**1/13/21**

**Title:**

A mouse model of adipocyte mTORC1 activation increases milk fat.

**Author names and affiliations:**

Noura El Habbal1, Hannah J. Hafner2, JeAnna R. Redd1, Zach Carlson2, Molly C. Mulcahy1, Allison C. Meyer1, Holly Reynolds2, Brigid Gregg1,2, and Dave Bridges1

1. Department of Nutritional Sciences, University of Michigan School of Public Health, Ann Arbor, Michigan, U.S.A.
2. Department of Pediatrics, University of Michigan Medical School, Ann Arbor, Michigan, U.S.A.

**Corresponding author:**

Dave Bridges Email address: [davebrid@umich.edu](mailto:davebrid@umich.edu)

Postal address: 3866 SPH I 1415 Washington Heights Ann Arbor, Michigan 48109-2029 Telephone: +1 (734) 764-1266

**Declaration of Interests**

The authors have no competing interests to declare.

**Abstract:**

Changes in the mammary gland biology during pregnancy and lactation and the interplay between mammary epithelial cells and adipocytes remain largely unknown. Mammary adipocytes are thought to "disappear" during lactation, and their contribution to milk composition is still not fully understood. A major nutrient sensor in most tissues is the mechanistic target of rapamycin 1 (mTORC1). To understand the role of mammary glands in sensing maternal nutritional status, we aimed to understand the effect of adipocyte mTORC1 hyperactivation on mammary gland function, milk composition, and offspring outcome in an adiponectin-Cre *Tsc1/2* knockout mouse model. Knockout (KO) and wild type (WT) C57BL/J6 female mice were mated with KO or WT males at 6-8 weeks of age. Data on maternal body mass composition during pregnancy and lactation, pup survival and weight, litter numbers, milk volume production, milk composition and lipidomic analysis, and mammary gland weights and histology were assessed. We used RNA sequencing to investigate mammary gland gene expression. Our results show that female pups born to KO dams are heavier, with KO dams having higher milk fat composition. Additionally, KO dams had lower percentage saturated fatty acids, higher percentage of monounsaturated fatty acids, and lower milk omega6:omega3 ratio a. Gene expression showed 112 upregulated and 153 downregulated genes in the mammary glands and differentially expressed metabolic pathways. These results suggest suggests a key role of mammary adipocyte mTORC1 in mammary gland function, milk composition, and offspring health.

**Key words**: Mammary glands, Milk composition, Adipocytes, mTORC1, Obesity

**1.0 Introduction**

Maternal obesity is on the rise and has reached 38% in 2013 (1). Obesity can impact offspring health during *in utero* development and during early postnatal life through lactation. According to the Developmental Origins of Health and Disease theory, the health of the offspring is highly influenced by intrauterine and early postnatal exposures (2). Furthermore, obesity can affect milk composition and lactational capacity. However, how maternal obesity affects lactation and offspring health during the critical postnatal window of development and the mechanisms that mediate those effects remain less clear.

Maternal obesity can influence early postnatal development through its impact on mammary gland function. Maternal weight has been positively correlated with milk protein and fat contents and overall caloric value (3). Initiation of lactation is delayed in pre-pregnancy obese or overweight women (4), while the duration of breastfeeding for 6 months or more is reduced (5). The probability of early weaning at 3 months postpartum was highest for infants of obese mothers (6).

~~The macronutrients present in mammalian milk are lactose, protein, and lipids. Lactose, the main carbohydrate in milk, is synthesized in the Golgi of the alveolar epithelial cells (7, 8). Milk proteins are synthesized in the rough endoplasmic reticulum of the alveolar epithelial cells (7, 8). Lipids, almost exclusively in the form of triglycerides, are synthesized in the smooth endoplasmic reticulum by~~ *~~de novo~~* ~~synthesis from available glucose, or they are derived from maternal diet or fatty acids from adipose tissue stores (7–9). The mechanisms by which these triglycerides are packaged and transported into the milk remain elusive (9, 10).~~

The mammary gland is composed of adipocytes and alveolar cells. Mammary adipocytes form a major proportion of the mammary gland and are necessary for proper gland development and proliferation (11, 12). During pregnancy and lactation, the mammary adipocytes undergo transformation and almost disappear to support lactation and epithelial cell expansion then revert to their original state at weaning during the involution process (13). The mammary adipocytes within closer proximity to the alveolar epithelial cells are thought to provide a primary source of lipids for milk production (13). Although the mammary fat pad undergoes restructuring during pregnancy and lactation, the exact fate of these fat cells and the mechanisms regulating their transformation are less clear.

Mechanistic Target of Rapamycin Complex 1 (mTORC1) is a critical nutrient sensor and a main regulator of protein and lipid synthesis (14, 15). In the presence of anabolic signals like insulin, energy abundance, and amino acid availability, mTORC1 function is upregulated via the Akt pathway (16). mTORC1 promotes lipogenesis and adipogenesis and inhibits lipolysis (14, 17). Obese subjects that had excess fat mass showed increased mTORC1 activity (16). Hyperactivation of mTORC1 in mammary glands has been studied in the context of breast cancer (18), but little is known about its role in macronutrient synthesis in the mammary gland during lactation (8). We show that chronic mTORC1 activation in maternal adipocytes via deletion of its upstream negative regulator, *Tsc1*, causes increased milk fat composition but a healthier lipid profile with lower omega6:omega-3 ratio, reduced gene expression of immune response pathways in the mammary glands, and increased weight of lactating offspring.

**2.0 Materials and Methods**

**2.1 Animals**

All mice were purchased from the Jackson Laboratory. All mice were fed a normal chow diet with *ad libitum* access to food and water.

To hyperactivate adipocyte mTORC1 and generate an adipose-specific *Tsc1* knockout, we used the Cre-loxP recombination technology. *Tsc1* fl/fl mice with flanked *Tsc1* gene exons 17 and 18 were crossed with *Adipoq*-Cre mice expressing the adipocyte-specific constitutive Cre recombinase controlled by adiponectin gene promoter. The parental strains (F0) for this experiment were 6-8 week old male *Tsc1* fl/fl ;Tg/+ or *Tsc1* fl/fl ;+/+ crossed with 6-8 week old female *Tsc1* fl/fl ;+/+ or *Tsc1* fl/fl ;Tg/+, respectively. The offspring (F1) were a combination of knockout (KO, fl/fl;Tg/+) and phenotypically wild-type (WT, fl/fl;+/+) at an expected ratio of 1:1. The knockout of the floxed alleles are driven by Adiponectin-Cre (19, 20), which is expressed in all adipocyte lineages (brown, white and mammary adipocytes) as shown in Wang et al. (21). As such one limitation of this approach is that all adipocytes are affected, not just mammary adipocytes (for which there is no known specific Cre driver). All mice were bred in our facility at the University of Michigan. All animal procedures were carried out in accordance with the National Institute of Health guide for the care and use of laboratory animals and was approved by the University of Michigan Institutional Animal Care and Use Committee prior to the work being performed.

Mice had *ad libitum* access to normal chow diet and water. Male breeders were removed from the cage after 16 days of mating to avoid the occurrence of a second pregnancy.We checked for litters on a daily basis after 2.5 weeks of mating. The number of pups born was recorded to determine maternal fertility and pup viability. After delivery (delivery day denoted as postnatal day 0.5, PND0.5), the dams continued to have *ad libitum* access to food and water.

Pups were sexed and culled to four animals (2 females and 2 males, when possible) per litter at PND2.5. The offspring were weighed at PND0.5, PND7.5, 14.5, 16.5. The pups underwent body composition analysis by echoMRI at PND16.5 then immediately sacrificed.

**2.2 Body Composition and Indirect Calorimetry**

In all groups, the dams underwent body mass assessment three times a week during pregnancy and lactation and on the day of delivery using magnetic resonance (EchoMRI 1100, EchoMRI, Houston, TX) to assess body composition. Mice were weighed by dynamic weighing to capture accurate weight using a digital scale. The weight was recorded along with the mouse ear tag number. The mouse was then gently placed in the MRI tube with the plunger slightly compressing along the mouse’s body to ensure restrained movement during the measurement. Fat, lean, free water and total water mass (g) were recorded for each animal. We weighed offspring and performed body mass assessment using MRI at PND16.5.

## **2.3 Sacrifice and Tissue Collection**

All animals were sacrificed using anesthetic gas inhalation (5% isoflurane drop jar) at PND16. Cervical dislocation was conducted as a secondary method to confirm euthanasia. The mice were pinned on a dissection board in a supine position. We dissected KO and WT dams by a midline incision of the skin from the rectum to the diaphragm and extracted thoracic, abdominal and inguinal mammary glands. Briefly, the peritoneum was pulled apart from the skin. The lower glands were excised carefully then weighed. Portions of the upper and lower glands were embedded in paraffin for histology, while the remaining tissue from the lower left and right mammary glands were collected in 2ml tubes and snap frozen in liquid nitrogen and later stored at -80C for molecular studies. Offspring of dams were sacrificed without tissue extraction at PND16 after body assessment measurements.

## **2.4 Determining Milk Output Volume**

At PND10.5, considered the peak of lactation (22), we determined milk output volume for the WT and KO dams. To determine milk volume, we used the weigh-suckle-weigh technique (23). Briefly, we weighed the dam separately then determined the aggregate weight of the pups. The dam and pups were then separated for two hours. During the two-hour separation, the pups were placed in a new cage and were kept warm using a heating pad. In the meantime, the dam remained in its initial cage with *ad libitum* access to normal chow diet and water. After the two-hour separation period, the dam was weighed again and the aggregate weight of the pups was measured. The pups were then returned to the dam’s cage and were allowed to nurse for one hour undisturbed. At the end of the nursing timepoint, the dam was weighed again and the aggregate weight of the pups was determined. After this one-hour nursing period, milk volume was determined as the weight change of the pups after nursing and after the 2-hour separation. The difference in the dam’s weight after nursing and after the 2-hour separation was calculated to further ascertain the dam’s milk supply.

## **2.5 Determining Milk Composition**

On PND16.5, we collected milk samples (~0.5ml) from the nursing dams. Briefly, we anesthetized the dam after two hours of separation from the pups by intraperitoneal injection of Ketamine (0.1275g/kg body weight). We then performed an intraperitoneal injection of oxytocin into the forelimb (2U/dam) to induce milk production. The dam’s nipples were manually squeezed to promote milk letdown, and the milk was collected into a 1.5 ml tube via suction. After milking was complete, the dam was immediately sacrificed using isoflurane and a secondary measure of cervical dislocation. We then dissected the dam by a midline incision of the skin, extracted thoracic, abdominal and inguinal mammary glands. The lower mammary gland pads were weighed. A small section of the lower mammary glands was saved for paraffin embedding for histology while the rest was snap frozen in liquid nitrogen and cryopreserved. Milk protein composition was analyzed using SDS-PAGE gels and diluted milk samples (4-fold dilution).

## **2.6 Determining Milk Protein Concentrations**

Milk samples collected from WT and KO dams were assessed for protein content. Milk was diluted to a factor of 4 (1:3 in PBS+EDTA). Skimmed milk was collected after centrifuging. Samples were heated to ~95C and loading cocktail was added onto the plastic plate with the gel along with a ladder. Gels were stained by Coomassie blue and quantified by near-infra-red imaging. Imaging was done using LiCOR Odyssey to determine protein levels of whey acidic protein, alpha casein, beta casein, lactoferrin, and serum albumin identified based on known molecular weights.

## **2.7 Determining Milk Fat Content**

Milk samples collected from WT and KO dams were assessed for fat content by the creamatocrit method using a hematocrit centrifuge (24). Briefly, samples were diluted to a factor of 4 (1:3 in PBS). Diluted samples were transferred into plain micro-hematocrit glass capillary tubes. The tubes were sealed from one end using Critoseal. The tubes were later placed in CritSpin mini-creamatocrit spinner. Samples were centrifuged for 8 cycles of 120 seconds per cycle for a total spin time of 16 minutes. The capillary formed layers of white fat and non-fat milk. The distance of the fat layer was measured in millimeters (mm) accurately using a 150 mm dial caliper (General Tools and Instruments 6” Dial Caliper, 2011). The total volume of milk (fat + non-fat milk) was also measured in mm. Percentage of fat was determined with respect to the total milk volume.

## **2.8 RNA Sequencing**

Using the lower right mammary gland tissues collected from the dams, we assessed whole-transcriptome RNA expression using five wild-type and six knockout samples. RNA samples were prepared from the mouse tissues using the PureLink RNA Mini Kit. Briefly, tissues were cut on dry ice to ~50mg samples then homogenized and treated to collect the purified RNA. The RNA was quantified using a nanodrop and purity was verified by an Agilent Bioanalyzer. All samples had a RNA integrity number (RIN) higher than 7.5. Library preparation and next generation sequencing was conducted by the Advanced Genomics Core at the University of Michigan. Paired-end poly-A mRNA libraries were generated and sequenced to an average depth of 57M (range 46M-69M) reads/sample on Illumina NovaSeq platform. Reads were aligned to the mouse reference genome GRCm38.p6 using Salmon v 1.3.0 (25) with the gc-bias and validateMappings flags. Mapping efficiency was 54.8% (sample range 53-56.6%). Transcript-level data was reduced to gene-level data via tximeta v1.8.4 (26) and txiimport v1.18.0 (27) prior to analysis by DESeq2 v1.30.1 (28). To determine differential expressed genes we evaluated 14242 genes, excluding those with low or no read counts, identifying 265 differentially expressed genes (q<0.05). Full gene expression results are reported in Supplementary Table 1. For gene set enrichment analyses, we used ClusterProfiler v3.16 after ranking genes by fold change. Gene set enrichment results are presented in Supplementary Table 2. Data are available from GEO at accession number XXXX

## **2.9 Western Blotting**

Using the lower right mammary gland tissues collected from the dams, we assessed TSC1/2 protein levels and mTORC1 activity to confirm knockout in mammary glands as a whole tissue and specifically in mammary adipocytes. To better determine the site of the knockout, a separate cohort of KO and WT dams were bred for this purpose. The mammary gland fat and stromal vascular fraction (SVF) were separated. Genotyping was separately done on the glandular and SVF compartments to verify knockout specificity in the mammary gland. Briefly, a portion of the whole lower right mammary gland, the SVF, and the fat samples was boiled and loaded into different wells with a ladder control. Proteins were transferred to nitrocellulose overnight. The matrix was stained for total protein using Revert total protein and scanned by LiCOR to normalize against total protein. Samples were incubated with the primary then the secondary antibodies. Briefly, antibodies against TSC1/2, total and phosphorylated mTORC1 targets (S6K, 4EBP1, S6) and regulators (Akt, IRS) were used.

**2.10 Lipidomic Analysis**

Lipidomic analyses were done by the Biomedical Research Core Facilities at the University of Michigan. Briefly, milk samples were frozen at -80°C until analysis to prevent lipid hydrolysis. and peroxidation. Samples were quickly thawed once for lipidomic analysis without undergoing multiple freeze-thaw cycles. Long chain fatty acid concentrations were determined by gas chromatography using an assay developed and optimized to analyze human milk in consultation with the University of Michigan Regional Comprehensive Metabolomics Resource Core. Results were reported on 33 lipid classes from C14:0 to C24:1. Lipidomic analysis methods include sample extraction, semi-purification and derivitization followed by fatty acid measurement by gas chromatography using an Aglient GC equipped with flame ionization detector. The coefficient of variation for lipidomic analysis is 2.5-3.6%.

## **2.11 Histology**

Mammary glands collected from WT and KO dams were embedded in paraffin and Hematoxylin and Eosin (H&E) stained at the Rogel Cancer Center’s Tissue and Molecular Pathology. Slides were blindly assessed for alveolar count and adipocyte size and count.

## **2.11 Statistical Analysis**

All statistical analyses were performed using R, version 4.0. For longitudinal measurements including body composition, food intake, and pup weight gain, data were analyzed using mixed linear models. Statistical significance was designated at p<0.05. We tested for sex-differences in all outcomes and report modifying effects of sex when significant.

**3.0 Results**

To understand how activation of mTORC1 in adipocytes affects lactation we evaluated pregnant mice that were wild-type (*Tsc1fl/fl; Adipoq-CreTg/+*) andknockout(*Tsc1fl/fl; Adipoq-CreTg/+*). In this model all adipocytes, including all white and brown adipocyte depots including mammary adipocytes are ablated for TSC1 and are predicted to have activation of mTORC1. These mice were mated with a male and dams and their offspring were monitored throughout lactation (See Figure 1A).



Figure 1: Dams and pups were monitored throughout lactation. Pup births and weights were measured on postnatal day (PND) 0.5. Pups were culled at PND4. Pups weights were assessed on PND7.5, 14.5 and 16.5. Milk volume was measured on PND10.5 using the weigh-suckle-weigh method. On PND16.5,milk was collected from dams and mammary glands extracted. Maternal body composition was measured on PND0.5 after delivery and every Monday, Wednesday, and Friday thereafter until and including PND16.5.

## **3.1 Maternal Body Composition Was Similar during Pregnancy and Lactation in Adipocyte *Tsc1* Knockout Mice**

Dam body composition was measured every Monday, Wednesday, and Friday during pregnancy and lactation and on the days of delivery and sacrifice. Body weights were comparable between dams (Figure 2A). Lean mass was also comparable between KO and WT dams (Figure 2B). KO dams had a slightly lower fat mass during pregnancy and during lactation (Figure 2C). While WT dams lost fat mass gradually during lactation, KO dams gained fat mass (Figure 2D, d=0.125g/day, p<0.001). Consistent with this, KO and WT dams had similar food intake during pregnancy, however during lactation KO dams had lower food intake compared to WT dams (Figure 2E). The average litter size from KO and WT dams was similar (Figure 2F). Pups were culled to four pups per dam to normalize milk supply.

|  |  |
| --- | --- |
| A | B |
| C | D |
| E | F |

Figure 2: Maternal body composition during gestation and lactation, food intake, and litter size of WT and KO dams. (A) Maternal body weights. (B) Maternal lean mass. (C) Maternal fat mass. (D) Maternal fat mass change from the day of delivery until PND16.5. (E) Average weekly food intake. (F) Litter size of WT and KO dam.

## **3.2 Mammary Gland Weights of KO Dams were Lighter**

Based on these changes in milk composition, we next examined the mammary glands from the adipocyte *Tsc1* knockout dams. At PND16.5, the lower abdominal and inguinal mammary glands were collected and weighed from KO and WT dams. The right lower mammary glands of KO dams were significantly lighter than those of WT dams (Figure 3, d=20.68%, p=0.042). Left lower mammary glands of KO dams significantly weighed less than those of WT dams (Figure 3, d= 28.75%, p=0.001).

## 

Figure 3: Mammary glands collected from lactating WT and KO dams on PND16.5 showing significant reductions in weight of left and right mammary glands of KO dams.

## **3.3 Adipocyte Size and Count**

So far, the KO dams have more smaller-sized adipocytes compared to the WT. Additionally, the KO dams have fewer number of adipocytes in the lower right mammary gland.

## **3.4 Pups Born to Adipocyte *Tsc1* Dams are Heavier During Peak Lactation.**

To evaluate effects on the offspring we monitored growth of pups during lactation (Figure 4A) There was no significant difference in pup weight at birth (PND0.5). At PND7.5, females born to KO dams were heavier than females born to WT dams (Figure 4B, d=XX, p=0.**047**), but weights of males born to KO or WT mothers were not significantly different (Figure 4B). At PND14.5 and PND16.5, there were no weight differences between groups or sexes. We hypothesize that this is because at later time points mice are eating more chow-based food and relying less on maternal lactation.

|  |  |
| --- | --- |
| A | B |

Figure 4: Pup weights during lactation. (A) Weight trajectories of male and female offspring of WT and KO dams weighed on PND7.5, 14.5, and 16.5. (B) Weights of male and female offspring of WT and KO dams on PND7.5.

## **3.5 Adipocyte *Tsc1* Dams Produce Similar Volumes of Milk, but with Higher Milk Fat.**

Based on the changes in offspring weight trajectories, we calculated the mass of milk produced per dam via the weigh-suckle-weigh technique. This was calculated by a two-hour separation period and then as the difference between pup weight after nursing for one hour and pup weight before nursing. As shown in Figure 5A, this was not significantly different between groups. Similar data were obtained by measuring the weight of the dams pre- and post- lactation. To test milk composition, milk was extracted from dams at PND 16.5. Creamatocrit fat analysis revealed that milk of KO dams had higher fat percentage than milk of WT dams (Figure 5B, d=34.07%, p=0.024).Using a milk gel, we quantified total protein and major milk proteins based on known molecular weights. Milk proteins including alpha-Casein, beta-Casein, lactoferrin, whey alpha protein (WAP), and albumin were similar between milk of KO and WT dams. In terms of milk lactose XXX.

|  |  |
| --- | --- |
| A | B |

Figure 5: Milk production and fat composition. (A) Weight of milk produced by WT and KO dams assessed by pup weight gain after an hour of nursing was similar between pups of WT and KO dams. (B) Fat composition of milk from KO dams is higher than fat composition of milk from WT dams.

## **3.6 Adipocyte *Tsc1* Knockout Alters Fatty Acid Composition in Milk**

Lipidomic analysis of the milk samples collected from PND16.5 showed a healthier milk fat composition in the KO milk with lower percentage of saturated fatty acid (SFA) (Figure 6A, d=XX, p=0.008) and higher percentage of monounsaturated fatty (MUFA) (Figure 6B, d=XX, p=0.009) and similar percentages of polyunsaturated fatty acids (Figure 6C). Analysis of omega-3 and omega-6 quantities showed that KO milk had higher percentage of omega-3 (Figure 6D, d=XX, p=0.013), a similar percentage of omega-6 (Figure 6E), and a lower omega-6:omega3 ratio in KO milk (Figure 6F, d=XX, p=0.046).

## **3.7 RNA Sequencing Reveals Suppressed Expression of Adaptive Immune Markers and Increased Expression of Muscle Biosynthesis Genes**

To understand the mechanisms by which adipocyte mTORC1 activation affects mammary gland gene expression we performed bulk RNAseq on mammary gland explants from lactating wild-type and knockout dams. We identified 139 significantly differentially expressed genes between these groups (Figure 7A-B, and Supplementary Table 1). Among the most highly expressed genes in the mammary gland are secreted milk proteins. These transcripts are not significantly altered by adipocyte *Tsc1* knockout, but trend downwards (Figure 7C). Several markers of adipogenesis and PPARg were upregulated including *Plin4, Adipoq, Cav2,* and *Fabp4*, consistent with increased adipocyte numbers (Figure 7D). Furthermore, several genes involves in eicosonaoid signaling were also significantly upregulated including the enzymes *Plcb1*, *Ephx2*, and *Pla2g4a* as well as the prostaglandin receptor *Ptger3*, wheras *Ptgs1* was downregulated (Figure 7E). This is consistent with elevations in DHA in the breastmilk. Gene set enrichment analyses identified

**4.0 Discussion**

Here, we show that hyperactivation of mTORC1 in adipocytes increases weight of female offspring at PND7.5 and increases milk fat composition in KO dams with a healthier milk fat composition. We also demonstrate that mTORC1 hyperactivation in adipocytes causes reductions in mammary gland weight, number of mammary adipocytes, and size of mammary adipocytes in KO dams. Additionally, our model reveals differentially expressed pathways in the mammary glands which can influence offspring immunity.

Mammary adipocytes play a critical role in the development and successful functioning of mammary glands. At puberty, alveolar ducts expand at the expense of the fat pad in the mammary gland (29, 30). ~~A mouse model of lipodystrophy with underdeveloped fat tissues reveals smaller mammary adipocytes, accelerated ductal growth, and potential sloughing of the ductal epithelial cells into the lumen indicating suboptimal mammary gland function and growth compared to controls (31). During pregnancy and lactation, adipocytes have a unique supportive function. Recently, it has been determined that mammary adipocytes de-differentiate gradually during gestation and almost disappear entirely during lactation allowing more space for milk production by the mammary alveolar epithelial cells (13, 22). Adipocytes closest to the mammary epithelial cells de-differentiate quicker than those farther away in the cleared fat pad (30, 32). The alveoli expand at the expense of the fat pad almost entirely covering its area (33). It is hypothesized that the adipocytes in the body mobilize their fat stores and provide for the mammary epithelial milk lipid production, which explains the reduction in size of the adipocytes during lactation (33–35).~~ In our KO model, we observed increased emptying of mammary adipocyte content as evident by the smaller size of mammary adipocytes compared to the WT. We also observed a higher fat content in the milk produced from KO dams. This is consistent with the idea that these adipocytes could be emptying their content into the milk in KO dams at a higher rate than that of WT dams. Additionally, and supporting our finding, transgenic pregnant mice with activated AKT in the mammary epithelial cells showed distended alveoli during lactation and a higher lipid droplet composition and size in the mammary epithelial during gestation and lactation (36). Milk composition from these transgenic mice revealed higher fat percentage and a higher protein concentration compared to controls (36). AKT, upstream of mTORC1, may play a significant role in regulating mammary gland differentiation and lipid and protein synthesis (36) which is concordant with our findings that milk from dams with mTORC1 hyperactivation had increased milk fat composition. We see lower saturated fatty acid percentage the milk of KO dams which can be due to the higher monounsaturated fatty acid percentage potentially driven by increased gene expression of EPA to DHA converting enzymes. This can also explain the lower omega6:omega3 ratio which can be primarily driven by increased gene expression of EPA to DHA converting enzyme as DHA levels were higher in the milk of KO.

In addition to differences in milk composition and mammary adipocyte count and size, mTORC1 is a nutrient sensor and is crucial for proliferation and growth. Mice treated with rapamycin for 12 days starting at gestational day 19 had reduced mammary gland size and reduced epithelial tissue (37). Our data revealed that mTORC1 hyperactivation in adipocytes caused reductions in mammary gland weight. This could be explained by the increased fat content in milk from KO dams suggesting that mammary gland weights could be reduced due to the increased emptying of their content into the milk in KO dams. Our results are consistent with the idea that mTORC1 increases lipid synthesis which is incorporated into milk composition and can be causing reduced mammary gland weights.

Maternal obesity can influence the offspring health via pre-gestational, gestational and lactational exposures. Children of mothers with class III obesity are at 2.3 times higher risk of being large for gestational age (38). Children of overweight or obese mothers had increased weight gain at age 0-4 years and a higher BMI z-score compared to children of lean mothers (39). Our data revealed increased offspring weight of females at PND7.5 which is consistent with the human findings from previous studies.

**5.0 Conclusions**

We have shown that hyperactivation of mTORC1 activity in adipocytes of pregnant and lactating dams can impact milk composition and mammary glnd gene expression. These findings are crucial to better understand the effects of nutrient sensing in the mammary gland on milk production and composition. Our data supports our hypothesis that mTORC1 hyperactivation in adipocytes increases mammary adipocyte capacity to produce fat and secrete it into the produced milk. The mechanisms by which mTORC1 could be influencing mammary gland function and milk secretion is insightful for future research addressing the effects of maternal obesity on offspring health. We present data, for the first time, that the milk nutritional composition may reveal a higher energy density but a healthier overall lipid composition. This warrants further studies to unravel the mechanisms by which mammary adipocyte nutrient sensing pathways can affect offspring health.

**6.0 Acknowledgements**

This work was supported by funding from the NIH (R01DK107535 to DB and K01DK102526 to BG) and supported by core facilities at the Rogel Cancer Center (NIH P30CA046592).

**7.0 Author Contributions**

**8.0 References**

1. Chen, C., X. Xu, and Y. Yan. 2018. Estimated global overweight and obesity burden in pregnant women based on panel data model. *PLoS One*. **13**.

2. Barker, D. J. P. 2007. The origins of the developmental origins theory. *J. Intern. Med.* **261**: 412–417.

3. Bzikowska-Jura, A., A. Czerwonogrodzka-Senczyna, G. Olędzka, D. Szostak-Węgierek, H. Weker, and A. Wesołowska. 2018. Maternal Nutrition and Body Composition During Breastfeeding: Association with Human Milk Composition. *Nutrients*. **10**.

4. Rasmussen, K. M., and C. L. Kjolhede. 2004. Prepregnant overweight and obesity diminish the prolactin response to suckling in the first week postpartum. *Pediatrics*. **113**: e465-71.

5. Bider-Canfield, Z., M. P. Martinez, X. Wang, W. Yu, M. P. Bautista, J. Brookey, K. A. Page, T. A. Buchanan, and A. H. Xiang. 2017. Maternal obesity, gestational diabetes, breastfeeding and childhood overweight at age 2 years. *Pediatr. Obes.* **12**: 171–178.

6. Castillo, H., I. S. Santos, and A. Matijasevich. 2016. Maternal pre-pregnancy BMI, gestational weight gain and breastfeeding. *Eur. J. Clin. Nutr.* **70**: 431–436.

7. Anderson, S. M., M. C. Rudolph, J. L. McManaman, and M. C. Neville. 2007. Key stages in mammary gland development. Secretory activation in the mammary gland: it’s not just about milk protein synthesis! *Breast Cancer Res.* **9**: 204.

8. Rezaei, R., Z. Wu, Y. Hou, F. W. Bazer, and G. Wu. 2016. Amino acids and mammary gland development: nutritional implications for milk production and neonatal growth. *J. Anim. Sci. Biotechnol.* **7**: 20.

9. McManaman, J. L. 2009. Formation of milk lipids: a molecular perspective. *Clin. Lipidol.* **4**: 391–401.

10. McManaman, J. L. 2014. Lipid transport in the lactating mammary gland. *J. Mammary Gland Biol. Neoplasia*. **19**: 35–42.

11. Landskroner-Eiger, S., J. Park, D. Israel, J. W. Pollard, and P. E. Scherer. 2010. Morphogenesis of the developing mammary gland: stage-dependent impact of adipocytes. *Dev. Biol.* **344**: 968–78.

12. Machino, M. 1976. Growth and Differentiation, Vo1.

13. Zwick, R. K., M. C. Rudolph, B. A. Shook, B. Holtrup, E. Roth, V. Lei, A. Van Keymeulen, V. Seewaldt, S. Kwei, J. Wysolmerski, M. S. Rodeheffer, and V. Horsley. 2018. Adipocyte hypertrophy and lipid dynamics underlie mammary gland remodeling after lactation. *Nat. Commun.* **9**: 3592.

14. Cai, H., L. Q. Dong, and F. Liu. 2016. Recent Advances in Adipose mTOR Signaling and Function: Therapeutic Prospects. *Trends Pharmacol. Sci.* **37**: 303–317.

15. Wang, X., and C. G. Proud. 2006. The mTOR Pathway in the Control of Protein Synthesis. *Physiology*. **21**: 362–369.

16. Catania, C., E. Binder, and D. Cota. 2011. mTORC1 signaling in energy balance and metabolic disease. *Int. J. Obes.* **35**: 751–761.

17. Laplante, M., and D. M. Sabatini. 2009. An emerging role of mTOR in lipid biosynthesis. *Curr. Biol.* **19**: R1046-52.

18. Chen, Y., H. Wei, F. Liu, and J.-L. Guan. 2014. Hyperactivation of mammalian target of rapamycin complex 1 (mTORC1) promotes breast cancer progression through enhancing glucose starvation-induced autophagy and Akt signaling. *J. Biol. Chem.* **289**: 1164–73.

19. Kwiatkowski, D. J., H. Zhang, J. L. Bandura, K. M. Heiberger, M. Glogauer, N. el-Hashemite, and H. Onda. 2002. A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells. *Hum. Mol. Genet.* **11**: 525–34.

20. Eguchi, J., Q.-W. Yan, D. E. Schones, M. Kamal, C.-H. Hsu, M. Q. Zhang, G. E. Crawford, and E. D. Rosen. 2008. Interferon regulatory factors are transcriptional regulators of adipogenesis. *Cell Metab.* **7**: 86–94.

21. Wang, F., S. E. Mullican, J. R. DiSpirito, L. C. Peed, and M. a Lazar. 2013. Lipoatrophy and severe metabolic disturbance in mice with fat-specific deletion of PPARγ. *Proc. Natl. Acad. Sci. U. S. A.* **110**: 18656–61.

22. Wang, Q. A., A. Song, R. K. Gupta, B. Deplancke, and P. E. Scherer. 2018. Reversible De-differentiation of Mature White Adipocytes into Preadipocyte-like Precursors during Lactation. *Cell Metab.* **28**: 282-288.e3.

23. Boston, W. S., G. T. Bleck, J. C. Conroy, M. B. Wheeler, and D. J. Miller. 2001. Short Communication: Effects of Increased Expression of α-Lactalbumin In Transgenic Mice on Milk Yield and Pup Growth. American Dairy Science Association.

24. Collares, F. P., C. V. Gonçalves, and J. S. Ferreira. 1997. Creamatocrit as a rapid method to estimate the contents of total milk lipids. *Food Chem.* **60**: 465–467.

25. Patro, R., G. Duggal, M. I. Love, R. A. Irizarry, and C. Kingsford. 2017. Salmon provides fast and bias-aware quantification of transcript expression. *Nat. Methods*. **14**: 417–419.

26. Love, M. I., C. Soneson, P. F. Hickey, L. K. Johnson, N. Tessa Pierce, L. Shepherd, M. Morgan, and R. Patro. 2020. Tximeta: Reference sequence checksums for provenance identification in RNA-seq. *PLoS Comput. Biol.* **16**: 1–13.

27. Soneson, C., M. I. Love, and M. D. Robinson. 2016. Differential analyses for RNA-seq: Transcript-level estimates improve gene-level inferences [version 2; referees: 2 approved]. *F1000Research*. **4**: 1–23.

28. Love, M. I., W. Huber, and S. Anders. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**: 550.

29. Macias, H., and L. Hinck. 2012. Mammary gland development. *Wiley Interdiscip. Rev. Dev. Biol.* **1**: 533–57.

30. Hovey, R. C., and L. Aimo. 2010. Diverse and active roles for adipocytes during mammary gland growth and function. *J. Mammary Gland Biol. Neoplasia*. **15**: 279–90.

31. Li, R., A. E. El Zowalaty, W. Chen, E. A. Dudley, and X. Ye. 2015. Segregated responses of mammary gland development and vaginal opening to prepubertal genistein exposure in Bscl2−/− female mice with lipodystrophy. *Reprod. Toxicol.* **54**: 76.

32. Lawson, D. A., Z. Werb, Y. Zong, and A. S. Goldstein. 2015. The Cleared Mammary Fat Pad Transplantation Assay for Mammary Epithelial Organogenesis. *Cold Spring Harb. Protoc.* **2015**: pdb.prot078071.

33. Richert, M. M., K. L. Schwertfeger, J. W. Ryder, and S. M. Anderson. 2000. An Atlas of Mouse Mammary Gland Development.

34. Cinti, S. 2018. Pink Adipocytes. *Trends Endocrinol. Metab.* **29**: 651–666.

35. Flint, D., and R. Vernon. 1998. Effects of food restriction on the responses of the mammary gland and adipose tissue to prolactin and growth hormone in the lactating rat. *J. Endocrinol.* **156**: 299–305.

36. Schwertfeger, K. L., J. L. McManaman, C. A. Palmer, M. C. Neville, and S. M. Anderson. 2003. Expression of constitutively activated Akt in the mammary gland leads to excess lipid synthesis during pregnancy and lactation. *J. Lipid Res.* **44**: 1100–12.

37. Jankiewicz, M., B. Groner, and S. Desrivières. 2006. Mammalian Target of Rapamycin Regulates the Growth of Mammary Epithelial Cells through the Inhibitor of Deoxyribonucleic Acid Binding Id1 and Their Functional Differentiation through Id2. *Mol. Endocrinol.* **20**: 2369–2381.

38. Kim, S. S., Y. Zhu, K. L. Grantz, S. N. Hinkle, Z. Chen, M. E. Wallace, M. M. Smarr, N. M. Epps, and P. Mendola. 2016. Obstetric and Neonatal Risks Among Obese Women Without Chronic Disease. *Obstet. Gynecol.* **128**: 104–12.

39. Hu, Z., F. A. Tylavsky, J. C. Han, M. Kocak, J. H. Fowke, R. L. Davis, K. Lewinn, N. R. Bush, and • Qi Zhao. 2019. Maternal metabolic factors during pregnancy predict early childhood growth trajectories and obesity risk: the CANDLE Study. *Int. J. Obes.*