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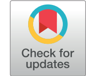
Original Research

Adipocytes Are the Only Site of Glutamine Synthetase Expression Within the Lactating Mouse Mammary Gland



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A B S T R A C T

Background: Glutamine in milk is believed to play an important role in neonatal intestinal maturation and immune function. For lactating mothers, glutamine utilization is increased to meet the demands of the enlarged intestine and milk production. However, the source of such glutamine during lactation has not been studied.

Objectives: We aimed to assess the effects of lactation on the expression of glutamine synthetase (GS) in the mammary gland and other tissues of lactating mice.

Methods: Mouse tissues were sampled at 4 time points: 8-wk-old (virgin, control), post-delivery day 5 (PD5, early lactation), PD15 (peak lactation), and involution (4 days after weaning at PD21). We examined the gene expression and protein concentrations of GS and the first 2 enzymes of branched-chain amino acid catabolism: branched-chain aminotransferase 2 (BCAT2) and branched-chain ketoacid dehydrogenase subunit E1α (BCKDHA).

Results: The messenger RNA (mRNA) expression and protein concentrations of GS in mammary glands were significantly lower at PD5 and PD15 compared with the control but were restored at involution. Within the mammary gland, GS protein was only detected in adipocytes with no evidence of presence in mammary epithelial cells. Compared with the control, mRNA and protein concentrations of BCAT2 and BCKDHA in mammary glands significantly decreased during lactation and involution. No changes in GS protein concentrations during lactation were found in the liver, skeletal muscle, and lung. In non-mammary adipose tissue, GS protein abundance was higher during lactation compared with the virgin.

Conclusions: This work shows that, within the mouse mammary gland, GS is only expressed in adipocytes and that the relative GS abundance in mammary gland sections is lower during lactation. This suggests that mammary adipocytes may be a site of glutamine synthesis in the lactating mouse. Identifying the sources of glutamine production during lactation is important for optimizing milk glutamine concentration to enhance neonatal and maternal health.

Keywords: mammary gland, adipocytes, glutamine, lactation, glutamine synthetase, BCAA, mouse

Introduction

Glutamine is the most abundant free α-amino acid in the human body with a body pool of ~80 g, most being held in skeletal muscle. Similarly, glutamine is the most abundant free α-amino acid in plasma, with an average turnover of 60–80 g/d where it is a major interorgan transporter of nitrogen, carbon, and energy. Glutamine amido nitrogen can give rise to purines, pyrimidines, glucosamine, NAD, and asparagine. The bulk of glutamine, however, is deamidated to glutamate and serves as a substrate for the Krebs cycle, the urea cycle, and gluconeogenesis [1–4]. Glutamine is also the primary respiratory fuel for enterocytes, immune cells, and proliferating cells [3,4].

Abbreviations: BCAA, branched-chain amino acids; BCAT2, branched-chain aminotransferase 2; BCKDHA, branched-chain ketoacid dehydrogenase subunit E1α; GS, glutamine synthetase; INV, involution; MECs, mammary epithelial cells; PD, post-delivery day.

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Enterocytes effectively metabolize all dietary glutamine and glutamate, meaning the body pool of glutamine is made endogenously by the action of glutamine synthetase (GS) [3,5]. In healthy conditions, skeletal muscle, lungs, adipose tissue, and liver show net glutamine release [3].

During pregnancy and lactation, there is a high demand for glutamine to support the enlarged intestine, the growing fetus, and milk production [2,6–9]. In all species studied to date, glutamine and glutamate account for 20% of the total amino acids in milk, both as free and protein-bound amino acids [10]. During the first 6 months of human lactation, free glutamine and glutamate increase ≤350% and 40%, respectively [11,12]. Glutamine plays a vital role in neonatal intestinal health and immunomodulation [12]. Glutamine concentrations in milk, however, may not be optimal because suckling piglets supplemented with glutamine, or glutamine and glutamate, showed improved immune and intestinal development [13]. Similarly, glutamine gavage in the form of L-glutamine or L-alanyl-L-glutamine twice a day for 1 week improved piglet growth and reduced intestinal damage when challenged with LPS [14]. It is, therefore, important to understand maternal glutamine metabolism during lactation to identify major sources of net glutamine production.

In pigs, cows, and goats, as much as 50% of milk glutamine is made within the mammary gland [15]. To our knowledge, glutamine metabolism in mice during lactation has not been examined. Here, we aimed to assess the effects of lactation on GS expression in the mammary gland and other tissues. Branched-chain amino acids (BCAAs) are precursors for endogenous glutamine synthesis, and this has been demonstrated in pig mammary glands in vitro. [16,17]. Thus, we also examined the first 2 enzymes of BCAA catabolism: branched-chain aminotransferase 2 (BCAT2) and branched-chain ketoacid dehydrogenase subunit E1α (BCKDHA) [18]. Understanding the changes in glutamine metabolism during lactation may lead to improvement of maternal diets and/or changes to formula compositions, ultimately enhancing neonatal health.

Methods

Study design

The summary of the study design is shown in Figure 1. Five- to 6-week-old C57BL/6J female mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, United States), fed a standard chow diet (5053 – PicoLab Rodent Diet 20), and kept under a regular 12:12 h light:dark cycle. Mice were mated at 8 weeks of age and maintained on breeder chow diet ad libitum (LabDiet 5015). Samples ($n = 5$, unless otherwise noted) were collected 5 d after delivery—early lactation (PD5) or 15 d—peak lactation (PD15), and 4 d following weaning on day 21 (involution [INV], $n = 4$). Inguinal adipose tissue samples of virgin 8-week-old female mice were used as the control (virgin). Animal care and experiments were conducted in accordance with the federal guidelines and approved by the Rutgers University Institutional Animal Care and Use Committee.

Tissue collection

Mice were anesthetized with an intraperitoneal injection of ketamine (10 mg/kg) and xylazine (12 mg/kg). Inferior vena cava blood was collected into heparinized tubes. Plasma was extracted and processed for amino acid analyses by high performance liquid chromatography (HPLC) [19]. Tissue samples (liver, lung, kidney, skeletal muscle, and retroperitoneal adipose tissue) were snap-frozen in liquid nitrogen and stored at −80°C until further processing. Samples for immunohistochemistry staining were fixed in 4% paraformaldehyde.

Immunoblotting

Total tissue protein was extracted from 200 mg frozen samples in 5 volumes of homogenization buffer (0.33M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM dithiothreitol (DDT), pH 7.4, and 1 μL/mL protease inhibitors) (Sigma P8340) using a TISSUE TEAROR (Biospec Products). Homogenates were frozen at −80°C, followed by thawing and centrifugation at 12,000 × g for 20 min at 4°C. The protein-containing infranatant was extracted and stored at −80°C for further use.

Protein concentration was determined with Bradford protein assay using Bio-Rad Protein Assay Dye Reagent Concentrate (#5000006) with bovine serum albumin as the standard. Gel electrophoresis was performed by separating an equal amount of protein on precast 4%–12% Bis-Tris gel (Thermo Fisher, NP0335BOX) as instructed by the manufacturer. Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (MilliporeSigma, IPVH00010) and stained with Ponceau-S (Sigma, P3504-50G) to confirm successful transfer. Membranes were blocked with 5% dry milk in 1× tris-buffered saline with 0.1% Tween-20 (TBS-T) for an hour at room temperature, followed by primary antibody incubation overnight at 4°C. Membranes were incubated in secondary antibody for an hour at room temperature, followed by visualization with ECL solution (Amersham ECL Western Blotting Select Detection Reagent, Cytiva, RPN2235) using a ProteinSimple FluorChem E Imaging

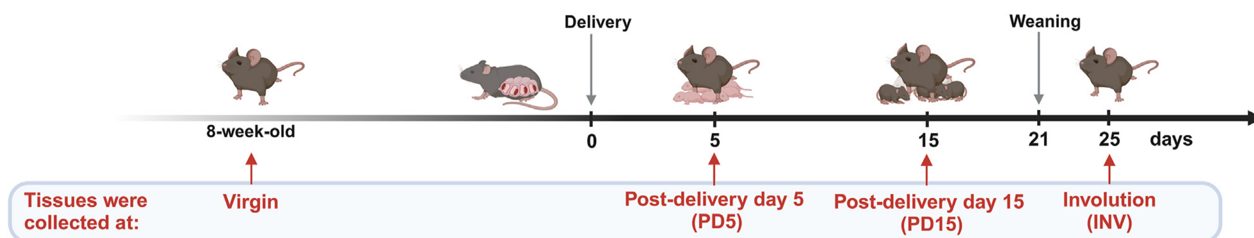

FIGURE 1. Schematic of study design. Tissues were collected from female mice at 4 time points: virgin (8-wk-old non-lactating virgin mice), post-delivery day 5 (PD5, early lactation), post-delivery day 15 (PD15, peak lactation), and 4 d after weaning at day 21 (involution [INV]). The figure was created with BioRender.

System. Densitometry analyses were performed using ImageJ, and values were normalized to total protein using Brilliant Coomassie Blue R-250 staining, as recommended by the American Society of Biochemistry and Molecular Biology [20,21], and expressed as fold of virgin group. In some blots, there were additional bands that we believe are nonspecific bands because they appeared in tissues in which the gene encoding GS (Glul) was ablated. All antibody information is provided in Supplemental Table 1.

Immunohistochemistry staining

Fresh samples were fixed in 4% paraformaldehyde before being processed by Rutgers Research Pathology Services. Five-micrometer thick paraffin-embedded mammary gland slides were stained with anti-GS antibody (Abcam, Ab197024, 1:1000). Liver samples were used as a positive control. Slides stained with secondary antibody only were used as a negative control.

RNA isolation and qRT-PCR

Isolation of total RNA from 10 to 20 μg frozen samples was performed according to the protocol of the commercial kit (Rneasy Lipid Tissue Mini Kit, Qiagen, 74804). The ratios of A260/280 and A260/230 were used to assess the quality of RNA. The integrity of 18S and 28S ribosomal bands was confirmed by agarose gel electrophoresis. cDNA was generated via reverse transcription of 1 μg of RNA (High-Capacity cDNA Reverse Transcription Kit, Thermo Fisher, 4368814) and stored at −20°C until use for qRT-PCR. Each sample was run in triplicate to analyze gene transcripts using a thermal cycler (StepOnePlus, Thermo Fisher Scientific). All primers were obtained from Integrated DNA Technologies, and the sequences are provided in Supplemental Table 2. The ΔΔCT method was used to compare gene expression of GLUL, BCAT2, or BCKDHA, normalized to the housekeeping gene 18S.

Statistical analysis

Statistical tests and graphs were generated using Microsoft Excel and GraphPad Prism ver.9.4.1. One-way ANOVA test followed by Tukey's post hoc analysis was used to compare among 4 experimental groups. The significance level was set to $\alpha = 0.05$. Data are presented in scatter plots with means $\pm$ SEM bars and individual values overlaid as dots.

Results

At PD15, dam weight was significantly higher than that of virgin (Supplemental Table 3). The average litter sizes were 6.2 ± 1.1 pups for PD5, 7.2 ± 0.7 pups for PD15, and 7.6 ± 0.7 pups for INV groups. The mean total litter weights were 20.3 ± 4.3 g and 52.7 ± 5.4 g, with average individual pup weights of 3.2 ± 0.3 g and 7.3 ± 0.4 g at PD5 and PD15, respectively. We chose not to standardize litter size because we did not anticipate litter size to have any effects on the outcomes studied, and

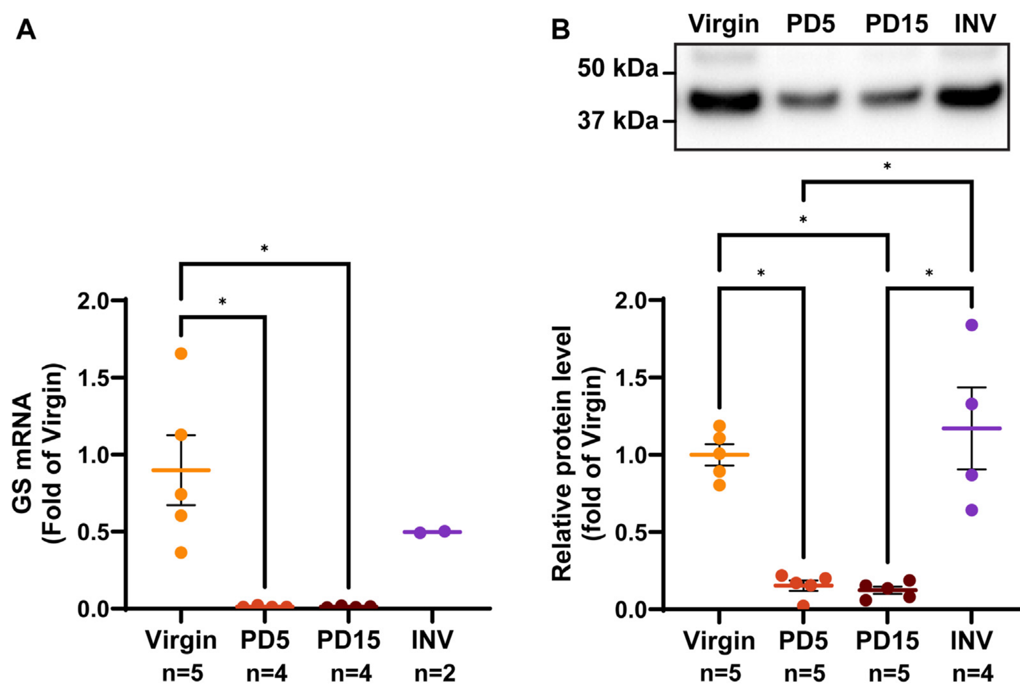

FIGURE 2. Glutamine synthetase is lower in the mammary gland during lactation. (A) mRNA expression and (B) relative protein abundance of GS in the mammary gland at 4 different time points. For Western blotting 10 μg of protein was loaded in each lane. Total protein stained by Coomassie Blue was used for loading control. A representative immunoblot is shown. Full immunoblots with total protein stained with Coomassie Blue corresponding to this figure are in Supplemental Figure 1. Relative GS abundance was normalized to total protein and expressed as fold of virgin group. *$P < 0.05$. Data are presented as mean ± SEM with each dot representing a data point. GS, glutamine synthetase; INV, involution; PD5, post-delivery day 5; PD15, post-delivery day 15.

normalizing litter size may alter various biological processes and milk composition [22].

GS in the mammary gland during lactation

Gene expression and relative abundance of GS protein in the mammary gland were significantly lower at PD5 and PD15 compared with the virgin but were restored at INV (Figure 2A, B, Supplemental Figure 1). Immunohistochemistry staining of mammary glands with anti-GS antibody was performed to assess where GS is expressed within the gland. In the virgin group, GS stained positive in the cytosol of adipocytes (Figure 3, virgin). At PD5 and PD15, mammary adipocytes decreased in relative number and size but consistently stained positive for GS (Figure 3, PD5 and PD15). The number of mammary epithelial cells (MECs, cluster of blue-stained cells surrounding the alveoli are seen in Figure 3) increased during lactation. Staining for GS, however, was restricted to adipocytes but was not detectable in MECs. At involution, the morphology of the mammary gland reverted to a virgin-like state with predominantly GS-positive adipocytes (Figure 3, INV).

A group of GS-positive cells were present surrounding a duct-like structure in the virgin sample (Figure 3, arrowhead). These cells also stained positive for UCP-1 (Supplemental Figure 2) indicating that they are brown adipocytes, as documented previously [23–25]. In our study, such cells were only present in the virgin samples, not seen during lactation or involution, and were not considered further.

Branched-chain amino acid catabolism in the mammary gland during lactation

Because BCAA are precursors for glutamine synthesis, we examined the first 2 enzymes of BCAA catabolism. The mRNA concentration of BCAT2 was lower at PD5, PD15, and INV (Figure 4A, Supplemental Figure 3) compared with the virgin. The protein concentration of BCAT2 showed similar low abundance during lactation and involution (Figure 4B). Although they did not reach statistical significance, BCAT2 mRNA and protein concentrations showed slight increases at INV compared with PD5 and PD15. The mRNA expression and protein abundance of BCKDHA in the mammary gland was significantly lower at PD5 and PD15 compared with virgin but was restored at involution (Figure 5A, B, Supplemental Figure 4).

GS in non-mammary tissues during lactation

Changes in GS expression during lactation may occur in other tissues, including non-mammary adipose tissues. Therefore, we examined GS expression in tissues known to show net glutamine synthesis. In liver, skeletal muscle, and lung, the abundance of GS protein showed no significant differences between Virgin and PD5, and therefore we did not investigate further (Supplemental Figure 5).

The size of non-mammary adipose tissue depots significantly decreased during lactation, and we were only able to

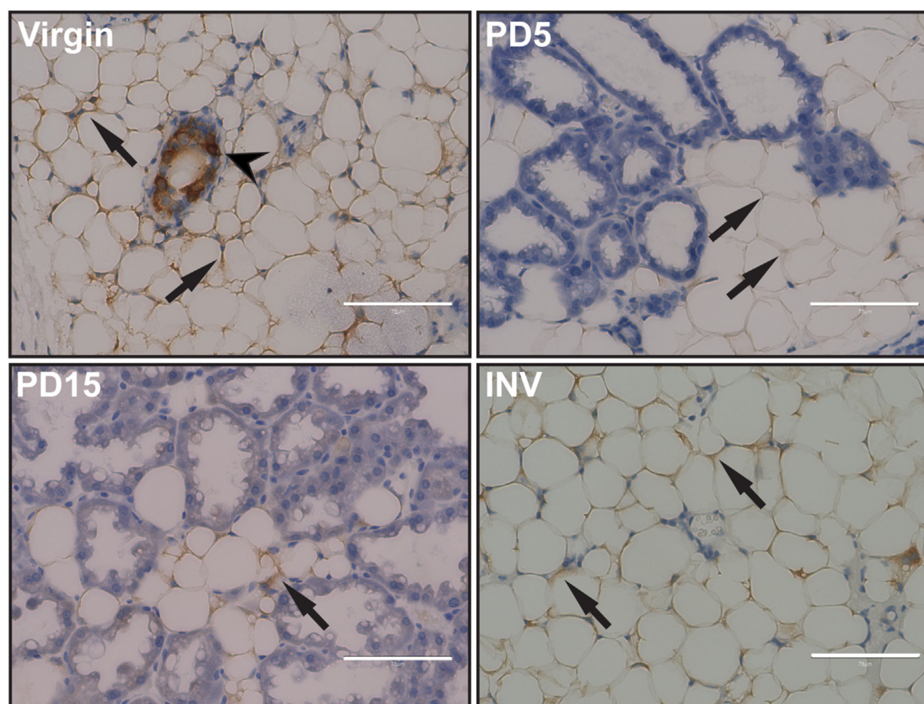

FIGURE 3. Glutamine synthetase protein is localized in mammary adipocytes. Immunohistochemistry staining of GS in the mammary gland at 4 time points. Arrows and an arrowhead point at the brown staining of GS. Nuclei are stained blue with 4′,6-diamidino-2-phenylindole (DAPI). The arrowhead indicates cells that were also stained for UCP1 in virgin samples. Clusters of blue nuclei surrounding alveoli present at PD5 and PD15 indicate mammary epithelial cells. Representative images are shown. Scale bars = 75 μm. GS, glutamine synthetase; INV, involution; PD5, post-delivery day 5; PD15, post-delivery day 15; UCP1, uncoupling protein 1.

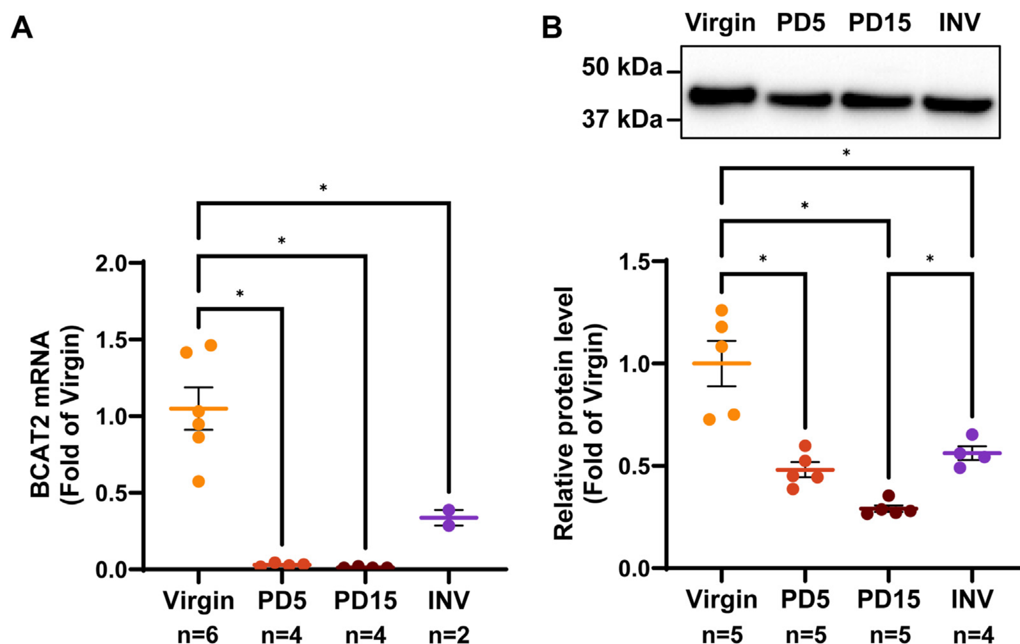

FIGURE 4. Branched-chain aminotransferase 2 is lower in the mammary gland during lactation. (A) mRNA expression and (B) relative protein abundance of BCAT2 in the mammary gland at 4 different time points. For Western blotting 25 μg of protein were loaded in each lane. Total protein stained by Coomassie Blue was used for loading control. A representative immunoblot is shown. Full immunoblots with total protein stained with Coomassie Blue corresponding to this figure are in Supplemental Figure 3. Relative BCAT2 abundance was normalized to total protein and expressed as a fold of virgin group. *$P < 0.05$. Data are presented as mean ± SEM with individual data points shown as dots. BCAT2, branched-chain aminotransferase 2, INV, involution; PD5, post-delivery day 5; PD15, post-delivery day 15.

collect retroperitoneal adipose tissue in limited amounts. Therefore, we could only examine GS protein abundance and not mRNA expression. The protein abundance of GS in retroperitoneal adipose tissue was significantly higher at PD15 than at virgin (Figure 6, Supplemental Figure 6). Although it did not reach statistical significance, there was a trend ($P = 0.067$) for GS protein abundance to be higher at INV compared with virgin (Figure 6).

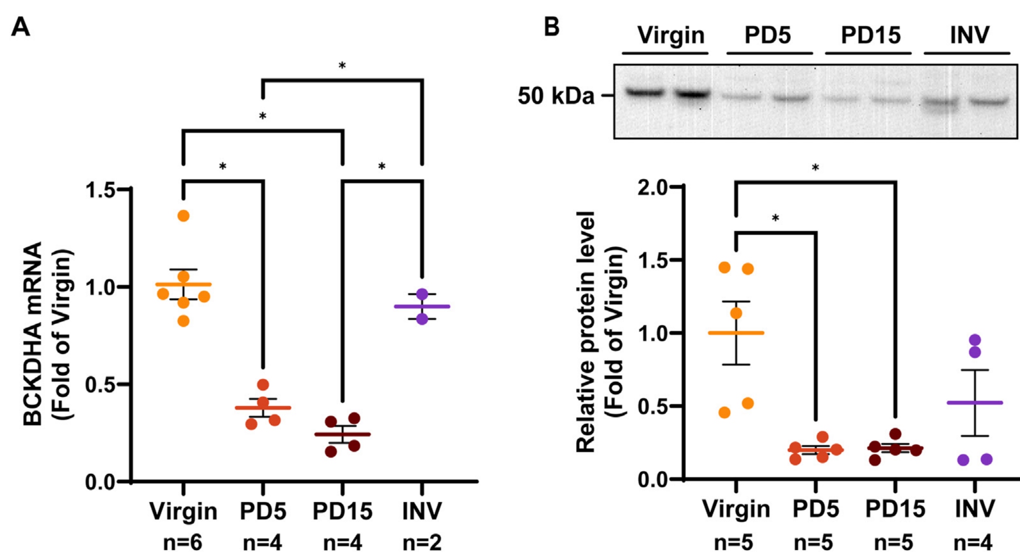

FIGURE 5. Branched-chain ketoacid dehydrogenase is lower in mammary gland during lactation. (A) mRNA expression and (B) relative protein abundance of BCKDHA in the mammary gland at 4 different time points. For Western blotting 30 μg of protein were loaded in each lane. Total protein stained by Coomassie Blue was used for loading control. A representative immunoblot is shown. Full immunoblots with total protein stained with Coomassie Blue corresponding to this figure are in Supplemental Figure 4. Relative BCKDHA abundance was normalized to total protein and expressed as a fold of virgin group. *$P < 0.05$. Data are presented as mean ± SEM with individual data points shown as dots. BCKDHA, branched-chain ketoacid dehydrogenase subunit E1α; INV, involution; PD5, post-delivery day 5; PD15, post-delivery day 15.

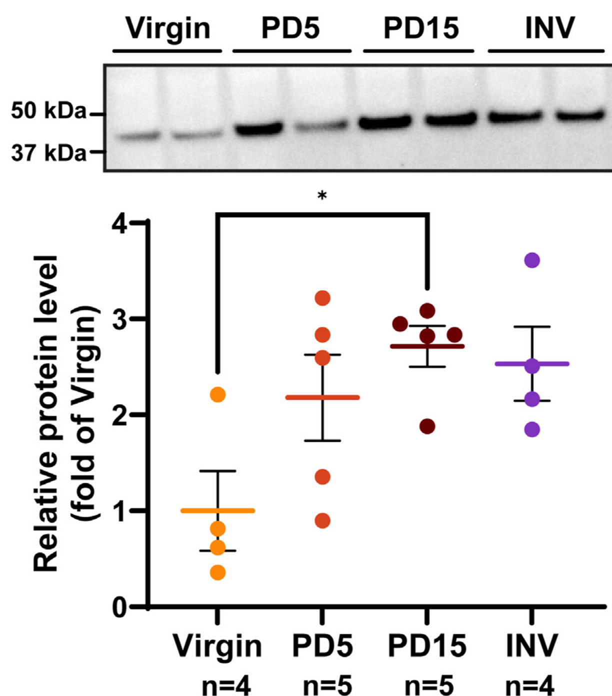

FIGURE 6. Glutamine synthetase protein abundance in retroperitoneal adipose tissue during lactation. Relative protein abundance of GS in retroperitoneal adipose tissue at 4 different time points. Ten micrograms of protein was loaded in each lane. Total protein stained by Coomassie Blue was used for loading control. A representative immunoblot is shown. Full immunoblots with total protein stained with Coomassie Blue corresponding to this figure are in Supplemental Figure 6. Relative GS abundance was normalized to total protein and expressed as a fold of virgin group. *$P < 0.05$. Data are presented as mean ± SEM with individual data points shown as dots. GS, glutamine synthetase, INV, involution; PD5, post-delivery day 5; PD15, post-delivery day 15.

Plasma amino acid concentrations during lactation

The average plasma concentration of glutamine in virgin mice was 749.0 ± 65.4 μM (Supplemental Figure 7). Plasma glutamine concentrations did not show significant changes during lactation and involution and remained at 783.8 ± 174.5 μM at PD5, 775.8 ± 111.6 μM at PD15, and 668.5 ± 64.8 μM at INV (Supplemental Figure 7). Similarly, the plasma concentration of glutamate did not alter significantly: 98.5 ± 4.8 μM at virgin, 107.3 ± 14.2 μM at PD5, 84.6 ± 7.7 μM at PD15, and 66.4 ± 11.3 μM at INV (Supplemental Figure 7).

Most other plasma amino acids showed no statistically significant changes in concentration from virgin to lactation and involution. Exceptions were lysine and aspartate. Plasma lysine concentrations were significantly higher at PD15 and INV compared with virgin (Supplemental Figure 7). The plasma aspartate concentrations were also higher in the PD5 group than in the Virgin and PD15 groups (Supplemental Figure 7). However, these statistically significant differences may not translate to biological significance given the limited sample size and relatively small changes.

Discussion

Glutamine is a non-essential amino acid that is abundant in a healthy, balanced diet. Dietary glutamine, however, is completely metabolized by the enterocytes, therefore the body pool of glutamine is synthesized endogenously by GS [3]. During pregnancy and lactation, maternal glutamine demand increases to support the enlarged intestine, the growing fetus, and milk production [2,6–8]. In milk of all species studied to date, glutamine and glutamate make up 20% of total amino acids (both as free and protein-bound amino acids) [10]. Free amino acids account for 5%–10% of total amino acids in milk. Of this amount, almost 70% are free glutamine and glutamate [12]. Free glutamine and glutamate in human milk significantly increase by ≤350% and 40%, respectively, within the first 6 months of lactation [12,26]. However, lactation is accompanied by a mild catabolic state in pigs, horses, mice, and humans, characterized by a loss of fat-free mass and decreased glutamine concentrations in plasma and skeletal muscle (indicative of endogenous proteolysis) [27–31]. Therefore, understanding maternal glutamine metabolism during lactation is important both to maternal and neonatal health.

Up to half of milk glutamine is proposed to be made in the mammary gland in pigs, cows, and goats [15]. We found, however, that the relative abundance of GS mRNA and protein in mouse mammary glands during lactation was lower compared with the nonlactating mammary gland. Using immunohistochemistry staining for GS, we observed that GS was only expressed in adipocytes within the mammary gland. The relatively lower GS protein abundance during lactation was due to the significant morphologic changes in the mammary gland. The virgin/non-lactating and lactating mammary glands are essentially 2 different tissues in terms of cell population. The non-lactating gland is primarily composed of adipocytes [32–34], with comparatively low-total protein but high GS expression. During pregnancy and lactation, there is a large proliferation and differentiation of MECs [34,35] with high-protein content but no GS expression. In this study, we sampled and analyzed the mammary gland that includes adipocytes, MECs, and other cells, resulting in our finding of lower GS expression during lactation. We did not determine the size or weight of the whole mammary gland, but it was visibly larger and is known to increase significantly during lactation in rats [36]. It is, therefore, possible that total GS expression is higher in the lactating mammary gland when expressed relative to mouse body weight.

Branched-chain amino acids are major substrates for endogenous glutamine synthesis. The enzyme BCAT transaminates BCAA to the corresponding branched-chain ketoacids and glutamate. Branched-chain ketoacid dehydrogenase (BCKDH) complex catalyzes the irreversible oxidative decarboxylation of branched-chain ketoacids. The glutamate produced by BCAT can be used as a substrate for GS. We found that during lactation the mRNA and protein concentrations of BCAT2 and BCKDHA in the mammary gland were significantly lower, similar to GS expression. In lactating rats, the mRNA, protein, and activity levels of BCAT2 and BCKDH subunit E2, but not E1α, have been reported to increase mammary glands compared with the virgin rats [18,

37,38]. The discrepancy between these findings and our study may be due to species differences, variations in experimental design, and/or reagents such as different antibodies used.

Known sites of net glutamine production are liver, skeletal muscle, lung, and adipose tissues [3]. We found that GS protein abundance during lactation did not show significant changes in the liver, skeletal muscle, and the lung (Supplemental Figure 5). Similarly, in a previous study in the rats, we observed no compensatory changes in GS activity or glutamine metabolism across the liver and hindquarters during lactation [39]. In the present study, however, we did find that GS protein abundance was significantly higher in retroperitoneal adipose tissue at peak lactation compared with virgin. Due to limited tissue availability during lactation, we were unable to examine mRNA expression in retroperitoneal adipose tissue or other adipose tissue depots.

The finding that within the mouse mammary gland, GS is only expressed in adipocytes has several implications. This could be a source of glutamine contributing to the body pool during lactation and a potential source of milk glutamine. Optimizing this glutamine production site may help combat the catabolic state associated with lactation and improve milk glutamine concentrations, thereby enhancing maternal and neonatal health. Future studies are warranted to evaluate the role(s) of mammary adipocyte–derived glutamine in milk production and MEC development during pregnancy and lactation.

Acknowledgments

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Author contributions

The authors' responsibilities were as follows – MW: conceived and designed research. HL, MN, HC, HM, MDW: conducted experiments. HL, MN, HC: analyzed data. HL, MN, MW: interpreted results. HL: prepared figures and drafted manuscript. HL, MW: edited and revised the manuscript and all authors: read and approved the final manuscript.

Conflict of interest

The authors report no conflicts of interest.

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Data availability

Data described in the manuscript will be made available upon request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cdnut.2024.102168.

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