**Specific Aim 2: Identify the relationship between time-dependent glucocorticoid exposure and mammary function.**

**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?

**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 one week to acclimatize with *ad libitum* access to normal chow diet and water. After acclimatization, one cohort of dams will be assigned to control or experimental arms. The control group will be on a normal chow diet with access to water. The experimental group 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, both 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. In another cohort, mice will be given one week to acclimatize. After acclimatization, mice will be simultaneously assigned to a control or experimental group and mated with age-matched males. Both groups in the second cohort will have *ad libitum* access to normal chow diet and water throughout gestation. The treatment group will be given dexamethasone in the drinking water at a dose of 1mg/kg/day. Dexamethasone exposure for this cohort will last throughout gestation and lactation until PND16.5. Male breeders will be removed from the cage after 18 days of mating to avoid a second pregnancy.

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. The offspring will be weighed at PND0.5, PND7.5, 14.5 and at 21.5.

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). We will weigh the dam and 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. Briefly, we will separate the dam and pups for 2 hours. We will anesthetize the dam by intraperitoneal injection of Ketamine (0.1275g/kg body weight). Once the mouse is under anesthesia, we will 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 mammary glands will be embedded in formalin for histology while the rest will be snap frozen in liquid nitrogen to determine mTORC1 expression as previously discussed via Western blotting. Milk macronutrient composition will be analyzed using milk gels and creamatocrit measurement.

**Rationale:** Glucocorticoids are important for proper mammary gland development (Anderson & Turner, 1956). In adrenalectomized rats, mammary gland size was reduced. 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). 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). Despite the need of both prolactin and glucocorticoids for normal development of mammary glands, the effects of glucocorticoid 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 are 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 of milk after glucocorticoid exposure in pregnancy and/or lactation is lacking in rodents and humans.

**Expected Results (Aims 2.1-2.2**): I hypothesize that dexamethasone will have a time-dependent effect, by which a prolonged exposure starting a week prior to conception will reduce mammogenesis and alveolar development. This will limit the mammary gland capacity to produce milk which will be evident in decreased milk output volume or the lack of milk production and nursing. Furthermore, I hypothesize that milk composition, if available, will consist of reduced macronutrients (lactose, protein and fat).

For dams exposed to dexamethasone on the day of conception, I hypothesize that mammary gland development will also be impaired but to a lesser degree than the preconception exposure. Milk output will be reduced as mammary gland development is impaired. I hypothesize that milk composition will have less macronutrients but to a lesser degree than the earlier exposure.

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

STAT5 and AKT, mTORC

Stat5 modulated akt pathway, and mtorc1 is downstream of akt, so if stat5 is reduced, mtorc should be reduced meaning milk macronutrient composition should be reduced compared to controls.

Potential Pitfalls and Alternate Approaches: 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.

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