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**Title:**

Activation of adipocyte mTORC1 increases milk fat in a mouse model of lactation.

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**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* knockout mouse model. Knockout (KO) and wild type (WT) C57BL/6J 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 KO dams having higher milk fat composition, more and larger mammary adipocytes, contributing to higher milk caloric density and heavier offspring weight during lactation. Additionally, milk of KO dams displayed a less saturated lipid profile with lower percentage saturated fatty acids, higher percentage of monounsaturated fatty acids, and lower milk omega6:omega3 ratio. Gene expression showed 139 differentially expressed genes in the mammary glands and 220 differentially expressed metabolic pathways. These results suggest a key role of mammary adipocyte mTORC1 in mammary gland function and morphology, milk composition, and offspring health.

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

**1.0 Introduction**

Maternal obesity increased by 11% from 2016 to 2019 and has reached 29% (1). Maternal weight can increase maternal mortality risk and pregnancy complications including gestational diabetes (2). According to the Developmental Origins of Health and Disease theory, the health of the offspring is highly influenced by intrauterine and early postnatal exposures (3). Maternal obesity can impact offspring health during *in utero* development and can increase risk of fetal macrosomia (4). During early postnatal life and the critical lactation window of development, maternal obesity can impair the ability to initiate and sustain breastfeeding and can alter milk composition (5). However, the mechanisms by which maternal obesity affects lactation ability and offspring health during lactation remain less clear.

Successful lactation requires the full development and differentiation of the mammary glands in preparation for milk production and secretion (6, 7). 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 (8, 9). During pregnancy and lactation, the mammary adipocytes undergo transformation and almost disappear due to de-differentiation to support lactation and epithelial cell expansion at the expense of the mammary fat pad (10). The mammary adipocytes within closer proximity to the alveolar epithelial cells are thought to provide a primary source of lipids for milk production (10). When the offspring is weaned or lactation is ceased, the mammary adipocytes then re-differentiate and expand reverting to their original form during the involution process (10). 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.

Given their role in maturation, development, and function of the mammary gland, mammary adipocytes are crucial for successful lactation. Maternal obesity can affect lactation onset and duration. Initiation of lactation is delayed in pre-pregnancy obese or overweight women (11) while the duration of breastfeeding for 6 months or more is shorter (12). The probability of early weaning at 3 months postpartum was highest for infants of obese mothers (13). In addition to altering lactation initiation and duration, maternal obesity can impact milk composition. Maternal weight, body mass index, fat mass, and fat mass percentage were positively correlated with total and true milk protein content at 3 months postpartum (14). Maternal weight and body mass index were positively correlated with milk fat content at 1 month postpartum (14). Furthermore, the fatty acid composition showed a higher omega6:omega3 ratio in milk of obese women compared to their non-obese counterparts (15).

Obese subjects that had excess fat mass showed increased activity of the mechanistic target of rapamycin complex 1 (mTORC1) in the visceral fat compartment (16). mTORC1 is a critical nutrient sensor and a main regulator of protein and lipid synthesis (17, 18). In the presence of anabolic signals like insulin, energy abundance, and amino acid availability, mTORC1 function is upregulated via the Akt pathway (19). mTORC1 promotes lipogenesis and adipogenesis and inhibits lipolysis (17, 20). Hyperactivation of mTORC1 in the mammary glands has been studied in the context of breast cancer (21), but little is known about its role in macronutrient synthesis in the mammary gland during lactation (22). Using a murine genetic model, we show that chronic mTORC1 activation in maternal adipocytes via deletion of its upstream negative regulator, *Tsc1*, increased milk fat composition and alters milk lipid profile, 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 (23, 24), which is expressed in all adipocyte lineages (brown, white and mammary adipocytes) as shown in Wang et al. (25). 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 (26), we determined milk output volume for the WT and KO dams. To determine milk volume, we used the weigh-suckle-weigh technique (27). 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 (28). 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. 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 (29) 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 (30) and txiimport v1.18.0 (31) prior to analysis by DESeq2 v1.30.1 (32). 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 and analyzing relative to Gene Ontologies. Similarities between enriched gene sets were calculated by Jaccard distances. Gene set enrichment results are presented in Supplementary Table 2. Data are available from GEO at accession number XXXX

**2.9 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 derivatization followed by fatty acid measurement by gas chromatography (GC) using an Agilent GC equipped with flame ionization detector. The coefficient of variation for lipidomic analysis is 2.5-3.6%.

## **2.10 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 adipocyte size and count with one slide per mouse.

**2.11 Mammary Gland Adipocyte Sizing and Counting**

Using an EVOS inverted fluorescent microscope, eight representative sections per slide were taken at a 10x objective and covered the entire tissue area. Mammary gland adipocytes were quantified using the software ImageJ. Using the 10x objective, the scale was measured at 1.21 pixels2 being equivalent to 1 µm2. This conversion was usedto assess adipocyte area in µm2. Briefly, the Adipocyte Tools Macros Plugin was installed to ImageJ. This plugin provided the parameters “p”, processing adipocyte segmentations options, and “s”, simple adipocyte segmentation options. In analyzing our images the parameter filters for “p” were set at minimum of 40 pixels, maximum of 1000 pixels, and dilates 30 pixels, and the parameters for “s” were set at minimum of 600 pixels and maximum of 1500 pixels. Adipocytes that were blurry, cut off, or below the 20 pixels threshold were excluded from the assessment as it was not feasible to select and measure them accurately. First, the processing segmentation option was used to process the image, and then the simple adipocyte segmentation option was subsequently used. Once these two parameters were set on the image, manual addition and deletion were performed to ensure adipocytes were properly selected. Once all the adipocytes were accounted for, they were analyzed using the “Measure” button within ImageJ software. The calculated parameters were normalized to the total mammary gland size. The numbers provided were then used to assess if there were any differences between KO and WT adipocytes within the mammary gland.

## **2.12 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 only 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/+*) as shown in Figure 1. In this model all adipocytes, including all white and brown adipocyte depots and mammary adipocytes are ablated for TSC1 and are predicted to have activation of mTORC1. Adipocyte *Tsc1* knockout (n=6) and wildtype (n=5) virgin dams were mated with a male having the opposite genotype. Each dam delivered and nursed offspring that were knockout and wildtype, and dams and their offspring were monitored throughout lactation. Experimental timeline and mouse models are shown in Figure 1.

## **3.1 Maternal Body Composition is 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 Adipocyte *Tsc1* knockout dams 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 during lactation, KO dams gained 71% more fat mass compared to WT dams (Figure 2D, p<0.001). However, KO and WT dams had similar food intake during pregnancy and lactation (Figure 2E).

## **3.2 Mammary Gland Weights of Adipocyte *Tsc1* Knockout Mice are 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.

Adipocyte *Tsc1* knockout dams had a 21% reduction in weight of right lower mammary glands (Figure 3A, p=0.042) and a 29% reduction in weight of the left lower mammary glands (Figure 3A, p=0.001) compared to the WT counterparts.

## **3.3 Adipocyte *Tsc1* Knockout Mice Have More and Larger Adipocytes**

After determining differences in mammary gland weights, we then performed histological analyses to assess the number and area of mammary gland adipocytes (Figure 3G-H). Using ImageJ Software, adipocyte *Tsc1* knockout mammary glands had 63% more adipocytes compared to the WT mice (Figure 3B, p=0.057), however, the average adipocyte area for KO and WT adipocytes was not significantly different (Figure 3C, p=0.36). We then assessed the adipocyte percentage area of the total mammary gland and found adipocyte *Tsc1* knockout to occupy nearly two folds more percentage area than the WT mammary adipocytes (Figure 3D, p=0.051). To further identify the effects of the adipocyte *Tsc1* knockout on the size of the mammary adipocytes, we analyzed the variability in adipocyte size. KO adipocytes had a significantly wider variability in adipocyte size distribution (Figure 3E, p<0.001). Given the larger variability in adipocyte size, we assessed the percent of adipocytes per 100 µm2 area range. Consistent the wider variability of adipocyte size seen in the KO in Figure 3E, KO adipocytes had 52% more larger sized adipocytes (200-300 µm2) compared to WT (Figure 3F, p=0.039). While the adipocytes across the other size ranges were not significantly different, adipocyte *Tsc1* knockout adipocytes had 46% fewer adipocytes in the smallest range of 0-100 µm2 compared to WT adipocytes (Figure 3F, p=0.060). Despite the similarities in the average adipocyte area, our results show clear histological differences between genotypes with the adipocyte *Tsc1* knockout having more adipocytes, larger adipocytes, and a higher percentage of total mammary gland area.

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

We assessed litter sizes by which the average litter size across genotypes was similar (Figure 4A). Pups were culled to four pups per dam to normalize milk supply.

To evaluate the effects of mammary gland morphological changes on the offspring we monitored growth of pups during lactation. There was no significant difference in pup weight at birth (PND0.5) but the pups born to adipocyte *Tsc1* knockout dams were 6% heavier than pups born to WT (Supplementary Figure 1, p=0.074).

At PND7.5, after adjusting for sex, pups born to adipocyte *Tsc1* knockout dams were 7% heavier than pups born to WT dams (Figure 4B, p=0.01). Females born to KO dams were 9% heavier than females born to WT dams (Figure 4B, p=0.044), 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 the pups are eating more chow-based food and relying less on maternal lactation.

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

Based on the changes in offspring weight and mammary gland size and histology, we calculated the mass of milk produced per dam via the weigh-suckle-weigh technique att. PND10.5. 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 adipocyte *Tsc1* knockout dams had 34% higher fat percentage than milk of WT dams (Figure 5B, 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 had similar concentrations between groups.

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

After determining a higher milk fat percentage in milk of KO dams, we assessed the specific fatty acid components of the milk fat using GC-MS. These analyses collected at PND16.5 showed a more desaturated and DHA-rich milk in the KO compared to the WT (full results in Supplementary Figure 4). Despite the average sum of long chain fatty acids being similar between genotypes (Supplementary Figure 3), the adipocyte *Tsc1* knockout dams produced milk with 11% lower saturated fatty acids (Figure 6A, p=0.008), 12% higher percentage of monounsaturated fatty (Figure 6B, p=0.009), but similar percentages of polyunsaturated fatty acids (Figure 6D). The MUFA/SFA ratio suggested a 24% higher rate of desaturation from SFA to MUFA as the diets are not changed (Figure 6C, p=0.004).

While PUFA’s overall were similar between groups, adipocyte *Tsc1* knockout milk had 28% higher level of n-3 (Figure 6E, p=0.013), driven mostly by a 42% increase in the n-3 fatty acid Docosahexaenoic acid (DHA) (Supplemental Figure 4, p=0.031). There was a similar percentage of n-6 fatty acids (Figure 6F), resulting in a 31% lower n-6:n-3 ratio (Figure 6G, p=0.008). Interestingly, the upstream precursors of DHA including ALA and EPA were largely unaffected, suggesting that ALA/EPA conversion into DHA or selective sparing of DHA occurs in the milk from adipocyte *Tsc1* knockout dams.

## **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). In spite of the observed differences in milk fat, and milk fatty acid composition, we were surprised that most fatty acid and triglyceride synthesis enzymes were unchanged (Figure 7C). Several markers of adipogenesis and PPAR were upregulated including *Plin4, Adipoq, Cav2,* and *Fabp4*, consistent with the observed increase in adipocyte numbers (Figure 7D). There were no detectable changes in PPAR transcripts. We also identified several genes involved in n-3 eicosanoid metabolism and including the enzymes *Cyp2e1*, *Gpx3*, *Ephx2*, and *Pla2g4a*, whereas the n-6 generating enzyme COX1 (*Ptgs1*) was significantly downregulated (Figure 7E).

In spite of the modest numbers of significantly differentially expressed genes, gene set enrichment analyses identified a 220 significantly differentially expressed biological pathways by GSEA (180 downregulated, 40 upregulated; Supplementary Table 3). By identifying overlap of the genes in these pathways, they fell largely into two clusters of significantly differentially expressed pathways, one set related to the downregulation of adaptive immune differentiation and function, and another related to upregulation of striated muscle function (Figure 7F). To further explore the potential effects on adaptive immune cell function, we examined the expression of the T-cell marker genes encoding for CD3 and the B-cell marker genes encoding for CD45 and CD19. Each of these markers were reduced 20-92% suggesting a potential reduction in adaptive immune cells in these mammary glands.

**4.0 Discussion**

Milk fat is the most variable macronutrient in human milk, and contributes the most to differences in energy content of milk (33). In this study we show that hyperactivation of adipocyte mTORC1 via adipocyte specific deletion of *Tsc1* alters milk fat composition and mammary gland adipocyte histology. Importantly, our approach is expected to activate mTORC1 in all adiponectin-expressing cells, including both peripheral and mammary adipocyte depots. The positive role of mTORC1 in adipocyte biology has been well established. mTORC1 is necessary for adipocyte differentiation in both peripheral (34, 35) and mammary adipocyte depots (36, 37). It is interesting that we observed increased adipocyte numbers, and elevated markers of differentiation in adipocyte *Tsc1* knockout mammary glands, as the Adiponectin-Cre is not expected to be activated until late in differentiation. It is also worth noting that there is evidence of de- and re-differentiation of post-involution mammary adipocyte, but to avoid this concern our studies focused on mice in their first pregnancy. The increased adipocyte hyperplasia could suggest a signal promoting mammary adipogenesis derived from peripheral adipocytes.

Once adipocytes are differentiated, mTORC1 is important for lipogenesis and the chronic absence of mTORC1 activity (by *Raptor* ablation) results in lipodistrophic mice (38, 39). Gain of function studies (via tissue-specific *Tsc1* knockout) of mTORC1 in adipocytes resulted in increased *in vitro* palmitate esterification in inguinal adipose tissue (40). In our adipocyte *Tsc1* knockout model, we were surprised that there were no obvious increases in lipogenic enzymes in the mammary gland, in spite of increased milk fat composition. This is consistent with data from *Raptor* knockout adipocytes which have elevated (not decreased) ACC, ACLY, and FASN protein levels (38). We propose two potential explanations, one is that there is increased peripheral lipid synthesis, which is then transported to the mammary gland adipocytes for storage and secretion. The other possibility is that the mechanisms by which mTORC1 drives lipid synthesis/export in mammary glands are distinct from those in adipocyte depots. This is consistent with elevated expression of the fatty acid transporter *Fabp4* (Figure 7C). In our adipocyte *Tsc1* knockout model, we showed reduced mammary gland weights despite increased mammary adipocyte number, size, and percentage of total gland area. Although our data shows histological differences in mammary glands in the adipocyte *Tsc1* knockout mice and increased offspring weight during lactation, the secreted milk volume measured at PND10.5 was similar across genotypes. This further confirms that the main driver of the increased pup weight can be explained by the increase in milk fat percentage. However, further studies using depot-specific activation of mammary adipocytes will be important to separate the roles of peripheral adipocytes from mammary adipocytes with respect to lactation.

Several studies in mammary epithelial cells are also consistent with a positive role of mTORC1 with respect to milk lipids (41). Transgenic activation of Akt (an upstream activator of mTORC1) in mammary epithelial cells resulted in higher milk fat percentage during lactation and larger milk lipid droplet size compared to the control mice (42). In a separate study, supplementation of the mTORC1 activating branched chain amino acid valine increased mammary gland lipogenic activity during lactation in a way that was reversible by the mTORC1 inhibitor rapamycin (43). Together these data suggest that mTORC1 activation may positively regulate milk lipids through multiple cell-types.

In addition to total lipids, we show an increase in both the relative desaturation of lipids, and the levels of DHA in milk of adipocyte *Tsc1* knockout mice. DHA is an essential omega-3 fatty acid important for infant growth and development and has been linked to cognitive performance and psychomotor development (44, 45). DHA and EPA levels are highly variable in human milk, and a better understanding of the physiological signals that control DHA levels in milk is important to optimizing the delivery of essential lipids to the infant. We examined the expression of the PC-DHA transporter *Mfsd2a* (46) but did not detect any differences in our mammary expression data. The DHA levels may also be linked to our observation of reduced gene expression of markers of adaptive immune cells. We show that several enzymes that convert DHA into bioactive lipids, including are upregulated in our lysates (Figure 7E). DHA-derived eicosanoids such as D-series resolvins and protectins could serve as negative signals to reduce the number of B and T cells in the mammary gland. This in turn could affect both mammary gland morphology, but also the secretion of antibodies into the expressed milk.

This is the first report that adipocyte mTORC1 activation alters the lipids in milk, and provides important new data towards our understanding of lipid metabolism during a critical developmental window. There are several strengths of our approach, including the use of matched diets, single parity and normalized litter sizes to comprehensively evaluate milk lipids and mammary gene expression. However, there are several limitations to this approach including the inability to exclude *in utero* effects on offspring growth, the inability to separate the roles of peripheral and mammary adipocyte depots, and the lack of a clear mechanism by which mammary (or peripheral) adipocytes result in increased milk lipids, milk fat saturation and milk DHA levels.

**5.0 Conclusions**

Our novel findings show that hyperactivation of mTORC1 activity in adipocytes of pregnant and lactating dams can impact milk composition, offspring weight, and mammary gland gene expression and morphology. These findings are crucial to better understand the effects of nutrient sensing in the mammary gland on milk production and offspring health. Our data supports our hypothesis that mTORC1 hyperactivation in adipocytes increases mammary gland capacity to produce fat and secrete it into the produced milk although the source of the fat composition remains less clear. The mechanisms by which mTORC1 could be influencing mammary gland function and milk secretion is insightful for future research addressing the effects of maternal excess nutrient signaling on lactation and infant health. We present data, for the first time, demonstrating 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 through lactational programming.

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**9.0 Figure Legends**

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.

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.

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.

Figure 4: Mammary glands collected from lactating WT and KO dams on PND16.5 and stained using hematoxylin and eosin for adipocyte counting, sizing, and percent area measurement. Adipocyte assessment shows increased adipocyte count in KO glands and a trend towards increased adipocyte total and percentage area.

Figure 5: Pup weights during lactation. Weights of male and female offspring of WT and KO dams at PND7.5.

Figure 6: 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.

Figure 7: Milk fat lipidomic analysis. (A) Lower average %SFA in milk of KO. (B) Higher average %MUFA in milk of KO. (C)Similar average %PUFA in milk of KO and WT. (D) Higher MUFA/SFA ratio in milk of KO. (E) Higher %omega-3 fatty acids in milk of KO. (F)Higher %Docosahexaenoic acid (DHA), an omega-3 metabolite, in milk of KO. (G) Comparable omega-6 fatty acid composition in milk of KO and WT. (H) Lower omega-6:omega-3 ratio in milk of KO.