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THE EFFECTS OF DIET INDUCED OBESITY ON SECRETION,
MILK COMPOSITION, AND MAMMARY GLAND
MICROENVIRONMENT IN A MOUSE MODEL

A Thesis in
Nutrition
by
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Abstract

Breast milk is the ideal food for infants, offering essential nutrients and energy for growth and proteins from the mother to develop immunity. Exclusive breastfeeding is therefore recommended for the infant during the first 6 months of life. Obese women however are less likely to initiate breastfeeding, breastfeed for shorter durations, and often suffer a delay in the onset of lactogenesis. 34% of reproductive age women are obese, thus obesity-impaired lactation is a significant problem. Recent research is focused on the impaired onset of lactogenesis with little information on how obesity affects milk secretion after the onset of lactogenesis and limited information on milk composition. DBA/2J mice were used to characterize how diet induced obesity (DIO) affects early, established lactation in a series of 3 studies. Female mice were fed either a high fat diet (HFD) with 45% kcal from fat or a control diet (CD) with 10% kcal from fat throughout the study. Mice were bred ~5 weeks after start of experimental diet and the body weights of the HFD group were significantly heavier than the control group. Experiments were performed during early lactation at LD 4-6. In study 1, we explored effects of DIO on secretion during established lactation. Milk secretion was measured by weigh-suckle-weigh, mammary gland morphology and the number of alveoli were visually assessed by hemoatoxylin and eosin (H and E) staining. The expression of major milk proteins was analyzed by real-time PCR and immunoblot. There was no significant effect of a high fat diet on milk secretion, mammary gland morphology, number of alveoli, or mRNA expression of secretory proteins β-casein and α-lactalbumin between mice fed HFD vs. mice fed CD. The mRNA expression of whey acidic protein (WAP) in the mammary gland was significantly lower (P<0.01) in mice fed HFD compared with mice fed CD, yet protein expression of WAP in the mammary gland was not different between groups. The protein expression of β-casein was significantly (P<0.05) greater in the mammary glands of mice fed HFD compared with mice fed CD. Study 1 determined that DIO does not impair secretion during established lactation as evidenced by no impairment in volume of milk secreted, no abnormalities in mammary gland morphology and no impairment in the expression of secretory proteins. These findings emphasize the importance of an obese mother receiving adequate lactational support in order to establish
the onset of lactogenesis as obesity should otherwise cause no secretory impairment. In
study 2, we explored effects of DIO on milk composition. Fat content, lactose and total
protein concentration, protein composition and zinc concentration were analyzed. There
was no significant effect of a high fat diet on % fat, lactose, or zinc concentration. Mice
fed HFD had significantly lower (P<0.001) total protein concentration in milk. When the
protein composition of milk was analyzed, serum albumin in milk was lower (P<0.05)
but expression of caseins was greater (α-casein, P = 0.065; β-casein, P < 0.01; γ-casein, P
< 0.01; ε-casein, P < 0.05) in milk from mice fed HFD. Study 2 determined that DIO
results in deceased total protein in milk but increased casein secretion, which could
impact infant health. This study emphasizes the necessity for additional research on the
effects of milk from an obese mother on infant growth and health. In study 3, we
explored effects of DIO on the mammary gland microenvironment, as the cells and
molecules that surround mammary epithelial cells may influence their function.
Mammary gland adiposity was compared between groups using H and E staining.
Macrophage infiltration was analyzed by immunohistochemistry (IHC) using the
macrophage marker F4/80 and the gene expression of pro-inflammatory cytokines and
Receptor Activator of Nuclear Factor κ B (RANK) was analyzed by real-time PCR. We
observed larger adipocytes and more macrophages present in mammary glands from mice
fed HFD compared with mice fed CD. The mRNA expression of pro-inflammatory
cytokines (TNF-α, P<0.05; MCSF, P<0.01; and IL-6, P=0.07) in the mammary glands of
mice fed HFD was greater compared with mice fed CD. The mRNA expression of
RANK was significantly higher (P<0.05) in mice fed HFD compared with mice fed CD.
Study 3 determined that DIO alters the mammary gland microenvironment by increasing
adiposity, macrophage infiltration and the expression of pro-inflammatory cytokines and
RANK. The altered cytokine milieu of an inflammatory lactating mammary gland
microenvironment could offer insight into the mechanisms that delay lactogenesis. In
conclusion, DIO produces an inflammatory microenvironment in the mammary gland,
which may impact the expression of proteins in the mammary gland and in milk. These
studies offer preliminary data for many new avenues in research including additional
regulatory mechanisms of secretory proteins, the safety of milk from an obese mother,
and RANK as a target for obesity-impaired lactation. Our studies also conclude that if an obese mother decides to breastfeed, she should be offered lactational support to allow her to initiate lactogenesis, as obesity should not cause any additional impairment on lactation.
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List of Abbreviations

Diet Induced Obesity (DIO)
High Fat Diet (HFD)
Control Diet (CD)
Hemoatoxylin and Eosin (H and E)
Immunohistochemistry (IHC)
Whey Acidic Protein (WAP)
Tumor Necrosis Factor Alpha (TNF-α)
Macrophage Colony Stimulating Factor (MCSF)
Interleukin (IL)
Receptor Activator of Nuclear Factor κ B (RANK)
Estrogen (E2)
Prolactin (PRL)
Monocyte Chemotactic Protein 1 (MCP-1)
Interferon Gamma (IFN-γ)
Epidermal Growth Factor (EGF)
Tumor Necrosis Factor Receptor (TNFR)
Immunoglobulin (Ig)
Transforming Growth Factor Betta (TGF-β)
T-Helper (TH)
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Literature Review
The Effects of Obesity on Lactation

For decades, as our nation developed, medical practice as well as policy was focused on under-nutrition as the greatest concern regarding maternal nutrition. The goal was to decrease the rate of infant mortality by decreasing the incidence of low-birth-weight births. Low-birth-weight (< 5lbs 8oz) and very-low-birth-weight (< 3lbs 4oz) infants have an increased risk of mortality during their first year of life, 2% and 24% respectively\(^1\). Risk factors for low- and very-low-birth-weights include low maternal pre-pregnancy weight and little weight gain during pregnancy\(^2\), supporting the need to prevent maternal under-nutrition. However, with rapidly rising rates of obesity including increasing pre-pregnancy weights, over-nutrition in expectant mothers is becoming a growing concern. According to current data, 34% of reproductive age women are obese\(^3\), suggesting maternal obesity affects a substantial portion of the population and is an issue that must be addressed.

Although scarce, there is a rising body of evidence that shows a strong correlation between obesity and inhibited lactation. This relationship was first described by Rutishauser and Carlin who observed a negative relationship between the duration of breastfeeding and maternal obesity\(^4\). We know very little about the causes of impaired lactation in obese women and for those women who do successfully lactate we know even less regarding the composition of their milk. To date there is very little research that examines the effect of maternal adiposity or a high fat diet on milk quality. Increased adiposity may have major implications for mammary gland development and function, which could in turn affect infant nutrition, growth and development. This review will explore the associations between obesity and poor lactation, possible causes for impaired lactation, how adiposity effects mammary gland development and function, the possible effects of obesity on milk composition, and the possible effects of obese mothers’ milk on infant health.
The Relationship Between Obesity and Lactation

Many studies show a negative association between breastfeeding success and BMI. The Australian National Health Survey found that women with a healthy BMI (20 – 25) had a greater rate of initiating breastfeeding than obese women (BMI >30). Hilson et al observed an increased risk of failure to initiate lactation in women with obese/overweight pre-pregnancy status. Kuyelka et al found an association between obesity and reduced initiation and duration of breastfeeding in Hispanic women. In a cohort of Danish women, Baker et al observed an association between high maternal BMI and shorter duration of breastfeeding. Dewey et al determined a positive association between high maternal BMI and a delay in the onset of lactogenesis. These studies show that a high maternal BMI is detrimental to breastfeeding success in many ways including: initiation of breast feeding, the timing of onset of lactatogenesis, and duration of breastfeeding. This evidence also comes from a variety of populations, suggesting regional influences such as culture are not a major contributing factor and that obesity is causing an underlying physiological impairment of lactation.

Socio-cultural and Psychological Effects of Obesity on Lactation

There are many social, cultural and psychological factors that influence how and if a woman breastfeeds. Amir and Donath performed a literature review and identified factors that are highly associated with obesity and low rates of breastfeeding. Obese women are more likely to belong to social groups that have lower rates of breastfeeding including low-socioeconomic status. Obese women have a lower intention to breastfeed, which may be related to the fact that obese women are less likely to have been breastfed themselves. The lower intention to initiate breastfeeding and maintain breastfeeding for the recommended 6 months may reflect a number of other factors including the inability to breastfeed due to time constraint (working long hours a day), not highly practiced in the family (lack of experience and support of other woman who have breast fed such as a mother) and frustration from impaired onset of lactogenesis (discussed later). Depression may also be a contributing factor for low breastfeeding rates as obese women have higher
rates of depression\textsuperscript{11} and depressed mothers are less likely to breastfeed than non-depressed mothers\textsuperscript{12}. These factors are undoubtedly influential in determining a mother’s intent and duration for breastfeeding, but the negative association between breastfeeding success and obesity exists in mothers who have chosen to breastfeed\textsuperscript{6} suggesting there are physiological factors determining lactational success.

**Anatomical Effects of Obesity on Lactation**

Obese women tend to have larger breasts and it has been observed throughout history that women with large breasts have more difficulties with breastfeeding. A breast that is large may be hard to handle as an inexperienced mother attempts breastfeeding for the first time. As reviewed by Amir and Donath, the mother’s view of the infant’s mouth may become obstructed and it may be difficult to position the infant appropriately\textsuperscript{10}. A heavy breast on the infant’s chest may not be conducive to successful feeding. Another issue is the inability of the infant to latch onto a larger breast. Excess periareolar adipose tissue can cause flattening of the areola and nipple\textsuperscript{5}, reducing the concave shape and leaving less surface area for the infant to latch onto. A successful latch made by the infant assures adequate stimulation of the nipple which is required to produce the necessary prolactin response to initiate lactogenesis II, the formation of mature milk in early lactation, and then produce milk for the next feeding. While larger breast size and body size of the mother may indeed make breastfeeding more difficult, it is unlikely to be the major reason for low lactation success in obese women who attempt to initiate lactation.

**Physiological Effects of Obesity on Lactation**

Lactation occurs in 2 stages, beginning with lactogenesis I. Lactogenesis I begins during mid-pregnancy, in response to high estrogen (E2) levels mammary epithelial cells differentiate to reach a fully secretory form\textsuperscript{13}. During this time serum prolactin (PRL) levels rise; PRL is necessary to initiate lactation and the production of mature milk, but the high concentration of progesterone secreted from the placenta inhibits the effects of
PRL, so lactation will not occur as long as progersterone levels are high. Prior to parturition however, an immature form of milk is produced by the mammary gland called colostrum. Colostrum is composed primarily of protein and minerals and is lower in carbohydrates, lipid and vitamins compared to mature milk. Colostrum contains important immunological factors for the infant such as lactoferrin and immuoglobulins which help impart passive immunity. Once parturition takes place it is very important that the infant begins suckling immediately – colostrum is an important source of nutrition for the infant because of the immunity it imparts and the PRL response from suckling is necessary for the onset of lactogenesis II.

Lactogenesis II occurs only after parturition and the expulsion of the placenta that reduces the plasma progesterone levels, allowing PRL to induce protein expression in the mammary gland and increase production of milk components. Lactogenesis II usually occurs within 72 hours postpartum, but can take up to 7 days. It is immensely important that the mother continuously attempts breastfeeding her infant during this time to stimulate the nipple with suckling and thus produce a PRL response. It is suggested that a new mother attempt breastfeeding every 3 hours after birth to ensure she provides adequate time to allow the infant to latch and begin suckling as early as possible. Suckling not only stimulates PRL secretion, but oxytocin secretion as well, which stimulates muscle contraction in the breast to initiate milk let down and in the uterus to limit postpartum bleeding and aid uterine involution. Figure 1 illustrates the actions of PRL and oxytocin during lactation.

Plasma PRL levels decline overtime, thus suckling is required to stimulate PRL secretion form the anterior pituitary in order to maintain lactation. If the mother does not initiate lactation, plasma PRL levels will fall to baseline in as little as 7 days postpartum. It is therefore very important that a mother continues to attempt breastfeeding during this time. Women who are having mechanical issues with breastfeeding must be supported by lactation consultants to remedy these issues before PRL levels fall too low resulting in failed lactation. If the infant is not latching properly, breast pumps may be employed
to stimulate the nipple, which could help it become erect and therefore easier for the infant to latch onto and it may be able to create the proper stimuli to produce a PRL response\(^5\).

Lactational failure has been associated with impaired onset of lactogenesis II. This was first described in animal models such as the cow. Fat cow syndrome occurs when cows are overfed and gain excessive weight, causing many reproductive issues, including some related to lactation such as milk fever, ketosis, and mastitis\(^{14}\). Although researchers have not looked specifically at the onset of lactogenesis II in these animals, excess weight is clearly related to poor milk production. Rasmussen et al has reported finding litters of obese dams that died and have no milk in their stomachs, suggesting these obese dams are not producing milk and cannot support their young\(^{14}\). There is little research on early lactation and the onset of lactogenesis II, but Rasmussen et al found plasma insulin levels and plasma PRL concentrations were abnormal in obese rats during early lactation compared with control rats\(^{14}\). In control rats, plasma insulin levels are high prior to parturition and decrease significantly by lactation day 3; obese rats maintain high insulin levels during this period and do not experience the decrease in plasma insulin seen in lean rats. During this same time period plasma PRL levels rise dramatically in control rats but not in obese rats. Adiposity is thought to alter the hormonal milieu in obese animals and may play a role in impaired PRL response.

The question remains, what may be causing impaired lactogenesis in obese women? Rasmussen et al found that overweight women (BMI >26) had a lower PRL response to suckling than healthy-weight women at 48h postpartum, but not at 7d postpartum, suggesting a cause of the delayed lactogenesis seen in overweight/obese women\(^{15}\). After adjusting for confounding factors such as time since delivery, only overweight/obesity remained significantly associated with decreased PRL response. Rasmussen et al then hypothesized the reason for a blunted PRL effect could be higher plasma progesterone and E2 levels that are concentrated in the excess adipose tissue of overweight and obese women\(^{15}\). Plasma progesterone and plasma E2 levels were measured at 48h and 7d postpartum, but there was no difference between
overweight/obese and control groups\textsuperscript{15}. We can conclude from these studies that obese women are at higher risk for delayed onset of lactogenesis II and have a lower PRL response to suckling, but this is not due to increased systemic progesterone or E2 levels. Together these studies in humans have highlighted a series of events that can explain obesity-impaired lactation: a large breast reduces an infant’s ability to latch and the mechanical stimulation necessary for PRL secretion, resulting in a blunted PRL response and delayed or failed onset of lactogenesis.

Animal studies have however shown that diet induced obesity will influence mammary gland development prior to pregnancy, the onset of lactogenesis, as well as the PRL response to suckling, suggesting that mechanisms other than a low PRL response may play a role in lactation impairment. A few animal studies have shown that obesity impairs mammary gland development. For example Rolls et al found that rats fed a high fat diet (HFD) had underdeveloped mammary glands and obese rats showed decreased lipogenesis\textsuperscript{16}. There are 2 studies in particular that examined mammary gland development in great detail in mice. The first study, conducted by Kamikawa et al studied the effects of diet induced obesity (DIO) on mammary gland development in prepubertal mice\textsuperscript{17}. C57BL/6J mice were put on a HFD (56.7\% kcal from fat) and control diet (CD) beginning at 4wks of age. Mice fed HFD had significantly heavier mammary glands, more mesenteric adipose, higher plasma glucose, insulin, and leptin. This study reported impaired ductal development in mice fed HFD with fewer branches and longer, more narrow ducts\textsuperscript{17}. This study thus associates increased adiposity with disrupted mammary gland development.

The second study, conducted by Flint et al studied the effects of DIO on mammary gland development and function during pregnancy and lactation\textsuperscript{18}. Outbred Swiss mice were fed a standard lab diet (CD) or standard diet with ad libitum access to high fat diet paste containing chocolate and peanuts starting at 4 mo of age. The initial rate of pup growth was lower for pups from dams on a HFD than pups from dams on the CD; this was interpreted by the authors as impaired lactogenesis. The growth rates of pups from dams fed HFD did however catch up to the growth of their control
counterparts over the next 3 days, exhibiting the delayed lactogenesis seen in obese humans. Mammary gland weights were significantly heavier in the mice fed HFD, but total DNA content of the mammary glands was the similar between groups, suggesting that the increase in weight is due to increased adiposity and thus increased adiposity within the mammary gland is associated with a delay in lactogenesis. To determine developmental effects of DIO, mammary gland whole mounts and histological sections were analyzed. These analyses showed less branching in the mammary glands of mice fed HFD during pregnancy and impaired alveolar development, because branches were elongated the authors interpret this as a defect in differentiation and not proliferation. Histological sections showed accumulation of lipid droplets in the epithelial cells of mice fed HFD, consistent with impaired milk secretion. This study provides valuable evidence that shows a clear connection between increased adiposity within the mammary gland and impaired differentiation of the epithelial cells resulting in reduced secretion and the functional outcome of delayed lactogenesis. Rather than focusing on systemic perturbations for impaired lactogenesis, such as low circulating PRL, increased mammary gland adiposity provides the first clue that local cellular dysregulation may be a contributing factor for delayed lactogenesis.
Increased Adiposity and the Mammary Gland

The lactating human breast is composed of glandular and fatty tissue in ~2:1 ratio respectively, compared to 1:1 for non-lactating women\textsuperscript{19}. Adipose is primarily located subcutaneously within the breast, but ~7% of breast tissue is adipose dispersed among alveoli (intra-glandular adipose)\textsuperscript{19}. Ramsay et al observed that there is variation in breast tissue composition between lactating women with some women having a ratio of 1:1 glandular to fatty tissue and the most variation within fatty tissue composition is found in intra-glandular adipose\textsuperscript{19}. Very little adipose is normally found within a 30 mm radius of the nipple\textsuperscript{19}, which suggests that obesity resulting in increased adipose surrounding the nipple may be responsible for impaired infant latch. There are however many ways other than the physical impediments of a large breast in which increased adiposity may affect lactation, such as the chemical signals produced by increased intra-glandular adipose. Mammary epithelial cells do not act in isolation, but rather function within an integrated network of adipocytes, connective tissue, vasculature, and the lymph system\textsuperscript{20}. Adipocytes in particular have a major influence over mammary gland function as they form a structural environment and play a regulatory role in mammary epithelial cell proliferation, differentiation and function\textsuperscript{21}. With such an active role in mammary gland function, increased adiposity likely changes the mammary gland microenvironment and may have consequences in mammary gland development and function. Little is known regarding how obesity and increased adiposity in the mammary fat pad influence lactation. The following section will examine the possible effects of increased adiposity in the mammary fat pad by reviewing our current knowledge of the role adipocytes play in mammary gland function.
**Adipocytes Secrete Paracrine and Endocrine Molecules**

Adipocytes produce a number of hormones, chemokines and cytokines, the concentrations and functions of which may be altered by increased adiposity. PRL for example is one such hormone secreted by adipocytes in the mammary gland. Interestingly, PRL secretion from sub-cutaneous adipose is significantly reduced in obese individuals whereas PRL secretion from visceral adipose is not affected. This suggests that effects of obesity on PRL secretion are depot-specific, perhaps affecting mammary adipose. The functions of adipose-derived PRL are not yet understood, but it could act in a paracrine fashion to regulate milk protein synthesis as systemic PRL does, thus providing another possible explanation for the association between obesity and impaired lactation.

Adipocytes express aromatase which catalyzes the conversion of androgens to E2, thus producing E2 locally and independent of ovarian regulatory factors. The regulation of aromatase is tissue specific, in the case of adipose, cytokines such as IL-6 and TNF-α promote E2 production. Simpson and Davis argue that local E2 acts in a paracrine fashion and that the levels of E2 in the tissue microenvironment are likely to exceed that of plasma E2. As well as hormones, adipocytes secrete pro-inflammatory cytokines, such as IL-6 and TNF-α. Increased adiposity results in increased secretion of pro-inflammatory cytokines, which could in turn up-regulate local E2 production. Kleinberg et al showed that E2 treatment inhibits the lactogenic effect of PRL and results in ~ 50% decrease in α-lactalbumin protein expression in primate mammary gland explants. This study illustrates the antagonistic effects of E2 on PRL and suggests that increased adiposity could in theory inhibit lactation through local antagonistic effects of E2.

**Chronic Inflammation**

Adipocytes secrete a number of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and MCP-1. While increased production of these cytokines have been detected in the systemic circulation of obese individuals this effect has been attributed to other cells within the adipose tissue such as macrophages. Macrophages can secrete a
number of cytokines in either an anti-inflammatory (M2) or pro-inflammatory (M1) manner. The M1, pro-inflammatory response of macrophages is stimulated by IFN-\(\gamma\) and can result in the secretion of IL-6 and TNF-\(\alpha\)\(^{28}\). Obesity results in the increased secretion of IFN-\(\gamma\) by T cells in adipose tissue\(^{29}\), which supports the M1 shift in adipose macrophages seen with obesity.

While macrophages contribute heavily to the increased production of pro-inflammatory cytokines, adipocytes have been shown to increase the secretion of the pro-inflammatory cytokines TNF-\(\alpha\) and MCP-1 in obese individuals\(^{24,26}\). TNF-\(\alpha\) and MCP-1 secreted from adipocytes act as chemoattractants, stimulating the infiltration of macrophages into adipose tissue\(^{30}\). Adipocyte hypertrophy is associated with increased necrosis and macrophages are often found localized to necrotic adipocytes, surrounding the cells to form ‘crown-like’ structures\(^{31}\). Thus as a result of obesity, macrophages recruited to adipose tissue through adipocyte necrosis and the increased secretion of TNF-\(\alpha\) and MCP-1. As shown in Figure 2, increased adiposity results in the increased secretion of TNF-\(\alpha\) and MCP-1 which leads to the recruitment of macrophages, contributing to the production of pro-inflammatory cytokines within the adipose tissue. The consequence of this self-perpetuating cycle is chronic inflammation. Chronic inflammation within the mammary gland would likely alter the mammary gland microenvironment and could impact mammary gland function, but this impact is not yet known.

**Mammary Gland Development and Function**

The mechanisms by which increased adiposity effect mammary gland development and function are not yet known, but an increasing number of studies have begun to shed light on this process. In order to determine how increased adiposity will affect the mammary gland it is necessary to understand how adipocytes influence mammary gland development. Using an adipocyte-depleted mouse model, Landskroner-Eiger et al examined effects of mammary specific adipocyte ablation on mammary gland development during puberty and adulthood\(^{32}\). In pubertal mice adipocyte depletion
ceased ductal outgrowth and reduced the number of terminal end buds. It is interesting to note that this same observation has been made with a diet-induced-obesity pubertal mouse model\textsuperscript{17,33}. This suggests that adipocyte-derived factors are necessary for mammary epithelial cell proliferation. In the adult mouse, adipocyte ablation resulted in thicker main ductal branches, a more tertiary branching pattern, alveolar structures similar to early pregnancy and increased expression of the milk protein β-casein\textsuperscript{32}. This study suggests that adipocyte derived factors may play an important role in mammary gland development during puberty and may suppress a lactogenic phenotype in adult mice, which would support the observations that excess adiposity impairs the lactational phenotype postpartum.

An interesting observation from the Kimikawa study was the infiltration of macrophages observed in the mammary glands of HFD fed mice\textsuperscript{17}. Landskroner-Eiger also noted that mammary gland adipocyte depletion resulted in the infiltration of macrophages due to adipocyte death and as mentioned before\textsuperscript{32}, these studies saw similar effects in ductal branching and terminal end bud number. This suggests that these changes may be the direct result of macrophage infiltration. Macrophages have been found to be present in the mammary gland during all stages of development. They are required for ductal elongation during postnatal development, are found around terminal end buds\textsuperscript{34}, and are believed to participate in the clearance of mammary epithelial cells during involution\textsuperscript{35}. Macrophages are not only associated with the epithelium within the mammary gland, but are dispersed among adipocytes and not all macrophages are associated with necrotic cells, suggesting another role macrophages play in the stroma\textsuperscript{36}. Macrophages likely participate in cross-talk with adipocytes resulting in the secretion of paracrine molecules that can regulate epithelial cell function.

TNF-α, an adipocyte and macrophage secreted cytokine, has been implicated in the regulation of proliferation and differentiation of mammary epithelial cells\textsuperscript{34}. Ip et al showed that TNF-α treatment of mammary epithelial cells in vitro, in the presence of epidermal growth factor (EGF), stimulated proliferation of epithelial cells and inhibited the expression of casein\textsuperscript{37}. In the absence of EGF, TNF-α stimulated alveolar and ductal
morphogenesis and low concentrations of TNF-\(\alpha\) stimulated the expression of caseins. Varela et al determined that the p55 TNF-\(\alpha\) receptor (TNFR) was predominantly expressed during pregnancy and early lactation, and mediated TNF-\(\alpha\) induced proliferation and inhibition of \(\beta\)-casein expression\(^{38}\). In contrast, the p75 TNFR was expressed throughout lactation and mediated TNF-\(\alpha\) stimulated \(\beta\)-casein expression. This shows that TNF-\(\alpha\) has a profound influence over mammary gland function and may play many regulatory roles throughout development and lactation.

Little is known regarding the effects of increased adiposity on cytokine production within the mammary gland microenvironment and how this would affect mammary epithelial cells. It is possible that increased adiposity would result in the increased expression of pro-inflammatory cytokines, with increased production as a result of larger adipocytes and more infiltrating macrophages in response to adipocyte necrosis. Higher concentrations of pro-inflammatory cytokines such as TNF-\(\alpha\) would result in a highly inflammatory mammary gland microenvironment; the consequence of which could be impaired mammary gland development and impaired milk production. Few studies have been performed examining the effects of a pro-inflammatory mammary gland microenvironment on mammary gland development and function – most of these studies conducted in non-pregnant mice or in vitro. Very little is known regarding the impact of increased adiposity and increased inflammation on lactation and subsequently on milk composition and thus warrants further investigation.
Possible Effects of Obesity on Milk Composition

Breast milk is the perfect food for infants, offering essential nutrients in a well-balanced supply and protection against many illnesses that provide the infant the opportunity for optimal health, growth, and development\textsuperscript{39}. Although some variation exists among mothers, breast milk composition is relatively consistent. There is very little research aimed to understand how obesity effects breast milk composition. As discussed thus far, obesity alters the mammary gland microenvironment and impairs the onset of copious milk production. With such influence over mammary epithelial cell function it is likely that the production and secretion of milk products may be altered. The following section will discuss normal milk composition and the possible changes that can occur in response to obesity in light of the limited evidence available.

Normal Milk Composition

Human milk is a complex fluid composed of thousands of compounds\textsuperscript{40}. The major classes of macronutrients that compose human milk are fat (3.8% by weight), protein (1%) and carbohydrates (lactose 7%)\textsuperscript{41}. Table 1 shows the representative values for many of the constituents of human milk.

Carbohydrate

Lactose is the primary carbohydrate found in milk with an average concentration of 68g/L and the most abundant compound next to water\textsuperscript{42}. Lactose concentration is important because it maintains the caloric density of milk required by infants as well as the volume due to osmolarity. Lactose concentration is considered to be the most stable compared to other macronutrients with no significant effects from nutritional status, for example a 65 year old malnourished Nigerian woman fell within normal range for milk lactose\textsuperscript{43}. Variation within milk lactose concentrations is primarily attributed to variation among individuals rather than external pressures such as nutritional status or diet. The onset of lactogenesis II is marked by the precipitous increase in milk lactose.
concentration that occurs within the first 72 hours postpartum when paracellular pathways in the alveoli are open\textsuperscript{44}. Due to its effects on molarity of milk, lactose correlates positively with milk volume and negatively with whey protein content once lactogenesis II is established and tight junctions are closed. While lactose is the major carbohydrate, glucose is present in breast milk at concentrations of approximately 0.02g/L and there are also a number of nucleotide sugars, glycolipids, glycoproteins and oligosaccharides found in milk\textsuperscript{42}. Lactose is also positively associated with the protein α-lactalbumin. α-lactalbumin couples with galactosyltransferase to form lactose synthase for the production of lactose in the mammary gland\textsuperscript{45}.

Protein

α-lactalbumin is a major milk protein that represents 25-30\% of total milk protein\textsuperscript{46}. Total protein in milk is highest in colostrum (~15.8 g/L) and will decline gradually throughout lactation (~8-9 g/L or 1\% by weight in mature milk\textsuperscript{41,42}. Proteins can be found in milk as cell constituents, as part of the milk fat globule membrane, as mammary derived milk proteins (for example, casein) and as serum proteins (for example, serum albumin)\textsuperscript{47}. Milk proteins are often divided into two classes, casein and whey which can be separated by centrifugation. Together, casein and whey provide most of the protein in milk with fat globular membranes and cells in milk contributing 1-3\% of the total protein\textsuperscript{47}. The casein fraction contributes 10-50\% of the total protein and is comprised of casein micelles (includes α, β, κ subunits), which contain calcium phosphate, giving milk its white color\textsuperscript{47}. β-casein is the major casein protein in human milk and evidence suggests that β-casein facilitates the absorption of Ca\textsuperscript{2+} and possibly other divalent cations such as zinc in the infant gut\textsuperscript{48}.

α-lactalbumin, the most abundant whey protein, is also thought to aid Ca and other mineral absorption in infants as it will increase the absorption of Zn and Fe in infant rhesus monkeys\textsuperscript{48}. The whey fraction contributes 50-90\% of total protein and aside from α-lactalbumin, contains a number of proteins of physiological significance for the infant, for example folate-binding protein, vitamin B12-binding protein, and vitamin D-binding protein are present in milk and likely aid in vitamin uptake in the infant gut\textsuperscript{47}. 
Lactoferrin is an Fe binding protein present in the whey fraction that facilitates the uptake of Fe by intestinal cells as well as forms a bactericidal peptide during digestion \(^{48}\). Immunoglobulins are also present in the whey fraction with IgA as the major type for humans; found at highest concentration in colostrum (~1-2 g/L), IgA will remain at a concentration of 0.5-1 g/L for up to 2 yrs of lactation \(^{48}\). Immunoglobulins provide the infant with some immunity from the mother and can protect from pathogens from E.coli to HIV \(^{48}\). Factors that influence milk immunoglobulin levels include maternal malnutrition, however this only occurs during early lactation suggesting the effect represents lower maternal plasma levels \(^{49}\). Total milk protein concentration is not significantly affected by malnutrition, but will change in response to maternal protein intake.

**Lipid**

Lipids are the primary energy source in milk and total milk fat ranges from 30-50 g/L (45-55% of total energy in kilojoules) \(^{42}\). Milk lipids are primarily triacylglycerides found in the core of milk fat globules (along with sterols and fat-soluble vitamins) which form an emulsion in the aqueous phase \(^{50}\). Breast milk lipids provide energy for the growing infant, are essential for the development of retinal and neuronal tissue and are a rich source of essential fatty acids (linoleic acid 8-17%, a-linolenic 0.5-1%, arachidonic acid 0.5-1% and DHA 0.2-0.5% of fatty acids). Lipids are the most variable milk component; total fat content will vary within a feeding, with the duration of lactation, with diurnal rhythm, between breasts, with gestational age, with maternal health and importantly for this discussion, with diet and maternal adiposity \(^{50}\).

**Increased Adiposity and Milk Macronutrients**

To date there are few studies that examine the effects of obesity on milk composition. Studies that look at factors affecting milk composition primarily use healthy weight women, but there is some evidence to suggest that increased adiposity can affect milk composition. Nommsen et al looked at factors that correlate with differences in milk composition and found that maternal fatness, measured as % of ideal body weight
(IBW), correlated significantly with milk lipid concentration suggesting increased adiposity will increase milk lipid concentration\textsuperscript{51}. Other studies had similar findings. Barbosa et al found that milk fat concentration is positively correlated with maternal body fat as measured by deuterium dilution\textsuperscript{52}. Michaelsen et al found a significant increase in milk fat with high maternal BMI (BMI >27 compared to BMI <21)\textsuperscript{53}. These studies suggest that an obese women would have higher milk fat concentrations than a healthy weight or even overweight women. More research is needed to determine the relationship between maternal obesity and milk composition as higher milk fat concentrations could result in greater energy density in milk, which could impact the infant’s risk of obesity.

A change in breast milk macronutrient concentration is consequential for infant growth and risk of obesity. Breast milk lactose and protein concentrations tend to remain constant in the face of dietary insult such as malnutrition\textsuperscript{43,47}, which implicates milk fat as the macronutrient of greatest concern for changes caused by obesity. Some animal studies however, suggest that obesity related impaired secretion could result in low protein or lactose milk concentrations, such as the study conducted by Flint et al, which found $\alpha$-lactalbumin mRNA expression significantly lower in animals fed HFD through day 10 of lactation\textsuperscript{18}, suggesting milk total protein and/or lactose concentration could be reduced, milk composition however was not analyzed. The pup growth rate in this study was similar between groups (aside from lactation day 1) suggesting that overall milk energy content is similar. In a cafeteria diet rat model, milk volume was reduced in obese rats, milk fat was higher, and milk protein concentration was lower\textsuperscript{54,55}. These studies suggest that obesity may impair milk protein synthesis and milk secretion, resulting in reduced milk volume, but higher fat content may adjust for the deficit in energy content, resulting in infant energy intake and growth similar to those nursed on milk from a healthy weight mother. There is a substantial amount of data to support an increased risk of obesity for the child of an obese mother\textsuperscript{56,57}, although these studies primarily focus on fetal programming. There is evidence from animals which suggests a maternal high fat diet during lactation will increase the offspring’s risk of obesity, which will occur with or without a high fat diet during gestation\textsuperscript{58}. This suggests maternal diet during lactation
alone may have a significant impact on offspring obesity, thus signifying the need for more research on the composition and health effects of an obese mother’s milk.

**Alterations in Hormonal and Cytokine Profiles**

Obesity has the potential to alter the hormone and cytokine profiles of milk as well as macronutrient content. The levels of hormones E2 and PRL, adipokines leptin and adiponectin, and a number of cytokines are likely or are known to be different in the breast milk of obese mothers. As discussed previously, E2 and PRL are produced endogenously in the mammary gland and obesity likely results in increased endogenous production of E2 and decreased endogenous production of PRL. It is not known whether a change in endogenous production of these hormones would be reflected in milk concentration. There is little evidence regarding E2, however it is present in breast milk in greater concentrations than plasma\(^{59,60}\), suggesting endogenous production may influence milk concentration. PRL is also present in human milk, and the relationship between plasma PRL and milk PRL is unclear; however, milk PRL has been reported at lower values than maternal plasma\(^{61,62}\). There is evidence that PRL in milk originates from maternal circulation\(^{63}\). Regardless of its source, milk PRL concentrations are likely reduced in obese women as endogenous mammary gland production may be compromised and obese women have reduced circulating PRL in response to suckling during early lactation\(^{15}\). In light of this limited evidence it is likely that PRL would be lower and E2 would be higher in milk from obese women compared to healthy weight women.

The adipokines leptin and adiponectin have been studied extensively. Leptin is an adipocyte-derived hormone and circulating levels are associated with the degree of adiposity of adult humans and mice\(^{64}\). Leptin is found in milk and is produced by mammary epithelial cells as well as transferred from maternal circulation\(^{64,65}\). Breast milk leptin is significantly correlated with maternal BMI\(^{66}\) which suggests an obese mother will have higher milk leptin than a healthy weight mother. Interestingly, although adiponectin is only produced by adipocytes, circulating levels are inversely associated
with adiposity, yet milk adiponectin is positively associated with adiposity\textsuperscript{67,68}. This suggests that obese mothers will have higher milk adiponectin as well as higher milk leptin than healthy weight mothers.

There is a lack of research regarding breast milk cytokine concentration and maternal obesity, but evidence suggests that obese mothers will have increased levels of pro-inflammatory cytokines in their milk. A comparison can be made between the mammary gland and placenta as the former nourishes the offspring postpartum and the latter, in utero. Maternal obesity in ewes results in increased mRNA expression of macrophage markers and pro-inflammatory cytokines TNF-\(\alpha\), IL-6, IL-8 and IL-18 in the placenta\textsuperscript{69} suggesting macrophage infiltration of the placenta and a resulting inflammatory response. Kimikawa et al found similar results in a mouse model of DIO resulting in macrophage infiltration and a slight increase in TNF-\(\alpha\) mRNA expression in non-pregnant mouse mammary glands\textsuperscript{17}. A change in cytokine profiles in an obese mother in the mammary gland or in milk is yet to be reported, but it is possible that increased adiposity would result in an inflammatory response within the mammary gland, producing higher level of pro-inflammatory cytokines that would be reflected in milk concentrations.
Altered Milk Composition and Infant Health

Understanding how obesity impacts the cytokine and hormonal milieu in milk is imperative for infant health as these molecules are powerful modulators of infant immunity, growth, and development. As discussed in the previous section, pro-inflammatory cytokines, hormones and adipokines are present in milk and obesity could change the concentrations of these molecules in milk. This section will discuss the possible impact obesity related changes in cytokine, hormone, and adipokine concentrations will have on infant health.

Impact of Altered Milk Cytokine Profile

A wide variety of cytokines are present in human milk\textsuperscript{70,71} and although their functions are not clear, there is evidence to suggest they participate in priming the infant immune system. The infant immune system is immature and requires the influence of maternal factors believed to be attributed to breast milk to develop a healthy immune response. Macrophages in milk for example, are thought to activate infant B and T cells and contribute sIgA\textsuperscript{72}. M-CSF is thought to impact the proliferation and differentiation of milk macrophages\textsuperscript{71}, which could be altered by maternal obesity. It is not known whether an increase in mammary gland inflammation or macrophage infiltration would influence milk macrophage levels, but this may be possible. Maternal cytokines such as IL-10 and IL-6 are believed to contribute to the maturation of the infant immune system through the differentiation of IgA producing cells\textsuperscript{72}. It is believed that chemokines such as IL-8 may have chemotactic activity for intestinal intraepithelial lymphocytes in the infant gut and may aid in defense against bacterial infections\textsuperscript{71}. A shift toward the production of pro-inflammatory cytokines in the mammary gland caused by obesity could reduce the levels of IL-10 and IL-8 in milk, leaving the infant more susceptible to infection.
The infant immune system also requires the development of tolerance or priming of an immune response. Anti-inflammatory cytokines such as IL-10 and TGF-β are thought to promote tolerance through an immunosuppressive action when the infant is exposed to a dietary antigen. The infant immune system tends to maintain a T-helper 2 (TH2) response due to the sterile environment of the uterus and it is this state that promotes tolerance to antigens. The balance between a TH2 and TH1 (cellular immunity utilizing macrophages and cytotoxic CD8 T cells) response must be maintained within milk and factors such as IL-10, lactoferrin, and osteoprotegrin are believed to inhibit pro-inflammatory cytokines induced by the TH1 response. It is possible that higher milk concentrations of pro-inflammatory cytokines could overwhelm this system and result in a TH1 response, contributing to inadequate tolerance to antigens.

Impact of Altered Milk Hormones

PRL is present in breast milk and is biologically active with post-translational modifications from the mammary gland that are thought to protect PRL from proteolytic degradation in the infant’s gut. Evidence suggests that PRL can modulate the neonatal immune system by directing the maturation of lymphocytes in the intestine and is required for the proliferation and differentiation of thymocytes and splenocytes. There is also evidence that suggests PRL is necessary for proper neuroendocrine and reproductive development. As discussed above, the milk of an obese mother is likely low in PRL which could result in a deficit in immune, neuroendocrine, and reproductive development. This hypothesis however requires extensive research as the links between milk PRL and infant health require more study as does the relationship between obesity and milk PRL.

Impact of Altered Milk Adipokines

A number of studies have found a positive correlation between maternal adiposity and breast milk leptin, a positive correlation between breast milk leptin and infant
circulating leptin, and a negative correlation between breast milk leptin and infant BMI\textsuperscript{75,76}. This evidence suggests that leptin could be protective against obesity in the offspring, but again this was studied in non-obese women, warranting the examination of such correlations in obese women. There is however, direct evidence that shows a protective effect of ingested leptin against obesity. Rats that were given oral doses of leptin during lactation had a lower body weight, decreased adiposity and consumed fewer calories than their controls, whether on a HFD or CD\textsuperscript{77} suggesting leptin in breast milk can help regulate appetite in the infant and reduce the infant’s risk for obesity even in light of an obesogenic environment.

Adiponectin is another adipokine that may have protective effects against obesity. In adults there is an inverse relationship between circulating adiponectin and adiposity, but adiponectin will improve insulin sensitivity in obese individuals\textsuperscript{78}. Milk adiponectin is positively correlated with maternal BMI, and infant serum adiponectin is positively related to milk adiponectin\textsuperscript{67}. There is a negative association between milk adiponectin levels and infant weight-for-age and weight-for-length Z-scores at 6 months of age\textsuperscript{79}. This suggests that although high circulating levels of adiponectin are negatively associated with obesity in adults, increased levels of adiponectin in breast milk are reflected in circulating levels in the infant and may be protective against obesity. This suggests that adiponectin may play a vital role in early infant development, priming the infant for healthy glucose metabolism and weight status. Other functions of adiponectin include the inhibition of TNF-\(\alpha\) and IL-6 production by macrophages and can enhance the production of anti-inflammatory cytokines\textsuperscript{80}. Higher adiponectin in the breast milk of an obese mother may therefore be protective for her child. Another study found a positive correlation between milk adiponectin levels and increased risk for obesity at age 2, this study however, found no association between maternal BMI and milk adiponectin\textsuperscript{81}. Although evidence suggests that increased adiponectin may be protective against obesity in infants, more research is needed, especially examining the effects of milk adiponectin from obese mothers as other factors in the milk could play a role in modulating the effects of adiponectin.
Conclusions

Obesity impairs lactation and with increasing numbers of obese women giving birth it is critical to understand how obesity impairs lactation and how obesity-impaired lactation impacts infant health. The current body of literature has revealed that obesity is associated with lower intent to breastfeed, reduced duration of breastfeeding, and increased failure to initiate lactogenesis, suggesting that the infants of obese women are at increased risk for inadequate nutrition and disease. There is evidence that suggests increased adiposity results in increased aromatase expression and thus increased E2 production in the mammary gland which has the potential to inhibit local PRL production and ultimately lactation. This hypothesis is a novel explanation for obesity impaired lactation. Evidence also suggests that increased adiposity will cause chronic inflammation in the mammary gland and macrophage infiltration, resulting in increased pro-inflammatory cytokines creating an inflammatory microenvironment for the mammary gland. Increased adiposity, inflammation, and impaired secretion may impact milk composition and thus infant health. Maternal obesity may have many effects on infant health including a change in milk protein composition and an increase in milk pro-inflammatory cytokines which may both negatively affect infant immunity. Alternatively, an increase in milk leptin and adiponectin may decrease the infant’s risk of obesity. Understanding the full impact of maternal obesity on breastfeeding is necessary to determine the proper recommendations to be made for obese mothers. Should the breast milk of obese mothers prove to be harmful for infant health current recommendations should be reconsidered and a recommendation formed specifically for obese mothers. However, should obese mothers’ milk prove to have benefits beyond those of healthy weight mother’s milk or simply no negative impacts on infant health, additional programs to support obese mothers in successful breastfeeding would be warranted.
Figure 1. The Production and Action of Prolactin and Oxytocin during Lactation.
Suckling of the infant stimulates mechanoreceptors in the nipple, resulting in the
secretion of oxytocin from the posterior pituitary and prolactin secretion from the anterior
pituitary. Oxytocin stimulates the contraction of myoepithelial cells in the mammary
gland, resulting in milk ejection. Prolactin binds the prolactin receptor in mammary
epithelial cells, which activates the production of secretory products, thus stimulating
milk production for the next feeding.
Figure 2. Inflammation in Adipose Tissue.
Obesity results in the enlargement of adipocytes and this hypertrophy can lead to necrosis. Macrophages often aggregate around necrotic adipocytes and release cytokines such as TNF-α. Adipocytes secrete the pro-inflammatory cytokines IL-6, IL-1b, TNF-α and MCP-1, which are found at elevated levels in the circulation of obese individuals. Macrophages will infiltrate adipose tissue in response to MCP-1 and TNF-α secreted by adipocytes. This produces the self-perpetuating cycle that results in chronic inflammation.
Table 1. Select Representative Values for Constituents of Human Milk

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Early Milk (g/L)*</th>
<th>Mature Milk (g/L)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>20-30</td>
<td>67</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.2-1.0</td>
<td>0.2-0.3</td>
</tr>
<tr>
<td>Oligosaccharides</td>
<td>22-24</td>
<td>12-14</td>
</tr>
<tr>
<td><strong>Total Protein</strong></td>
<td><strong>16</strong></td>
<td><strong>9</strong></td>
</tr>
<tr>
<td>β-casein</td>
<td>2.6</td>
<td>4.4</td>
</tr>
<tr>
<td>κ-casein</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>α-lactalbumin</td>
<td>3.62</td>
<td>3.26</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>3.53</td>
<td>1.94</td>
</tr>
<tr>
<td>Serum Albumin</td>
<td>0.39</td>
<td>0.41</td>
</tr>
<tr>
<td>sIgA</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>IgM</td>
<td>0.12</td>
<td>0.2</td>
</tr>
<tr>
<td>IgG</td>
<td>0.34</td>
<td>0.05</td>
</tr>
<tr>
<td><strong>Total Lipids</strong></td>
<td><strong>20</strong></td>
<td><strong>35</strong></td>
</tr>
<tr>
<td>Triglyceride</td>
<td>97-98†</td>
<td>97-98†</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.7-1.3†</td>
<td>0.4-0.5†</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>1.1†</td>
<td>0.6-0.8†</td>
</tr>
<tr>
<td>Saturated FAs</td>
<td>43-44†</td>
<td>44-45†</td>
</tr>
<tr>
<td>MUFA†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PUFA†</td>
<td>13†</td>
<td>14-15†</td>
</tr>
<tr>
<td>Total ω-3</td>
<td>1.5†</td>
<td>1.5†</td>
</tr>
<tr>
<td>Total ω-6</td>
<td>11.6†</td>
<td>13.06†</td>
</tr>
<tr>
<td>Thiamin</td>
<td>20 (µg/L)</td>
<td>200 (µg/L)</td>
</tr>
<tr>
<td>Niacin</td>
<td>0.5 (mg/L)</td>
<td>1.8-6 (mg/L)</td>
</tr>
<tr>
<td>Retinol</td>
<td>2 (mg/L)</td>
<td>0.3-0.6 (mg/L)</td>
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<tr>
<td>Carotenoids</td>
<td>2 (mg/L)</td>
<td>0.2-0.6 (mg/L)</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>0.33 (µg/L)</td>
<td></td>
</tr>
<tr>
<td>Vitamin E</td>
<td>8-12 (mg/L)</td>
<td>3-8 (mg/L)</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.25</td>
<td>0.2-0.25</td>
</tr>
<tr>
<td>Iron</td>
<td>0.5-1 (mg/L)</td>
<td>0.3-0.9 (mg/L)</td>
</tr>
<tr>
<td>Zinc</td>
<td>8.12 (mg/L)</td>
<td>1.3 (mg/L)</td>
</tr>
</tbody>
</table>

*Concentration in g/L unless otherwise stated
†Expressed as % of total lipids
‡Expressed as % weight of total lipids

Introduction
It has been well established that breast milk is the optimal source of nutrition for the infant, providing all necessary nutrients in easily digested and bioavailable forms\textsuperscript{41}. Breast milk also provides immunological factors that protect infants from infection and help prime the infant immune system\textsuperscript{60,72}. Breastfeeding has been associated with decreased risk for many diseases during infancy\textsuperscript{82} and increased IQ and academic performance in childhood\textsuperscript{83}. Therefore exclusive breastfeeding is recommended for the infant during the first 6 months of life by the American Academy of Pediatrics as well as many other organizations\textsuperscript{84}. Breastfeeding has such a profound impact on the health of both the infant and mother that the surgeon general recently released a ‘Call to Action’ to support breastfeeding, outlining strategies to help mothers meet their breastfeeding goals\textsuperscript{85}.

Emerging research has implicated overweight/obesity as a risk factor for lactational impairment. There are a number of studies that show an increased risk of failure to initiate lactation with overweight/obesity\textsuperscript{6,7,86}. This could be the result of a number of complications that delay breastfeeding attempts including extended recovery time after cesarean birth, which is more common among obese women\textsuperscript{87}. Larger breast size may also contribute to impaired breastfeeding initiation as excess periareolar adipose tissue can cause flattening of the areola and nipple\textsuperscript{5}, reducing the concave shape and leaving less surface area for the infant to latch onto. Increased maternal fatness is also associated with a delay in the onset of lactogenesis (the onset of copious milk production) in both humans\textsuperscript{9,14,88} and animal models\textsuperscript{14}. A blunted PRL response is associated with the delay in lactogenesis II in obese women\textsuperscript{15}, but beyond this information there is little evidence to explain how obesity impairs lactation. Animal studies have shown that obesity results in disrupted mammary gland development\textsuperscript{17,18,89} which has been associated with a delay in lactogenesis II\textsuperscript{18}. There is very little comprehensive evidence to suggest a mechanism for obesity-impaired lactation. Decreased duration of breastfeeding is associated with increased BMI\textsuperscript{7,8}. Obese women are more likely to stop breastfeeding after 1 week postpartum\textsuperscript{86} which may reflect an impairment in the onset of lactogenesis II. Frustrations caused by delayed lactogenesis or the inability of the infant to latch properly can result in an obese mother choosing to stop breastfeeding and use
formula instead. Obese women also intend to breastfeed for shorter periods of time than non-obese women, which may reflect social or cultural influences\(^6\).

The mechanisms by which obesity impairs lactation are not known. Animal studies show that DIO can alter mammary gland development\(^{17,18}\) and suggest that DIO impairs secretion during early lactation\(^{18}\). Mammary epithelial cells do not act in isolation, but rather function within an integrated network of adipocytes, connective tissue, vasculature, and the lymph system\(^{20}\). Adipocytes in particular have a major influence over mammary gland function as they form a structural environment and play a regulatory role in mammary epithelial cell proliferation, differentiation and function\(^{21}\). With such an active role in mammary gland function, increased adiposity likely changes the mammary gland microenvironment and may have consequences in mammary gland development and function. Adipocytes secrete a number of pro-inflammatory cytokines, including TNF-\(\alpha\)\(^{24}\). TNF-\(\alpha\) influences mammary gland development by stimulating epithelial cell proliferation and inhibiting the expression of the secretory proteins \(\beta\)-casein and WAP\(^{37,90}\). Altered mammary gland microenvironment and increased pro-inflammatory cytokines also have the potential to influence milk composition. Obesity has been shown to result in lower total milk protein and higher fat\(^{55}\), but the effects on individual proteins has not been reported. This suggests that altering the mammary gland microenvironment, such as increasing the expression of pro-inflammatory cytokines can impact mammary gland development and alter milk composition.

In order to explore the impact of obesity on lactation, we aimed to determine 1) how DIO affects secretion once lactation has been established, 2) how obesity affected milk composition, and 3) how obesity altered the mammary gland microenvironment using a mouse model in a series of 3 studies. In study 1 we hypothesized that once lactation is established, DIO will not impair secretion during established lactation, emphasizing the importance of lactational support for obese women postpartum. In study 2 we hypothesized that DIO will result in altered milk composition, warranting further research to determine if breast milk from an obese mother is as healthy for an infant as milk from a non-obese mother. In study 3 we hypothesized that DIO will result in altered
mammary gland microenvironment, characterized by increased adiposity of the mammary gland accompanied by macrophage infiltration and increased pro-inflammatory cytokines which could offer insight into the mechanisms involved in obesity-impaired lactation.
Materials and Methods
**Mice.** This study was approved by the IACUC Committee at The Pennsylvania State University, which is accredited by the American Association of Laboratory Animal Care. Male and female DBA/2J mice were obtained commercially (Jackson Laboratories) at 3 weeks of age and were housed individually in polycarbonate cages. Mice were maintained on a 12 hr light/dark cycle under controlled temperature and humidity. At 4 weeks of age female mice were randomly assigned to either a high fat (HFD) or low fat control (CD) diet. Two studies were conducted with a sample size of 30 mice in the first and 80 mice in the second. Females were fed commercially available purified diets (Research Diets Inc.) identical in composition except for fat content. The HFD (D12451) contained 45% kcal from fat with additional fat from lard and CD (D12450B) contained 10% kcal from fat. Mice were fed these diets for 5 weeks. Food intake and body weight were measured once a wk. Once mice fed HFD were >20% heavier than mice fed CD (~5 wk), females were mated and allowed to deliver naturally (M-Fig. 1). Some litters were weighed and counted on day of birth and at LD5.

**Weigh-suckle-weigh.** Weigh-suckle-weigh was preformed as described by McDonald et al with slight modification. Briefly, on LD 4 or 5, pups were removed from dams for 1 hr and then returned to dams to suckle for 2 hr, this emptied the mammary glands and normalized the prolactin response between dams. Pups were then removed from dams for an additional 2 hr which allowed the milk supply to replenish and pups to grow hungry. Pups were then weighed as a litter, returned to dams and suckled for 2 h, then weighed again. The difference between the final and initial litter weight represents milk yield.

**Milk and tissue collection.** On LD5 dams were removed from pups for 2 h, anesthetized by inhalation of isofluorane and injected subcutaneously with 0.02 IU oxytocin. Milk was manually expressed and kept on ice until storage at -20°C. Mice were euthanized by CO₂ inhalation. Only thoracic mammary glands #4 and #9 were used for analysis. Mammary glands used for protein and zinc analysis were frozen on dry ice and stored at -
80° until analysis. Mammary glands used for RNA were stored in RNeater (Sigma) at -20° C until analysis. Mammary glands from mice that did not have milk removed previously were collected for histology and were fixed in 2% buffered paraformaldehyde overnight. Mammary glands for whole mount analysis were spread on glass slides and fixed in Carnoy’s solution (6 parts ethanol, 3 parts chloroform, 1 part glacial acetic acid) overnight.

**Histology.** Paraformaldehyde-fixed mammary glands were serially dehydrated in ethanol, embedded in paraffin, and sectioned (5µm) onto positively charged glass slides as previously described. Sections were deparaffinized in xylene then serially rehydrated in ethanol (100% twice for 2 min, 95% twice for 2 min, 70%, 1 min). Sections were stained with hematoxylin and eosin (H and E) by staining 4 min with hematoxylin solution (0.5% hematoxylin 5% ethanol, 10% ammonium aluminum sulfate, 0.037% sodium iodate in H2O), washing with 1% acid ethanol and incubating 2 min in blueing solution (1.22% lithium carbonate in H2O) to achieve a bluing of the nuclei. Sections were then counterstained 1 min in eosin solution (0.33% eosin, 75% ethanol, 0.5% glacial acetic acid). Sections were mounted with toluene Mounting Medium (Thermo Scientific). H and E stained sections from mice fed CD and HFD were visually compared to assess differences in mammary gland morphology. The number of alveoli were compared by counting the alveoli in a 10x view (n=3) and the number of alveoli is expressed as the mean±SD per unit area. Immunohistochemistry was performed as previously described. Deparaffinized sections were incubated overnight with commercially available anti-mouse P4/80 purified monoclonal antibody (AbD Serotech), diluted 1:1000 in buffer (10%goat serum, 1%BSA, 0.05% Tween-20 in PBS) and detected with biotinylated goat anti-rat IgG (Vector Labs). The antigen-antibody complex was visualized using ABC Vectastain kit (Vector Labs) according to manufacturer’s instructions and counterstained with toluidine blue (EMD). Sections were mounted with toluene Mounting Medium (Thermo Scientific). Cells stained positive for P4/80 were quantified by counting in a 10x view and macrophage number is expressed as mean±SD.
per unit area. Sections were imaged using a Leica DM IL LED microscope and LAS V3.6 softwear.

**Whole mount analysis.** Mammary glands fixed in Carnoy’s solution were serially rehydrated in ethanol (70% twice for 15 min, 50% twice for 10 min, 30% twice for 10 min, 10% twice for 10 min) and stained with carmine alum stain (0.2% carmine, 0.5% aluminum potassium sulfate in H2O) overnight. Mammary glands were destained (70% ethanol, 6% hydrochloric acid) for 30 min to minimize background. Whole mounts were serially dehydrated with ethanol (70% twice for 15 min, 95% twice for 15 min, 100% twice for 15 min) and incubated in xylene for 24-48hr until adipose was cleared. Whole mounts were mounted with toluene Mounting Medium (Thermo Scientific). Whole mounts from mice at LD5 that were fed CD and HFD were visually assessed for differences in extent of branching, alveolar development and presence of macrophages. Whole mounts from non-pregnant mice were used to visually detect macrophages. Whole mounts were imaged using a Leica DM IL LED microscope and LAS V3.6 softwear.

**Real time relative RT-PCR.** Total RNA was isolated from mammary glands homogenized in TriZOL reagent following manufacturer’s instructions (Invitrogen). RNA integrity was assessed following electrophoresis through agarose and ethidium bromide staining. cDNA was generated with ImProm-II Reverse Transcription System (Promega) following manufacturer’s instructions. Real-time PCR was performed to determine relative mRNA expression as previously described94 using iQ SYBR Green Supermix (BioRad) and the DNA Engine Opticon 2 System real-time thermocycler (BioRad) with Opticon 2 System softwear. Primer specificity (Table 1) was validated by assessment of single temperature dissociation peak (data not shown). Each sample was measured in duplicate and normalized to β-actin using the following equation $\Delta C_{t_{\text{gene}}} = C_{t_{\text{gene}}} - C_{t_{\beta-\text{actin}}}$ . Fold change in expression was calculated for each sample using the
equation: $2^{\Delta \Delta Ct}$, where $\Delta \Delta Ct = (\text{mean of normalized Cts for mice fed HFD}) - (\text{mean of normalized Cts for mice fed CD})$. Data is expressed as mean fold change ± SD.

**Immunoblot analysis.** Mammary gland tissue homogenates were prepared as described by Gorivodsky et al.$^{95}$ Total protein was determined using a Bradford assay (Biorad). Tissue homogenate (30 µg protein) was diluted in Laemmli sample buffer (BioRad) containing DTT (100mM) and incubated at 95°C for 5 min. Proteins were electrophoresed by SDS-PAGE at 200 V using a 12% gel and immunoblotted as previously described.$^{94}$ Proteins were transferred onto Hybond ECL nitrocellulose membrane (GE Healthcare) for 60 min at 100 V. For the detection of major secretory proteins (β-casein and WAP) in the mammary gland, the primary antibodies rabbit anti-mouse β-Casein (1:1000; FL-231, Santa Cruz Biotechnology) and goat anti-mouse WAP (1:1000; M-16, Santa Cruz Biotechnology) were used coupled with secondary antibodies, donkey anti-rabbit IgG-HRP (1-25000, GE Healthcare UK Ltd.) and donkey anti-goat IgG-HRP (1:25000, Santa Cruz Biotechnology) respectively. In order to compare the expression of epithelial cell specific protein e-cadherin in mammary glands from mice fed a CD vs HFD, the primary antibody mouse anti-mouse e-cadherin (1:5000; Abcam) was detected by sheep anti-mouse IgG-HRP (1:20000, GE Healthcare UK Ltd.). The expression of β-actin was used as a loading control with primary the antibody mouse anti-mouse β-actin (1:10000, Sigma) and sheep anti-mouse IgG-HRP (1:20000, GE Healthcare UK Ltd.). Proteins were visualized with Super Signal West Femto Chemiluminescent Substrate (Thermo Scientific) or Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) and exposed to autoradiography film. Relative band density was quantified using Carestream Gel Logic 212 Pro.
Milk analysis.

**Creamatocrit:** Frozen whole milk samples were thawed to room temperature, warmed to 37°C and vortexed to mix thoroughly. Milk was drawn into a 60 mm micro-hematocrit tube (VWR), sealed with Critoseal (Fischer Scientific) and centrifuged in a hematocrit microcentrifuge for 10 min at 2500 rpm. Length of total milk and cream layer were measured in duplicate. %fat was calculated (cream /total volume * 100).

**Zinc:** Whole milk (37-137 μl) was diluted in 1 ml nitric acid (0.78 N) and digested at room temperature for 1 week. Milk was then wet-ashed in concentrated nitric acid as described by Clegg et al\(^9^6\). Briefly, concentrated nitric acid (~ 1 ml) was added to partially digested milk and boiled down to ~1 ml liquid. Wet-ashed milk was diluted in 2 ml double deionized water and zinc concentration was determined by atomic absorption spectrophotometry.

**Skimmed milk:** Whole milk was centrifuged at 2000 x g for 15 minutes at 4°C. The cream layer was scraped away using a pipette tip which allowed the skimmed milk to be removed by pipette and transferred to a new tube.

**Lactose:** Lactose concentration was measured using commercially available colorimetric Lactose Assay Kit (Abcam) according to manufacturer’s instructions. Skimmed milk was diluted 1:9 with assay buffer for analysis. The O.D. was measured at 570nm on the EPOCH microplate reader (Biotek). Samples were measured in duplicate and lactose concentration was calculated by standard curve using Gen5 softwear (BioTek). Lactose concentration was determined by the presence of galactose after the addition of lactase enzyme to the sample, therefore a control was run for every sample with the omission of lactase to correct for any endogenous galactose, this value was subtracted from the mean concentration for each sample.

**Total protein:** Skimmed milk was diluted in 2 volumes of buffer (50mM Na\(_2\)PO\(_4\), 150 mM NaCl, 50 mM EDTA) and centrifuged twice at 11,600xg for 15 minutes at 4°C. Protein in supernatant was measured by Bradford assay (Biorad).
Protein composition: Proteins in skimmed milk (0.5µl milk or 15µg protein) were diluted in Laemmli sample buffer (BioRad) containing DTT (100mM) and incubated at 95°C for 5 min; then electrophoresed by SDS-PAGE at 200 V for 1 hr using a 13% gel. The gel was fixed in (50% methanol, 10% acetic acid) for 1 hr, stained with Coomasie Brilliant Blue R250 (Fisher Biotech) (0.25% R250, 50% methanol, 10% acetic acid) for 1-3 hr, and de-stained first with fixative buffer (1 hr), followed by destaining buffer (5% methanol, 7.5% acetic acid) overnight. Relative band density was quantified using Carestream Gel Logic 212 Pro.

Cytokine Array. A cytokine array was performed in order to determine the protein expression of cytokines in mammary gland homogenates from mice fed a CD or HFD. The commercially available Proteome Profiler Mouse Cytokine Array kit (Panel A, R&D Systems) was used according to the manufacturer’s instructions. One mammary gland from a mouse fed CD and 1 mammary gland from a mouse fed HFD were compared. Protein expression was visualized with Super Signal West Femto Chemiluminescent Substrate (Thermo Scientific) and exposed to autoradiography film. Relative spot density was normalized to a positive control and quantified using Carestream Gel Logic 212 Pro.

Statistical Analysis. Results are presented as mean ± standard deviation. Statistical comparisons between HFD and CD groups were performed using Student’s t-test (Prism Graph Pad software) and significance was demonstrated at p < 0.05.
Methods: Tables and Figures

M-Fig. 1. Experimental Design. Female mice were fed either HFD or CD for ~5 weeks then females were housed with males for 2 weeks. Samples were collected 4-6 days after birth.

Table 1. Real-Time PCR primers

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<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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All primers were designed using the Primer3 Input v.0.4.0 (http://frodo.wi.mit.edu/primer3)
GenBank accession number follows primer name. Primer sequences presented as 5’ to 3’.
Results
Study 1: The Effects of DIO on Secretion During Established Lactation

Model. Female mice were maintained on either HFD or CD for ~5 weeks prior to breeding. At the initiation of breeding mice fed HFD were significantly heavier (P<0.05) than mice fed CD (Sup.Fig. 1). Food intake did not differ significantly between groups (Sup.Fig. 2), suggesting the excess weight gain seen in mice fed HFD resulted from increased dietary fat. We noted discrete differences in reproductive success between mice fed HFD and mice fed CD. We determined that mice fed HFD and mice fed CD had a similar number of births (36 and 38 births respectively), however mice fed a HFD had a significantly lower pup survival rate compared to mice fed CD (P=<0.001)(Fig. 1) This reflects the effects of obesity on the failure to initiate lactation\textsuperscript{14}.

The objective of this study was to analyze the secretory capacity of mice fed a HFD once lactation was established therefore dams were allowed to nurse young until LD 4-5 before experimentation began.

Milk secretion. Milk secretion was measured by weigh-suckle-weigh, where the difference in litter weights before and after suckling reflects milk secretion. There was no significant difference in milk secretion between groups (Fig. 2).

Mammary gland morphology. To determine if mammary gland morphology is altered by DIO we assessed mammary gland morphology using H and E staining and whole mount analysis. MG whole mounts showed similar morphology between groups with large alveolar clusters filling most of the fat pad and displaying extensive branching (Fig. 3). The ‘alveolar area’ from the mammary gland of a mouse fed HFD was compared with the mammary gland of a mouse fed CD to estimate the area of mammary gland fat pad infiltrated by ductal branching. Preliminary data shows that the mammary gland area for a mouse fed HFD was 1.5cm\textsuperscript{2} compared with 1.98 cm\textsuperscript{2} for the mammary gland of a
mouse fed CD (a 32% difference in size), suggesting that the mammary glands from mice fed HFD may potentially have fewer alveoli than mammary glands from mice fed CD. To determine the relative number of alveoli, MG sections from mice fed HFD and CD were stained with Hand E and the number of alveoli from 3 sections per group were counted at 10X magnification. There was no significant difference between the number of alveoli in mammary glands from mice fed HFD (208±28.31) and mammary glands from mice fed CD (200±14.01). This was verified by noting similar protein expression of e-cadherin and mRNA expression of K18, both epithelial cell-specific proteins (Sup. Fig. 3). Although the number of alveoli was not altered by DIO, alveolar development may have been affected, to determine if DIO alters the morphology of alveolar structures, H and E stained mammary gland sections for mice fed HFD and CD were visually compared. Mammary gland sections from LD1-LD5 for mice fed CD were prepared in order to compare the mammary gland morphology of mice fed HFD at LD5 to determine if mammary gland development is delayed (Fig 4). There were no obvious differences between the mammary gland morphology of mice fed HFD at LD5 suggesting no delay in mammary gland development or morphological abnormalities associated with DIO.

**Expression of major secretory proteins.** To further explore effects of DIO on secretion, we evaluated differences in mRNA and protein expression of major secretory proteins in the MG. The mRNA expression of WAP (P<0.01), and β-casein (P=0.094) were lower in the mammary gland of mice fed HFD, although only effects on WAP were significant (Fig. 5a). No effect on α-lactalbumin expression was noted. The protein abundance for WAP and β-casein in the mammary gland was analyzed and β-casein expression was significantly greater (P<0.01) while no significant differences were noted for WAP (Fig. 5b).
Summary of Study 1. DIO does not impair secretion during established lactation. Whole mounts reveal that ductal branching and alveoli clusters are visually similar in size and infiltration of the mammary fat pad in mice fed HFD and CD. Preliminary data suggested that the area of the mammary gland covered by alveoli may be smaller in mice fed HFD compared to mice fed CD, but analysis of H and E stained mammary gland sections determined there was no difference in alveoli number. This was also supported by similar expression of epithelial specific proteins. DIO had no effect on alveolar morphology at LD5. Similar morphology and number of alveoli between mice fed HFD and CD suggests there is no difference in secretory capacity, which was consistent with the finding that milk secretion at LD5 is not affected by DIO. The mRNA expression of WAP is lower in the MG as a result of DIO, but this is not reflected in protein expression. β-casein mRNA expression is slightly lower in mice fed a HFD, but mammary gland protein expression is significantly greater than mice fed CD.
Study 2: The Effects of DIO on Milk Composition

Milk zinc concentration. To determine if DIO alters milk zinc concentration, milk zinc was measured by atomic absorption spectrophotometry. There was no significant difference in milk zinc concentration of mice fed HFD (10.84±3.74 μg/ml) and mice fed CD (12.49±4.29 μg/ml).

Milk fat. To determine if DIO alters milk fat, we measured % fat in milk by creamatocrit. There was a trend towards higher % milk fat in mice fed HFD (P=0.1412) (19.49±6.80%) compared with mice fed CD (17.03±8.56%).

Milk lactose concentration. Lactose is an important milk constituent as it drives the water content and thus regulates milk volume. To determine the effect of DIO on milk lactose composition, lactose was measure using a colorimetric assay. There was no effect of DIO on milk lactose concentration, as lactose concentration for mice fed HFD was 17.21±2.04 mg/ml compared with 17.05±2.08 mg/ml for mice fed CD.

Protein concentration and composition. To determine if DIO affects total protein concentration, we measured total milk protein by modified Bradford assay. Total protein concentration was significantly lower (P<0.001) in milk from mice fed HFD compared to milk from mice fed CD (Fig.6) consistent with previous reports. To determine if DIO alters milk protein composition, milk proteins were separated by SDS-PAGE and analyzed in two-different ways. 1) In order to determine the relative abundance of protein consumed by the offspring, the protein composition of equal volumes of milk were compared; 2) In order to determine the relative abundance of protein expressed and secreted by the mammary gland, the protein composition in equal amount of milk protein
were compared.. Proteins identified in milk samples include lactoferrin (~76 kDa), serum albumin (~64 kDa), α-casein (~40 kDa), β-casein (~28 kDa), γ-casein (~24 kDa), ε-casein (~22 kDa) and WAP (~14 kDa) (Fig. 7a and c). When analyzed by equal milk volume, there were no significant differences in individual protein abundance between mice fed CD and HFD (Fig. 7b), except for β-casein abundance which was greater (P=0.069) in milk from mice fed HFD compared with milk from mice fed CD. When analyzed by equal protein amount there were no differences in individual protein abundance except for serum albumin, which was significantly (P<0.01) lower in milk from mice fed HFD compared with milk from mice fed CD (Fig. 7d). Proteins analyzed by equal protein loading were then normalized to serum albumin in order to determine the relative abundance of secreted proteins by the mammary gland. Serum albumin is not expressed by the mammary gland and thus serves as an internal standard for the effects of DIO on the abundance of secreted proteins. When normalized to serum albumin the abundance of caseins were greater in milk from mice fed HFD compared with mice fed CD (α-casein, P=0.065; β-casein, P<0.01; γ-casein, P<0.01; ε-casein, P<0.05) (Fig. 7e). The greater abundance of secreted β-casein in the milk of mice fed HFD supports the finding that β-casein is also more abundant in the milk of mice fed HFD when analyzed by volume, even though total protein in the milk from mice fed HFD is lower than that of mice fed CD.

**Summary of Study 2.** Taken together, these data show that DIO results in lower milk protein concentration and altered protein composition. Zinc concentration, %fat, and lactose concentration in milk was not affected by DIO. Total protein was lower in the milk of mice fed HFD; however, there was a greater abundance of casein proteins compared to the milk of mice fed CD.
**Study 3: The Effects of DIO on Mammary Gland Microenvironment**

**Mammary gland adiposity.** In order to determine if DIO increased the number of adipocytes within the mammary gland fat pad, adipocytes were counted on H and E stained mammary gland sections. There was no significant difference between the mean number of adipocytes in any given area of the mammary glands from mice fed HFD (672±149.37) compared with mammary glands from mice fed CD (772±93.13). However, the adipocytes in mammary glands from mice fed HFD were, ~2-fold larger than adipocytes in the mammary glands of mice fed CD (Fig.8). Mammary glands from mice fed HFD (0.65±0.14 g) were significantly (P<0.01) heavier than the mammary glands of mice fed CD (0.41785±0.06 g).

**Macrophage infiltration.** To determine if macrophages were present in MGs, we looked for the signature “crown like structures” in mammary gland whole mounts. The aggregation of macrophages around adipocytes stain darker than other stromal cells with carmine and have been described as resembling a crown. Macrophages could not be detected in lactating mammary gland whole mounts as the alveoli were too dense for macrophage visualization. Whole mounts from non-pregnant mice were then used to identify macrophages. Numerous ‘crown like structures’ were identified in mammary glands from non-pregnant mice fed HFD while none were seen in mammary glands from non-pregnant mice fed CD (Sup.Fig. 4). To determine the relative abundance of macrophages in lactating mammary glands, IHC was used to detect the macrophage specific marker F4/80 (Fig. 9a). Macrophage abundance was significantly greater (1.6-fold) in mammary glands from mice fed HFD compared with mammary glands from mice fed CD (P<0.001) (Fig. 9b).
**Expression of pro-inflammatory cytokines and RANK in the mammary gland.** In a preliminary experiment we used a cytokine array to evaluate differences in the protein abundance of a number of cytokines in mammary glands from mice fed HFD compared with mammary glands from mice fed CD. Our data indicated that IL-1b, IL-17, and IL-23 were only expressed in the mammary glands of mice fed HFD (Sup.Fig. 5). Expression of TIMP-1, CD54, MIG, MCSF, IL-6, and IL-1a was higher in the mammary glands of mice fed HFD compared to mice fed CD. INF-y expression was lower in mammary glands from mice fed HFD compared to mice fed CD. We next measured the mRNA expression of pro-inflammatory cytokines TNF-α, IL-6, IL-1b, IFN-y, and MCSF. The expression of TNF-a (P<0.05) and MCSF (0.01) were significantly higher in the mammary gland of mice fed HFD compared to mice fed CD (Fig. 10). No other significant differences were noted, although there was a trend towards higher IL-6 expression in mammary glands of mice fed HFD (P=0.07). In order to determine the effects of DIO on the expression of RANK, a vital cell signaling protein implicated in mammary gland development and function, mRNA expression was measured. RANK expression was significantly higher (P<0.01) in the mammary glands of mice fed HFD compared with the mammary glands of mice fed CD (Fig. 11).

**Summary of Study 3.** Taken together, these data show that DIO alters the mammary gland microenvironment. DIO results in larger adipocytes within the mammary gland, which is reflected in greater mammary gland weight. DIO results in the infiltration of macrophages in the mammary gland and the increased expression of pro-inflammatory cytokines, particularly TNF-α and MCSF. In addition, the expression of RANK is greater in the mammary glands of mice fed HFD.
Results: Figures

**Figure 1. The Effects of DIO on Pup Survival**
The survival rate of pups was compared between mice fed CD (CD) and mice fed HFD (HFD). The Survival rate is presented as the percent of pups born that survive to lactation day 5. *(P<0.001). Mean±SD was graphed.

**Figure 2. The Effects of DIO on Milk Secretion**
Milk secretion was compared between mice that were fed CD (CD) and mice fed HFD (HFD). Milk secretion was measured as the weight gain in pups after suckling and expressed as milk (mg) consumed per pup. Mean±SD was graphed.
Figure 3. Comparison of Whole Mount Mammary Glands during Lactation
Representative carmine stained whole mount mammary glands from mice fed CD (CD) compared with mice fed HFD (HFD). Note the densely packed alveoli in both mammary glands. Images taken at 5x view.
Figure 4. The Effects of DIO on Morphology of the Lactating Mammary Gland
Morphology of the lactating mammary gland is visualized by Hand E staining. Sections from mice fed CD are presented from lactation day 1 (LD1) to lactation day 5 (LD5) (A) for comparison with the morphology of the mammary gland at lactation day 5 from a mouse fed HFD (HFD) (B). Sections imaged at 40x are presented on the right while sections imaged at 10x are presented on the left (A and B). Note the lumens of alveoli filled with secretory products indicative of normal secretion (arrows in A and B).
Figure 5. The Effects of DIO on Expression and Abundance of Secretory Proteins in the Mammary Gland

A) The mRNA expression of whey acidic protein (WAP), α-lactalbumin (aLac) and β-casein (bCas) in the mammary glands of mice fed CD (CD) was compared with that of mice fed HFD (HFD). Expression is presented as the fold change from CD mean. *(P<0.01)

B) The protein abundance of β-casein (b-Cas) and whey acidic protein (WAP) in the mammary gland of mice fed CD (CD) were compared to that of mice fed HFD (HFD). Protein abundance was normalized to β-actin. *(P<0.01). Mean±SD was graphed.
Figure 6. The Effects of DIO on Protein Concentration in Milk
The protein concentration in milk from mice fed CD (CD) was compared with milk from mice fed HFD (HFD). Mean±SD was graphed. *P<0.001.
Figure 7. The Effects of DIO on Protein Composition in Milk
Coomassie stained gels loaded with milk by equal volume (A) and equal concentration of protein (C). Molecular weight markers are located on the left of the gel and proteins are labeled at the right. The quantitative analysis of band density for milk loaded by volume (B), milk loaded by protein concentration, *P<0.01 (D). Band intensity was normalized for serum albumin abundance for milk loaded by protein, *β-casein, P<0.01; γ-casein, P<0.01; ε-casein, P<0.05 (E). Mean±SD was graphed.
Figure 8. The Effects of DIO on Relative Adipocyte Size
The visual comparison of a representative H and E stained mammary gland sections for mice fed CD (CD) and mice fed HFD (HFD). Both images are taken at 40x magnification and the red line in both images is the same length, demonstrating how two adipocytes from a mouse fed CD are roughly the same size as one adipocyte from a mouse fed HFD.
Figure 9. The Effects of DIO on Number of Macrophages in the Mammary Gland
A) Macrophages were detected by IHC using the macrophage specific marker P4/80 and visualized with DAB staining (black) in mammary gland sections from mice fed CD (CD) and mice fed HFD (HFD) at lactation day 5.
B) The quantification of macrophages presented as number of macrophage positive staining (#) per unit area. Mean±SD was graphed. *P<0.001.
Figure 10. The Effects of DIO on Cytokine mRNA Expression in the Mammary Gland

The mRNA expression of TNF-α (TNFa), MCSF, IL-6, IL-1b, and IFNγ (IFNy) in the mammary glands of mice fed CD (CD) was compared with that of mice fed HFD (HFD). Expression is presented as the fold change from CD mean. *TNFa, P<0.05; MCSF, P<0.01.

Figure 11. The Effects of DIO on the mRNA Expression of RANK in the Mammary Gland

The mRNA expression of RANK in the mammary glands of mice fed CD (CD) was compared with that of mice fed HFD (HFD). Expression is presented as the fold change from CD mean. *P<0.01.
Discussion
Obesity impairs the secretion of milk at the onset of lactogenesis which is the onset of copious milk production\textsuperscript{14}. In animal studies, the failure to initiate lactogenesis is characterized by empty stomachs and early pup death\textsuperscript{14}. Flint et al noted the accumulation of lipid droplets in mammary epithelial cells and lower expression of $\beta$-casein, WAP, and $\alpha$-lactalbumin mRNA in the mammary glands of mice fed high fat diet at lactation day 1, indicative of impaired secretion\textsuperscript{18}. Although defects responsible for impaired lactation are not understood, obesity-impaired mammary gland development has been reported in non-pregnant\textsuperscript{17} and pregnant\textsuperscript{18} mice, both exhibiting a lower degree of branching, which would suggest reduced secretory capacity.

However, during mid-lactation, Flint et al observed that the mammary gland morphology of mice fed a high fat diet was similar to that of mice fed a control diet, with secretory products appropriately expressed and filling the lumen of alveoli indicating that lactogenesis II had been established\textsuperscript{18}. The current literature suggests that DIO does not impair secretion beyond a delay in lactogenesis, but this has not been determined. Our study aimed to determine if DIO impairs secretion during early (lactation day 5), established lactation. Upon preliminary examination of mammary gland whole mounts, we noted a smaller area of the mammary gland covered by alveoli in mice fed a high fat diet compared with controls, suggesting impaired development and a possible reduction in the secretory capability of the mammary gland. However, we found no differences in the number of alveoli between mice fed a high fat diet and mice fed a control diet, suggesting that the secretory capacity was normal, which was confirmed using weigh-suckle-weigh. We also determined that the abundance of milk proteins in the mammary gland was not impaired by DIO. Together, our data indicate that secretion is normalized by day 5 of lactation which is consistent with reports from Flint et al who found no abnormalities in mammary gland morphology or differences in pup weight with maternal DIO after lactation day 3\textsuperscript{18}. Our data suggest that once lactogenesis II has been established obese women suffer no impairment in secretion, thus emphasizing the importance of adequate support during early attempts at breastfeeding and the initiation of lactogenesis.
There is very little research examining the effects of obesity on milk composition. Flint et al found lower mRNA expression of key secreted proteins, $\alpha$-lactalbumin, $\beta$-casein, and WAP, in the mammary glands of mice fed a high fat diet compared with mice fed a control diet; however, neither protein abundance or milk protein composition was assessed. $\alpha$-lactalbumin is a key determinant in secretion as it interacts with galactosyltransferase to form lactose synthase to produce lactose in the mammary gland\(^ {45}\), therefore inhibited expression of $\alpha$-lactalbumin would likely result in low milk lactose as well. Lactose also drives osmotic pressure, thus low milk lactose may result in low milk volume. One might therefore expect milk protein, lactose or volume to be lower in the milk of an obese mother. We found no difference in milk lactose concentration in the milk from mice fed a high fat diet compared with milk from mice fed a control diet consistent with previous reports in rats\(^ {55}\). In contrast to reports from Flint et al\(^ {18}\), we saw no difference in $\alpha$-lactalbumin mRNA expression in the mammary gland or milk volume consumed by pups, both consistent with normal lactose concentration. Low $\alpha$-lactalbumin expression noted by Flint et al at lactation day 1 is consistent with their finding that milk production is reduced\(^ {18}\). Flint et al observed that $\alpha$-lactalbumin expression had increased in mice fed high fat diet by lactation day 10 and although the expression was still significantly lower than controls, pup growth was no longer delayed\(^ {18}\), suggesting milk secretion was not impaired and thus lactose concentration was likely not impaired.

Rolls et al noted a higher fat content in the milk of obese rats\(^ {55}\). We determined that DIO did not have a significant effect on %fat in milk, which is not consistent with other findings\(^ {55}\). This discrepancy may be the result of difference in species, but more likely is an effect of differences in diet. Milk fat content as well as composition is greatly affected by maternal diet\(^ {50}\). The rats in the study conducted by Rolls et al were fed a standard rodent chow supplemented with high fat foods such as salami and cookies\(^ {55}\), the use of such foods makes it difficult to determine nutrient intake compared to a formulated diet, this could result in differences in weight gain as well as milk fat. There was a trend
(P=0.1412) toward higher milk fat in our mice fed high fat diet comprised of lard as the only source of additional fat, but the variation was so great, the difference was not significant.

Rolls et al noted a lower protein concentration in the milk of obese rats. We examined protein in milk and found total protein in milk from mice fed a high fat diet was significantly lower than protein in milk from mice fed a control diet, consistent with previous reports. Our evaluation of milk protein was extended to analyzing the protein composition of milk from mice fed a high fat diet, which to our knowledge has not been previously reported. We first noted that milk from mice fed a high fat diet contained less serum albumin than the milk of mice fed a control diet. Serum albumin is not produced by the mammary epithelial cell, but transcytosis across the epithelium; our data suggests that there may be an impairment somewhere along the transcytotic pathway. Serum albumin has been shown to colocalize with IgA in the mammary gland and is believed to share the same transcytotic pathway as IgA. This suggests that if there is an impairment of the transcytotic pathway resulting in lower serum albumin in milk, the concentration of IgA and other immunoglobulins may be compromised as well, which would compromise the immune function and health of the infant. It is important to note that the transcytotic pathway is completely separate from the secretory pathway of milk proteins such as casein. We found the abundance of caseins was higher in milk from mice fed a high fat diet, indicating that mammary gland abundance and secretion of casein is elevated with DIO. This was consistent with our finding that β-casein protein abundance was elevated in the mammary glands of mice fed high fat diet. This could have a negative impact on infant health as a high ratio of casein:whey has been shown to disrupt the bioavailability of micronutrients in milk. Casein binds divalent metals such as zinc, and zinc from milk with higher casein levels has been determined to be less bioavailable than milk with a lower casein:whey ratio. We measured milk zinc concentration and found no effect of DIO, but higher casein content in milk suggests that zinc may be less available to the infant, thus DIO may pose a risk to infant health by reducing the availability of essential nutrients. Alternatively, the binding of β-casein has been shown to enhance the absorption of iron in milk while α-casein inhibits iron
availability\textsuperscript{99}. This suggests that the milk from an obese mother may have more available iron for the infant. Our data suggests DIO may alter the bioavailability of micronutrients in milk and thus warrants additional research.

Obesity is a state marked by chronic inflammation. Adipocytes secrete a number of pro-inflammatory cytokines such as IL-1\textbeta, IL-6, TNF-\alpha, and MCP-1\textsuperscript{24,26}. TNF-\alpha and MCP-1 secreted from adipocytes act as chemoattractants, stimulating the infiltration of macrophages into adipose tissue\textsuperscript{30}. Macrophages can in turn secrete pro-inflammatory cytokines such as TNF-\alpha and IL-6\textsuperscript{28}, thus producing a self-perpetuating cycle of chronic inflammation. To our knowledge there are no published reports of obesity-induced inflammation in the lactating mammary gland. Kimikawa et al have examined the infiltration of macrophages in the mammary glands of non-pregnant mice fed high fat diet\textsuperscript{17}. In addition to the presence of macrophages, Kimikawa et al found a higher mRNA expression of TNF-\alpha, suggesting an inflammatory response\textsuperscript{17}. We hypothesized that increased adiposity will alter the mammary gland microenvironment by an inflammatory response. We showed that although the number of adipocytes in the mammary gland were not different between mice fed high fat diet and mice fed control diet, the adipocytes in the mammary glands of mice fed high fat diet were larger, which was reflected in the greater weight of mammary glands from mice fed high fat diet. This indicates that DIO will increase adiposity within the mammary gland, consistent with previous reports\textsuperscript{17}. We also that determined mammary glands of mice fed high fat diet were infiltrated with macrophages, suggesting an inflammatory response to increased adiposity. The mRNA expression of pro-inflammatory cytokines TNF-\alpha, MCSF, and IL-6 were increased in the mammary glands of mice fed high fat diet, indicating that DIO produces an inflammatory mammary gland microenvironment. The consequences of an inflammatory microenvironment in the mammary gland are not fully understood; however, one possibility is that the pro-inflammatory microenvironment may alter expression of milk proteins. TNF-\alpha plays a prominent role in mammary gland development, inducing proliferation and inhibiting the expression of \beta-casein and
WAP\textsuperscript{90}. Our study determined DIO increased TNF-\(\alpha\) mRNA expression and decreased WAP and \(\beta\)-casein mRNA expression, suggesting TNF-\(\alpha\) may be inhibiting the expression of WAP through the induction of transcription factor NFkB as previously suggested\textsuperscript{90}. In our study however, increased expression of TNF-\(\alpha\) was not associated with the reduced abundance of \(\beta\)-casein or WAP proteins, in fact the amount of \(\beta\)-casein produced by the mammary gland and secreted into milk were higher. This suggests that a separate mechanism induced by DIO is maintaining \(\beta\)-casein protein and WAP protein abundance, perhaps post-translational modifications that enhance protein stability. For example, it has been proposed that Akt stimulates the translation of \(\beta\)-casein protein\textsuperscript{100}. RANK is a major activator of Akt signaling\textsuperscript{101}, thus implicating a role for enhanced RANK expression on \(\beta\)-casein modulation.

RANK is regulated temporally in the mammary gland with higher expression during pregnancy in order to maintain cellular proliferation and a decrease in expression beginning in late pregnancy to allow the differentiation and maturation of alveoli\textsuperscript{102}. The expression of RANK decreases during late pregnancy and is reduced during lactation, allowing the differentiation of mammary epithelial cells to a secreting phenotype\textsuperscript{102}. Overexpression of RANK and increased RANKL/RANK signaling will result in the impaired accumulation of secretory products in vitro and will prevent the differentiation of mammary epithelial cells in vivo\textsuperscript{102}. Interestingly, the expression of RANK is stimulated by MCSF\textsuperscript{103}, therefore, it is possible that increased adiposity will result in macrophage infiltration, and the increased production of MCSF which will in turn increase the expression of RANK which could inhibit lactation. We determined the mRNA expression of MCSF is elevated with DIO, as was the expression of RANK. Our study thus provides preliminary evidence to suggest the RANK signaling pathway as a mechanism involved in obesity-impaired lactation. Despite the increased expression of RANK, our mice fed a high fat diet were able to successfully lactate, suggesting other mechanisms in play which may have an antagonistic effect on RANK signaling. Further studies are needed to address this.
In conclusion, our data indicate that DIO alters the mammary gland microenvironment, resulting in increased expression of pro-inflammatory cytokines which have the potential to impact many aspects of lactation. The pro-inflammatory microenvironment has the potential to impact the immunological factors as well as bioavailability of micronutrients in milk and thus may negatively impact infant health. Further research is needed to examine the effects of the inflammatory mammary gland microenvironment caused by DIO as pro-inflammatory cytokines have the potential to influence milk composition and mammary gland function.
Appendix A: Supplementary Figures

Sup.Fig. 1. The Effects of DIO on Maternal Body Weight
The growth curves for mice fed CD (CD) and HFD (HFD). 2 separate cohorts of mice were used, the growth curves depicted for each (1 or 2). Mean±SD was graphed. The mean weight of mice fed CD was significantly (P<0.05) lower than the mean weight for mice fed HFD by the initiation of breeding (week 5) for both cohorts of mice.

Sup.Fig. 2. The Effects of DIO on Maternal Food Consumption
Weekly food consumption (g) was compared between mice fed CD and mice fed HFD (presented is the data for cohort 1, the results for cohort 2 were similar). There was no significant difference in amount of food consumed, except for week 5, P<0.001. Mean±SD was graphed.
Sup. Fig. 3. The Effects of DIO on Expression and Abundance of Epithelial Cell Specific Proteins

A) The protein abundance of e-cadherin in mammary glands of mice fed CD (CD) and HFD (HFD). P=0.9949. Mean±SD was graphed.

B) The mRNA expression of K18 in the mammary glands of mice fed CD (CD) was compared with that of mice fed HFD (HFD). Expression is presented as the fold change from CD mean. P=0.472.
Sup.Fig 4. Identification of Macrophages in Mammary Gland Whole Mounts of Non-Pregnant Mice.
Mammary gland whole mounts were visually assessed for the presence of macrophages by locating the ‘crown like structures’ of darker carmine staining (A) at 40x. (B) A 10x view of the image in Panel A. Examples of macrophages identified in 3 different mice fed HFD at 40 x(C). Macrophages were not seen in whole mounts from mice fed CD.
Sup.Fig 5. The Identification and Relative Abundance of Pro-Inflammatory Cytokine in the Mammary Gland
The quantitative analysis of cytokine protein abundance in the mammary gland of a mouse fed CD (CD) and a mouse fed HFD (HFD). Abundance normalized to internal control (Corrected Expression).
References


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