**Specific Aim 2: Identify the relationship between time-dependent 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. Our hypothesis is that maternal glucocorticoid exposure will act in a time-dependent manner impairing mammary gland development and reducing milk output and milk macronutrient composition ultimately leading to reduced offspring weight and impaired metabolic health and fat mass. To test this, we will identify how a) timed dexamethasone exposure affects mammary gland size and development, b) how timed dexamethasone exposure affects milk output volume and lactose, protein and fat composition, and c) the effect of the exposure on maternal and offspring health via assessing body composition and insulin tolerance.

**Rationale:** 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). Dexamethasone was found 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). 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?

**Methods (Aims 2.1-2.3):** To assess the effects of glucocorticoids on milk production and milk volume, we will obtain 8-week old C57Bl6/J mice from Jax laboratories. Mice will be given two weeks to acclimatize with *ad libitum* access to normal chow diet and water. After acclimatization, the first cohort of dams will be assigned to one group of the following: control 1, control 2, experimental 1, or experimental 2. The two control groups will have *ad libitum* access to normal chow diet and water. The two experimental groups will have *ad libitum* access to normal chow diet with dexamethasone administration in the drinking water at a dose of 1mg/kg/day. After one week of treatment, all groups in this cohort will be mated with age-matched male mice. The dexamethasone exposure will start one week prior to conception and will last all throughout gestation and lactation until postnatal day (PND) 16.5 and 21.5 for experimental groups 1 and 2, respectively.

In a second cohort, mice will be given two weeks to acclimatize. After acclimatization, mice will be simultaneously assigned to one of the following four groups: control 1, control 2, experimental 1, or experimental 2. Dams will then be mated with age-matched males after acclimatization. Both control 1 and control 2 groups in the second cohort will have *ad libitum* access to normal chow diet and water throughout gestation. Experimental groups 1 and 2 will be given dexamethasone in the drinking water at a dose of 1mg/kg/day at the beginning of mating. Dexamethasone exposure for experimental groups 1 and 2 will last throughout gestation and lactation until PND16.5 and PND21.5, respectively.

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 both cohorts, the dams will undergo body mass assessment three times weekly 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. The offspring will be weighed at PND0.5, PND7.5, 14.5, 16.5, and at 21.5 depending on their group.

At PND10.5, we will determine milk output volume for both cohorts. 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 dam and the pups.

On PND16.5, we will collect milk samples (~0.5ml) from the nursing dams in groups control 1 and experimental 1 from both cohorts. 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. 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. Briefly, we will determine protein expression of mTORC, phoshph-mTOR, S6K, phosphorylated S6K, AKT, phosphorylated AKT, S6, phosphorylated S6, 4E-BP1, and phosphorylated 4E-BP1 using respective protein antibodies. Milk protein composition will be analyzed using milk gels and diluted milk samples (by a factor of 5, 1:4 ratio). We will check for whey acidic protein, alpha casein, beta casein, lactose, serum albumin. Milk fat composition will be analyzed by the creamatocrit method using a hematocrit centrifuge.

Dams in groups control 2 and experimental 2 from both cohorts will undergo an insulin tolerance test (ITT) being challenged with 0.75g/kg insulin after a 6-hour fast with access to water or dexamethasone and with their pups allowed to nurse during the fasting period. The effect of glucocorticoid treatment on dam’s insulin sensitivity will be determined. After the ITT is done, dams will have *ad libitum* access to chow again. The pups will be weighed at PND16.5 and will be remain in their respective cages until weaning at PND21.5. Upon weaning, pups will be weighed and weaned into new cages according to their sex. Pups will have *ad libitum* access to water and normal chow diet at weaning. Their water and food intake will be reported weekly. Weaned pups will undergo body composition analysis at weaning and 3 times weekly thereafter. At 6 weeks of age, offspring will undergo glucose tolerance test (GTT) to determine metabolic health. Within the same week and at 6 weeks of age, offspring will be sacrificed and their fat pads (eWAT and iWAT) will be collected for molecular studies to assess mTORC1 protein expression via Western blotting as described earlier.

**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 hyperglycemic 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 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 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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