Imaging will be done using Licor machine to determine protein expression. Briefly, we are interested in whey acidic protein, alpha casein, beta casein, lactose, and serum albumin.

## Determination of Milk Fat

Milk samples collected from control and experimental groups of PND0.5-16.5 will be assessed for fat content by the creamatocrit method using a hematocrit centrifuge. Briefly, samples will be diluted to a factor of 3 (1:2 in PBS) into well-sealed capillary tubes. The tubes will be placed in CritSpin mini-creamatocrit spinner. Samples will be centrifuged for 8 cycles of 120 seconds. The capillary will form layers of white fat and non-fat milk. The distance of the fat layer will be measured in millimeters (mm) accurately. The total volume of milk (fat + non-fat milk) will be measured in mm. Percentage of fat will be determined by formulas on Excel software.

## Glucose Tolerance Test

Weaned offspring in groups control and experimental PND0.5-21.5 will undergo a glucose tolerance test (GTT) being challenged with 1g/kg of body weight after a 6-hour fast with *ad libitum* access to water.The effect of maternal glucocorticoid exposure during lactation on offspring adolescent glucose sensitivity will be determined. Briefly, after the fast, the tail will be cut to allow for blood sampling via AccuCheck Advantage Glucometer. Tail vein blood will be immediately measured at 0minutes after the 6-hour fast to denote fasting blood glucose. Mice will be injected by a syringe into the interperitoneal cavity with the appropriate glucose dosage. The timer will be set as to allow for blood collection every 15 minutes. Blood will be collected at 5, 30, 45, 60, 75, 90 and 120 minutes after injection. After the GTT is done, mice will have *ad libitum* access to normal chow diet and water again.

## Western Blotting

Using the fat tissues collected from offspring of groups PND0.5-21.5, gWAT and iWAT will be assessed for mTORC1 activity. Briefly, a portion of the sample will be boiled and loaded into different wells with a ladder control. Proteins will transfer to a second matrix 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 antibodies then the the secondary antibodies. Briefly, antibodies against mTORC, phoshph-mTOR, S6K, phosphorylated S6K, AKT, phosphorylated AKT, S6, phosphorylated S6, 4E-BP1, and phosphorylated 4E-BP1 will be used.

## Histology

Mammary glands collected from control and experimental groups PND0.5-16.5 will be embedded in paraffin. Slides will be assessed for branching and for ductal size. As described by Plante et al, to assess branching, we will count the number of ramifications along portions of the main duct (Plante *et al.*, 2011). The length of the primary duct will also be measured in mm to determine the development of the gland.

# Expected Results

Expected Results (Aims 2.1)

As high doses of glucocorticoids given to adrenalectomized rodents caused mammary gland regression, I hypothesize that dexamethasone will have a time-dependent effect, by which a prolonged exposure starting a week prior to conception (cohort 1) will reduce mammogenesis and alveolar development more so than when introduced at conception (cohort 2). This will be evident in the reduced size of the glands and the reduced branching.

Expected Results (Aims 2.2)

As I expect that mammary gland development will be impaired, this will limit the mammary gland capacity to produce milk. Consistent with findings of reduced milk output after glucocorticoid treatment during lactation, I expect a bigger decrease in milk output volume or the lack of milk production and nursing in cohort 1 than cohort 2. Furthermore, I hypothesize that milk composition, if available, will consist of reduced macronutrients (lactose, protein and fat) more so in cohort 1 than cohort 2. This is consistent with the results seen when high doses of glucocorticoids were given during lactation.

Expected Results (Aims 2.3)

As glucocorticoids cause hyperglycemia (Tamez-Pérez *et al.*, 2015; Suh & Park, 2017), I hypothesize that dams exposed to dexamethasone in both cohorts will be insulin resistant during the ITT compared to their respective controls. Given the effects of antenatal glucocorticoid exposure on offspring health, I hypothesize that offspring from both cohorts will have reduced weights at birth with cohort 1 having higher reductions than cohort 2. Given the expected reduced milk output and macronutrient composition, I expect offspring weights to remain lower than their control counterparts. Fat mass in experimental offspring is expected to be higher despite an overall less body weight indicating impaired metabolic health of the offspring. When offspring undergo the GTT at 6 weeks of age, I expect that experimental offspring to be hyperglycemic with impaired insulin sensitivity. Fat pads collected from these offspring are expected to have higher weights than controls. mTORC1 activity in offspring adipose tissue is expected to be higher in experimental offspring, as mTORC1 activity promotes lipogenesis and is increased in obesity, which in our case is reflected in a thin-fat phenotype in the offspring (Khamzina *et al.*, 2005).

Potential Pitfalls and Alternate Approaches (Aims 2.1-2.3)

It is possible that the dams will give birth to inviable pups or pups that die during lactation and thus will not nurse. In that case, we will have to alter our exposure to determine which developmental window is most critical for viability and lactation. It is also possible that the chronic dexamethasone exposure will abolish mammary gland capacity to produce milk, making it impossible nurse pups and ultimately leading to pup death. In that case, we will alter dexamethasone exposure in new cohorts with treatment windows as follows: one week prior to mating only, one week at mating in the first trimester, one week during the second trimester, and one week during the third trimester. This will allow us to determine more accurately when the effects of dexamethasone are most drastic.

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