LACTATION AND CHOLINE INTAKE INFLUENCE BIOMARKERS OF CHOLINE METABOLISM

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ABSTRACT

This study examined the influence of lactation, and choline intake during lactation, on biomarkers of choline metabolism. Lactating (n=27) and control (n=21) women were randomized to 480 or 930mg choline/d for 10-12 wks; Blood and urine were collected for choline metabolite (and enrichment) measurements while blood leukocytes and mammary epithelium were collected for gene expression analysis of choline metabolic genes. Lactating (vs. control) women exhibited elevated plasma choline and betaine; decreased urinary excretion of betaine and dimethylglycine; and increased leukocyte transcript abundance of *CHDH*. A higher choline intake by lactating women yielded higher plasma betaine and dimethylglycine, higher serum sarcosine, and higher milk glycine. These data collectively suggest that lactation induces metabolic adaptations to increase the supply of betaine perhaps secondary to its role as an osmolyte, and that extra choline during pregnancy increases metabolites that may be important in milk synthesis.

Dedicated to Him

BIOGRAPHICAL SKETCH

Crystal Davenport grew up in the southeastern United States, predominately in South Carolina and North Carolina. She attended Clemson University and graduated in 2010 with a B.S. in Animal and Veterinary Science. During college, Crystal worked in various research labs, spending the majority of her time in those dedicated to poultry nutrition. On the advice of a mentor at Clemson she applied in Molecular Nutrition to the Division of Nutritional Sciences at Cornell University, and began work in the Fall of 2010. Crystal did several lab rotations through funding provided by the Presidential Life Sciences Fellowship, finally settling on Dr. Marie Caudill's lab in the spring of 2011. She has been working with Dr. Caudill on research projects investigating choline metabolism and choline dietary requirements. In 2012, her research was recognized by the NIH and she was awarded an NIH Training Grant in Translational Science.

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TABLE OF CONTENTS ***Page***

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Choline is an essential water-soluble micronutrient with adequate intake (AI) recommendations of 425 mg/d for adult women, 450 mg/d for pregnant women, and 550 mg/d for lactating women (IOM 1998). Choline functions in the body as the precursor molecule for an array of metabolites with critical roles in human health (see **Figure 1**). Derivatives of choline include the cell membrane and lipoprotein constituent, phosphatidylcholine (PC); the methyl donor betaine; and the cholinergic neurotransmitter acetylcholine. PC biosynthesis can occur through one of two pathways: (i) the cytidine diphosphate-choline (CDP-choline) pathway in which free choline is phosphorylated and then attached to diacylglycerol; or (ii) the phosphatidylethanolamine *N*-methyltransferase (PEMT) *de novo* pathway involving the S-adenosylmethionine (SAM)-dependent sequential tri-methylation of phosphatidylethanolamine (PE) to PC. Although considered a route of *de novo* choline synthesis, choline itself can serve as a source of methyl groups for the methylation of PE to PC (Yan et al., 2011) and thus PEMT activity is influenced by choline supply (Yan et al. 2011, West et al. 2013). Betaine is the oxidized derivative of choline and is a major source of methyl groups (Zeisel et al., 2003), but also a potent osmolyte (Lever and Slow, 2010). Methyl donation by betaine remethylates homocysteine to methionine and generates dimethylglycine (DMG), methylglycine (sarcosine), and glycine in a sequential demethylation manner as follows: betaine → DMG → sarcosine → glycine.

The vital role of choline and its high demand in human development are increasingly recognized (Zeisel, 2013; Nyaradi et al., 2013; Boeke et al., 2013; Caudill, 2010). Human pregnancy has a profound effect on choline metabolism. Pregnant (vs. control) women exhibit

diminished plasma concentrations of choline derived methyl donors (Yan et al., 2012; Friesen et al., 2007) and increased obligatory losses of choline metabolites (Yan et al., 2012). In addition, a higher maternal choline intake during pregnancy may improve maternal and child health outcomes. Supplementing the diet of third trimester pregnant women with extra choline has been shown to: (i) decrease the placental production of sFLT1, an anti-angiogenic factor that increases the risk of placental dysfunction and preeclampsia (Jiang et al., 2013); and (ii) attenuate fetal response to stress by altering the methylation, and expression, patterns of genes that regulate fetal cortisol production (Jiang et al., 2012). Further, rodent studies have consistently demonstrated lasting beneficial effects of perinatal choline supplementation on offspring cognitive functioning (Corriveau et al., 2012; Monk et al., 2012; Williams et al., 1998; Jones et al., 1999; Pyapali et al., 1998; Montoya et al., 2000; Meck et al., 1997a; Meck et al., 1997b; Meck et al., 1999; Meck et al., 1993).

Less is known about the influence of lactation, and choline intake during lactation, on choline metabolism. However, substantial amounts of choline are present in human milk (Holmes-McNary et al., 1996) and lactation has been shown to deplete liver choline stores in rodents on a normal feed-pellet diet (Zeisel et al., 1995), indicating that PEMT activity is insufficient to meet choline demands during lactation. In addition, milk choline corresponds to dietary choline intake in lactating rats (Holmes-McNary et al., 1996) and with biomarkers of maternal choline metabolism in humans (Ilcol et al., 2005). Further, supplementation of the maternal diet with additional choline in the form of PC improves milk choline content (Fischer et al., 2010), which may be important for infant cognition (Aizawa et al., 2011).

To further understanding of the impact of lactation, and choline intake during lactation, on choline metabolism and dietary requirements, we conducted a dose-response feeding study in lactating and control women. A comprehensive panel of choline metabolites in maternal blood and urine were quantified together with the transcript abundance of several choline metabolizing enzymes in maternal leukocytes and the mammary epithelium. In addition, stable isotope methodology was employed to determine the metabolic flux and partitioning of choline and its methyl groups among choline-related metabolic pathways. Deuterium *methyl*-d9-choline was used as the tracer and has three deuterium labels on each of choline's three methyl groups (see **Figure 1**). Measuring the enrichment of choline metabolites enables the tracing of the intact molecule (d9 species) and its methyl groups (d3- or d6- species).

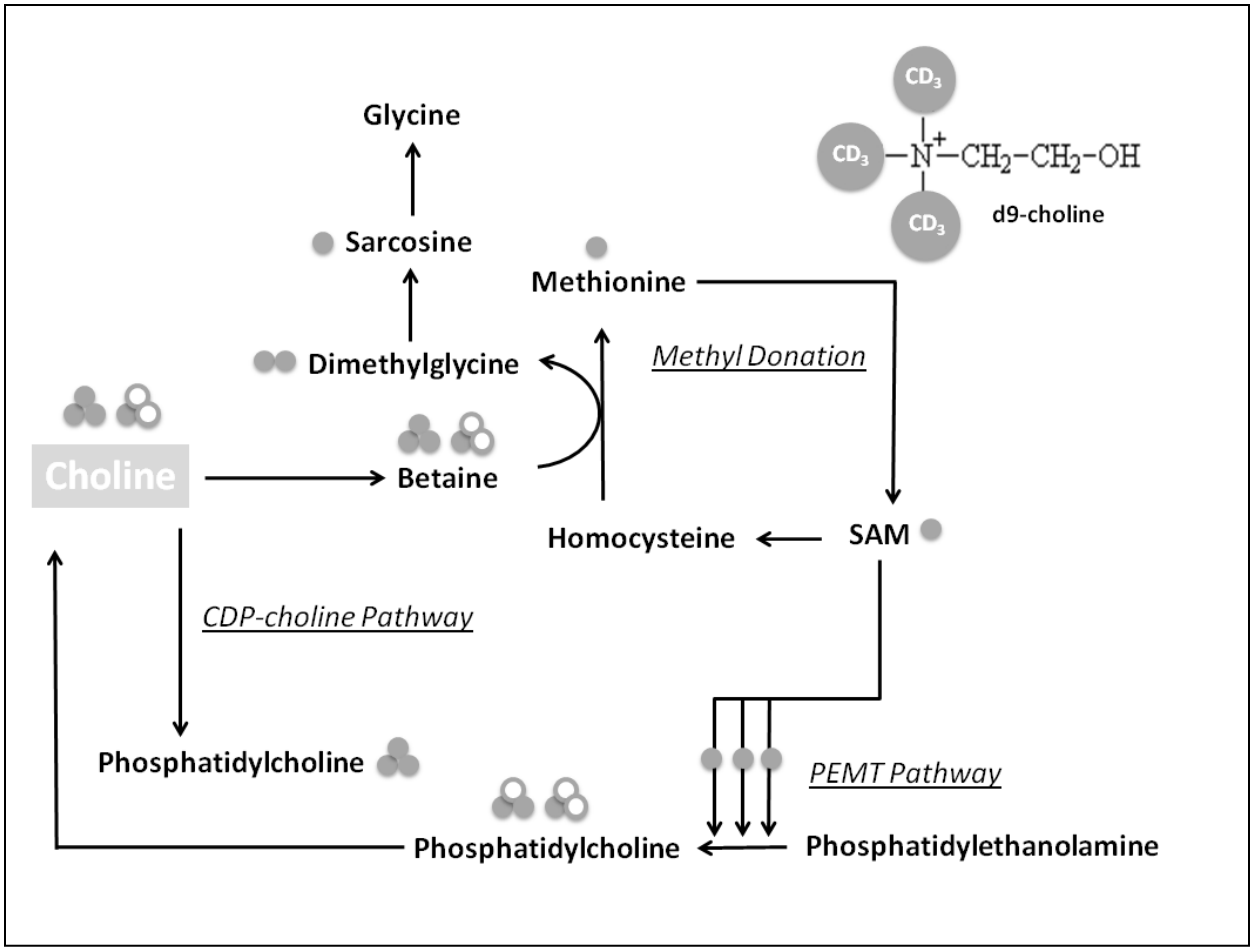

FIGURE 1. Choline metabolism and the metabolic fate of orally consumed deuterium-labeled choline. The d9-choline tracer (see top right) contained deuterium-labeled methyl groups thereby facilitating examination of the metabolic fate of choline-derived methyl groups in addition to the intact molecule. During methyl donation, d9-choline may be oxidized to d9-betaine, followed by conversion of d9-betaine to d6-dimethylglycine and donation of a labeled methyl group containing three deuterium labels to methionine. These donated labeled methyl groups may be used to produce d3 or d6-PC through the PEMT pathway. PEMT-PC labeled with one (d3-PC) or two (d6-PC) methyl groups may then be metabolized to form free choline resulting in d3-choline. d3-choline may be oxidized to d3-betaine, followed by another round of methyl donation. Alternatively, intact dietary choline containing all of its original methyl groups (d9-choline) may proceed down the CDP-choline pathway to produce d9-PC without entering methyl donation. Circles, methyl groups; grey circles, labeled methyl groups; white circles, unlabeled methyl groups.

METHODS

Study participants

Healthy lactating (5-wk postpartum) and control women (non-pregnant, non-lactating) aged 21-41 y were recruited from Ithaca, NY, and surrounding areas between January 2009 and October 2010 (West et al 2012.; Yan et al. 2012). Entry into the study was contingent on good health status as assessed by a blood chemistry profile, complete blood count, and medical history questionnaire. Additional inclusion criteria included no tobacco or alcohol use, no history of chronic disease, normal kidney and liver function, and a willingness to comply with the study protocol. For lactating women, this included the intention to exclusively breastfeed for the duration of the study. Exclusion criteria included the use of prescription medications known to affect liver function. The screening and experimental procedures were approved by the Institutional Review Boards for Human Participants at Cornell University and Cayuga Medical Center, and written informed consent was obtained prior to study entry. The study was registered at ClinicalTrials.gov as NCT01127022 (http://clinicaltrails.gov/) and the Consort flowchart has been reported (Yan et al., 2012).

Study design, diet and supplements

This was a dose-response feeding study in which lactating (n=28) and control (n=21) women were randomized to either 480 mg choline/d (n=15 lactating, n=10 control) or 930 mg choline/d (n= 13 lactating, n=11 control) for 10-12 weeks, as described previously (Yan et al., 2012). Our control cohort is the same group of women that served as the reference group for our prior study examining the effect of pregnancy on choline intake (Yan et al., 2012).

However, in the present study, biomarkers of choline metabolism in the control cohort were re-measured concurrently with measurements in our lactating cohort to ensure internal validity. Choline was derived from the study diet (380 mg/d) plus supplemental choline chloride (either 100 or 550 mg choline/d) for total choline intakes of 480 or 930 mg/d, respectively. During the last 4-6 wks of the study, participants consumed 22% of their total choline intake (480 or 930 mg choline/d) as *methyl*-d9-choline.

Throughout the 10-12 wk study, participants consumed a 7-d cyclic menu (Yan et al. 2012). All food items and beverages were provided by the investigators, and one meal/d was consumed on-site throughout the week. All other meals and beverages were provided as takeaways. In addition to the supplemental choline chloride (Balchem, New Hampton, NY, USA), study participants consumed a daily 200mg docosahexaenoic acid supplement (Neuromins; Nature's Way Products, Springville, UT, USA), a daily over-the counter choline free prenatal multivitamin supplement (Pregnancy Plus; Fairhaven Health, LLC, Bellingham, WA, USA), and a thrice-weekly potassium/magnesium supplement (General Nutrition Corp., Pittsburgh, PA, USA) in order to achieve recommended nutrient intake levels not met with the study diet (Yan et al. 2012). When eating on site, participants consumed supplements under the supervision of study personnel. Otherwise, supplements were provided in plastic bags along with takeaway meals, and participants were instructed to consume the supplements with a meal of their choice. Consumption of each menu and supplement item was verified through the use of a daily checklist provided by the investigators.

Sample collection and processing

Fasting blood samples were collected into serum separator gel and clot activator tubes (SST Vacutainer, Becton, Dickinson and Company) and EDTA coated tubes (Vacutainer) at study baseline (wk 0), study midpoint (wk 6), wk 9, and study-end (wk10) (defined as wk 10 for both lactating and control women). Blood samples were processed for plasma, serum, and buffy coat as previously described (Yan et al. 2012). 24 hr urine samples were collected throughout the 10 wk study, processed, and stored at -80°C (Yan et al., 2012). Breastmilk was expressed in the morning at the Human Metabolic Research Unit (HMRU) by pumping with a Medela electric breast pump (Medela Inc). Lactating women were fasted for breastmilk sample collection that occurred on the same morning as the corresponding week's blood collection. Breastmilk samples consisted of the full expression of one breast 2 hr after the first feed of the day. Women expressed the same breast throughout the study. On collection, breastmilk samples were immediately placed on ice, dispensed into 15 mL centrifuge tubes, and stored at -80C.

Extraction of RNA from buffy coat and mammary epithelium

Total RNA was extracted from blood leukocytes via a commercially available kit (RNeasy Mini kit; Qiagen) at the time of blood processing and stored at -80°C. DNase treatment (Turbo DNA-free DNase; Ambion) of leukocyte RNA was performed according to the manufacturer's protocol to remove genomic DNA contamination. Mammary epithelium total RNA was isolated from the breastmilk at the time of milk processing by a modification of the Trizol method (Simms et al., 1993). Briefly, breastmilk (15 mL) was centrifuged at 2000g for 10 min at 4°C and the fat layer was collected from the top of the tube and homogenized with 1.5ml Trizol. The

homogenized samples were vortexed with 200 ul chloroform and centrifuged at 12000rpm for 15 min at 4°C. The clear aqueous phase was subsequently removed and mixed with 0.75 ml isopropanol in a new tube. Tubes were inverted repeatedly and then incubated at room temperature for 30 min. Centrifugation was repeated at 12000 rpm for 15 min at 4°C. The supernatant was removed by pipetting and the RNA pellet at the bottom of the tube was washed with 1 ml 75% ice cold ethanol. Tubes were centrifuged again at 12000 rpm for 5 min at 4°C, and the supernatant was removed. Finally, the RNA pellet was resuspended in 50 ul of RNase free water and stored at -80°C.

RNA concentrations for both blood leukocytes and mammary epithelium were measured via Nanodrop (NanoDrop™ ND1000 Spectrophotometer, Thermo Scientific). Samples with a 260/230 ratio >1.8 were considered to have sufficiently pure RNA for assessment of transcript abundance.

cDNA production

Reverse transcription of blood leukocyte and mammary epithelium total RNA was performed on a MyCycler thermocycler (BioRad). For reverse transcription, RNA (2.5 µg) was pipetted into a PCR tube with 1.83 µl of RNase free water and subsequently incubated at 70°C for 10 min and 4°C for 5 min. The remainder of reverse transcription was performed via the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA). The reaction conditions were as follows: 25°C for 10 min; 42°C for 40 min; 95°C for 5 min, and quenching at 4°C for 5 min. Following reverse transcription, leukocyte cDNA was diluted 1/15 and mammary epithelium cDNA was diluted 1/5 based on prior RNA concentrations and stored at -80°C.

QPCR measurement of choline metabolism gene expression

Quantitative real-time PCR (qPCR) analysis was performed on a Lightcycler 480 II (Roche). Genes of interest in blood leukocytes and mammary epithelium included *phosphocholine cytidyl transferase* (*PCYT1A*), *acetylcholinesterase* (*ACHE*), acetylcholine muscarinic receptor (*CHRM4*), choline dehydrogenase (*CHDH*), and *phosphatidylethanolamine N-methyltransferase* (*PEMT*). *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used as the housekeeping gene for both blood leukocytes and mammary epithelium. All primers were designed using GeneRunner 3.01 (http://www.softpedia.com/). Primers were as follows: *GAPDH*, forward, 5'-CTCCACGACGTACTCAGCG-3', and reverse, 5'-TGTTGCCATCAATGACCCCTT-3'; *CHRM4*, forward, 5'-TGTGCTGATCTCATCATAGGC-3', and reverse, 5'-TCACCACGTAGTCCAGGGC-3'; *PEMT*, forward, 5'-GGGGTTCGCTGGAACTTTC-3', and reverse 5'-GCCCAGGTAGTTGGCTGTG-3'; *PCYT1A*, forward, 5'-CAGAAGGTGGAGGAAAAAAGCA-3', and reverse, 5'-TATGTTTCAGTGCTCCTTCCGG-3'; *ACHE*, forward, 5'-CGCTACAACCTTCCAGAGTG-3' and reverse 5'-ACACGTTGAGGTACAGGCAG-3'; *CHDH*, forward, 5'-GCAAGGAGGTGATTCTGAGTGG -3' and reverse 5'- GGATGCCCAGTTTCTTGAGGTC-3';. The quality of primers was assessed by the consistency, height, and tightness of the melting curves. Blood leukocyte derived cDNA was analyzed with the Roche SYBR Green system. The program for leukocyte analysis was as follows: preheat at 95°C for 5 min; amplification phase was 95°C for 30 sec, 60C for 30 sec, and 72°C for 30 sec, for a total of 45 cycles. Mammary epithelium derived cDNA was analyzed with the Maxima SYBR Green system (Fermentas). The program for mammary epithelium analysis was as follows: preheat 95°C for 10 min; amplification phase was 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec, for a total of 45 cycles. Data were

expressed by the delta delta Ct method, in which the expression level of the gene of interest was normalized by the expression level of the housekeeping gene as fold change before comparison between samples (Livak and Schmittgen, 2001).

Genotyping

Select genetic variants were ascertained in one-carbon metabolic genes that may impact choline metabolism, including *betaine-homocysteine N-methyltransferase (BHMT)* G742A (rs3733890), *choline dehydrogenase (CHDH)* G233T (rs12676), *methylenetetrahydrofolate dehydrogenase 1 (MTHFD1)* G1958A (rs2236225), *methylenetetrahydrofolate reductase (MTHFR)* C677T (rs1801133), *phosphate cytidylyltransferase 1, choline,* α *(PCYT1A)* (rs939883, T→A),*5-methyltetradhydrofolate-homocysteine methyltransferase (MTR)* A2756G (rs1805087), *5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR)* A66G (rs1801394), and *PEMT* G5465A (rs7946). *BHMT* G742A, *MTHFD1* G1958A, and *MTHFR* C677T polymorphisms were determined by sequencing the double-stranded DNA templates with an Applied Biosystems Automated 3730 DNA analyzer. All other genotypes were determined using fluorescent TaqMan probe commercially available kits (Applied Biosystems, Foster City, CA).

Measurements of plasma and urine choline metabolites

Phosphatidylcholine (PC) and sphingomyelin (SM) were extracted from plasma (study wks 0, 6, 9, and 10), along with the PC and SM isotopomers (d0, d3, d6 and d9), and subsequently quantified by liquid chromatography-tandem mass spectrometry (LC-MS)

according to the method of Koc et al. (Koc et al., 2012). The Koc et al. method was modified by our lab by using d4-PC, rather than d9-PC, as the internal standard (Yan et al., 2011). Ions with a m/z ratio of 184 were monitored for unlabeled metabolites (d0-PC and d0-SM), ions with a m/z ratio of 188 were monitored for d4-PC, and ions with m/z ratios of 187, 190, and 193 were monitored for d3 labeled metabolites (d3-PC and d3-SM), d6 labeled metabolites (d6-PC and d6-SM), and d9 labeled metabolites (d9-PC and d9-SM), respectively. Because the d4-PC internal standard yielded ions with m/z ratios of 188 (the ions monitored for d4-PC) and 190 (the ions monitored for d6-PC), a second run without the addition of d4-PC was conducted for plasma at study wks 9 and 10. The second run was used to determine enrichments of the PC isotopomers (i.e., d3-PC divided by the sum of d3-, d6- and d9-PC).

Free choline, betaine, and dimethylglycine (DMG) were extracted from plasma (study wks 0, 6, 9, and 10) and urine (study wks 0, 6, 9, 10). The isotopomers of these metabolites (d0, d3, d6 and d9) were quantified by LC-MS/MS according to the method of Holm et al. (Holm et al., 2003) with modifications including the use of d13-choline, d3-betaine, and d3-DMG, rather than d9-choline, d9-betaine, and d6-DMG, as internal standards. The ions with m/z ratios monitored for each metabolite were as follows: d0-choline, 104 → 60; d3-choline, 107 → 63; d6-choline, 110 → 66; d9-choline, 113 → 69; d13-choline, 117 → 69; d0-betaine, 118 → 59; d3-betaine 121 → 62; d6- betaine, 124→ 65; d9-betaine, 127 → 68; d0-DMG, 104 → 58; d3-DMG, 107 → 61; d6-DMG, 110 → 64. As the internal standards, d3-betaine and d3-DMG were also target metabolites for quantification, a separate run without the addition of internal standards was conducted to measure plasma and urine enrichments (as described above). Urine data are reported as total metabolite excreted, not as a concentration.

For analysis of choline metabolite abundance at wks 9 and 10, given that a percentage of the choline supplied was deuterium labeled, the deuterium label (d3,d6,d9) areas were combined with the unlabeled metabolite areas to calculate total metabolite abundance.

Measurements of plasma and urine amino acid metabolites

Homocysteine, cysteine, methionine, cystathionine, glycine, and sarcosine were extracted from serum (study wks 0, 6, and 10) and urine (study wks 0, 6, and 10). Gas chromatography-mass spectrometry (Stabler et al., 1987; Allen et al., 1993) was employed to quantify these amino acids. Urine data are reported as total metabolite excreted per 24-h, not as a concentration.

Isotopic enrichment calculations

Isotopic enrichment [labeled metabolite/(labeled + unlabeled metabolites)] was examined in blood and urine to monitor the flow of dietary choline (d9-choline metabolites) and its methyl groups (d3 and d6-choline metabolites) through the choline metabolic pathways. The isotopic enrichment percentage was calculated using the peak area under the chromatography curve of the labeled metabolite divided by the total area under the curve of all isotopomers of the metabolite. For example, d3-PC enrichment = peak area of d3-PC / peak area of (d0+d3+d6+d9)-PC × 100%. The enrichment of d3-PC indicates the proportion of PEMT-derived d3-PC in the total plasma PC pool. Metabolite enrichment ratios were examined as indicators of the flow of orally consumed choline through a specific portion of a pathway (i.e., enrichment of product/ enrichment of precursor) or its partitioning between pathways (e.g.,

CDP-choline pathways, choline oxidative pathway). Under the current labeling strategy, the amount of PEMT-derived d9-PC (~ 0.02% of plasma total PC) was lower than the detection limits (~ 0.1% of plasma total PC). Therefore, we assumed that all of the measured d9-PC was derived from the CDP-choline pathway.

Statistical Analysis

Plots and histograms of the residuals were used to assess normality and variance homogeneity. Data that deviated from the normal distribution were logarithmically transformed to achieve normality, and the transformed data were used in subsequent analyses. The presence of outliers within the data set was also assessed using box plots; values >3x the interquartile range of the boxplot of the data were excluded.

To test for baseline (wk 0) differences in the continuous variables, a two-factor ANOVA (choline intake, lactation, and their interaction) was performed, or in cases where variables were only available for lactating women, a one-factor ANOVA (choline intake) was conducted. To test for baseline (wk 0) differences in the categorical variables (e.g., genotype frequency, ethnicity, education) between lactating and control women and choline intake groups, chi-square tests, or when expected counts were less than five, Fisher's exact tests, were used.

To delineate the impact of reproductive state on the dependent variables (i.e. choline metabolite concentrations) through time, a mixed linear model (MLM) was constructed for each dependent variable. As lactation is a physiologic state, not a treatment, baseline (week 0) measures of dependent variables were included as a data point. Reproductive state, continuous time (e.g., wks 0, 6, 9, and 10 for plasma variables; wks 0, 6, and 10 for urine/serum variables),

choline intake, and their interactions were included as fixed effects; the sample identifier was included as a random effect.

To delineate the impact of choline intake on the dependent variables (i.e., choline metabolite concentrations and amino acid metabolite concentrations) among lactating women, a MLM was constructed for each dependent variable. As baseline (wk 0) measures may influence the response to choline treatment, measures of the dependent variables at wk 0 were included as a covariate. Choline intake, continuous time (e.g., wks 6, 9, and 10 for plasma variables; wks 6 and 10 for urine/serum variables), and their interaction were included as fixed effects; the sample identifier was included as a random effect; and the week 0 measure was included as a covariate.

To test for differences in mRNA abundance based on reproductive state in blood leukocytes, a 2-factor ANOVA (choline intake and lactation) was performed with the study-end (wk10) mRNA abundance as the dependent variable and choline intake, lactation, and their interaction as independent variables. To test for differences in mRNA abundance based on choline intake in blood leukocytes and mammary epithelium of lactating women only, a one-factor ANOVA (choline intake) was performed with the study-end (wk10) mRNA abundance as the dependent variable and choline intake as the independent variable; baseline mRNA abundance (wk0) was included as a covariate. Pearson's correlation analysis of the mRNA expression of choline metabolism genes in blood leukocytes of lactating women at study-end (wk10) versus the mRNA expression of the same genes in mammary epithelium at study-end (wk10) was also performed.

Finally, to test for differences in choline metabolite enrichment based on reproductive state in urine and plasma, a 2-factor ANOVA (choline intake and lactation) was performed with the study-end (wk10) choline metabolite enrichment as the dependent variable and choline intake, lactation, and their interaction as independent variables. To test for differences in choline metabolite enrichment based on choline intake in plasma and urine of lactating women only, a one-factor ANOVA (choline intake) was performed with the study-end (wk10) choline metabolite enrichment as the dependent variable and choline intake as the independent variable. Baseline enrichment (wk 0) was not included as a covariate because deuterium-labeled choline metabolites were not present.

Candidates for entry as additional covariates into all MLMs and ANOVAs were age, BMI, ethnicity, and the eight genetic variants in one-carbon metabolic genes. In addition, where possible, genotypes with a count of ≤2 at a given loci were combined together with the heterozygote genotype to yield samples sizes of ≥3 for each genotype in the model (all genotypes were combined in this manner except for *PCYT1A* (rs939883, T→A), *MTHFD1* G1958A (rs2236225), and *MTRR* A66G (rs1801394)). The covariates that did not achieve a significance of ($P<0.1$) were removed from the models in a stepwise manner.

All analyses were performed using SPSS 21.0 for Windows (SPSS Inc, Chicago, IL, USA). Differences were considered to be significant at values of $P\leq0.05$ and $P\leq0.10$ was considered to be indicative of trends. Values are presented as means ± standard error unless noted otherwise.

RESULTS

Participant Characteristics and Baseline Measures

Forty-eight women were included in the final analysis: 21 control women who completed 10 wks of the study and 27 lactating women who completed 10 wks (n=25) or 9-wks (n=2) of the study. Data were available for every time point (wks 0, 6, and 10 for serum; wks 0,6,9, and 10 for urine; wks 0, 6, 9, and 10 for plasma; and wks 0 and 10 or wks 0 and 9 for mRNA abundance) with the exception of those who ended the study early at wk9 (missing wk 10), three wk 9 plasma samples, one wk 10 sample for urine only, and 8 RNA wk0-wk10 pairs (7 blood leukocyte, 1 mammary epithelium) which were of insufficient quantity or poor quality

At baseline, lactating (vs. control) women used less (p=0.0001) oral contraception, consumed more (p=0.005) vitamin supplements, were less (p=0.04) physically active, and differed in the frequency of the PEMT (P=0.03) and PCYT1A (P=0.03) genotypes (see **Table 1**). Lactating (vs control) women also exhibited lower (p=0.01) leukocyte *PEMT* mRNA abundance, higher (p=0.001) leukocyte *CHDH* mRNA, lower (p=0.001) leukocyte *CHRM4* mRNA abundance, higher (p=0.0001) plasma choline, and excreted less urinary betaine (p=0.0001) and DMG (p=0.0001) (**Table 1**). Otherwise, the dependent variables of interest did not differ between the reproductive groups (**Table 1**). For choline intake, no differences at baseline were detected between the 930 and 480 mg choline/d intake groups for any of the dependent variables (data not shown) with the exception of plasma choline which was lower (p=0.02) in the 930 (6.3 ± 1.5 nM/mL) vs. 480 (7.3 ± 1.7 nM/mL) mg/d group. In addition, no interactions ($P \geq 0.1$) were detected between reproductive state and choline intake assignment for any of the dependent variables.

TABLE 1. *Baseline characteristics, mRNA abundance, and metabolite concentrations in control and lactating women.* [1]

Variables	Control Women	Lactating Women	P Value
Characteristics[2]			
Ethnicity (*Caucasian/African/ Latino/Asian/Other*)	14/2/2/1/2	19/1/3/1/3	1
Age (*yrs*)	28.8 (21-40)	29.1 (22-41)	0.8
Prepregnancy BMI	23.6 (18-30)	26.7 (18-42)	0.1
Education (*high school/associate/ bachelors/masters/ doctorate/other*)	6/0/5/5/5/0	7/3/10/7/0/1	0.06
Oral Contraceptive Use (*no/yes*)	12/9	27/1	0.001
Vitamin Supplement Use (*no/yes*)	14/7	7/20	0.005
Physical Activity (*low/medium/high*)	3/10/8	12/13/3	0.04
Genotypes (counts)			
MTHFR, rs1801133, 677 *(CC/CT/TT)*	9/9/3	11/15/1	0.5
MTHFD1, rs2236225, 1958 *(GG/GA/AA)*	3/15/3	6/17/4	0.9
BHMT, rs3733890, 742 *(GG/GA/AA/unknown)*	13/7/1/0	7/12/4/4	0.1

PEMT, rs7946, 5465 *(GG/GA/AA)*	5/10/6	4/5/18	0.03
CHDH, rs12676, 233 *(GG/GT/TT)*	12/7/2	16/9/2	1
PCYT1A, rs939883, *(TT/TA/AA)*	9/9/3	5/21/1	0.03
MTR, rs1805087, 2756 *(AA/GA/GG)*	14/6/1	18/9/0	0.7
MTRR, rs1801394, 66 *(AA/GA/GG)*	7/9/5	9/13/5	0.9
Blood Leukocyte mRNA expression (fold mRNA abundance relative to control women)[3]			
ACHE	1 ± 1.2	0.5 ± 1.1	0.3
PEMT	1 ± 1.2	0.1 ± 0.1	0.01
PCYT1A	1 ± 1.3	0.4 ± 0.2	0.5
CHDH	1 ± 0.6	2 ± 1	0.001
CHRM4	1 ± 1.5	0.2 ± 0.1	0.001
Mammary Epithelium mRNA expression (fold mRNA abundance relative to *GAPDH*)[4]			
PEMT		1.3 ± 0.1	
PCYT1A		1.3 ± 0.1	
CHDH		1.4 ± 0.1	
Plasma Choline Metabolite Concentrations[5]			
Choline (*nM/mL*)	5.9 ± 1.6	7.5 ± 1.4	0.0001
Betaine (*nM/mL*)	25 ± 10	27 ± 8	0.5
Dimethylglycine (*nM/mL*)	2.4 ± 2	1.9 ± 0.6	0.3
Phosphatidylcholine (*nM/mL*)	1586 ± 301	1652 ± 372	0.6

Sphingomyelin (*nM/mL*)	509 ± 92	551 ± 114	0.2
Urinary Choline Metabolite Excretion[5]			
Choline *(mg/d*)	2.5 ± 1.1	2.4 ± 0.9	0.9
Betaine (*mg/d*)	8.8 ± 5.6	3.9 ± 2.5	0.0001
Dimethylglycine (*mg/d*)	3.7 ± 2.2	1.6 ± 1.3	0.0001
Serum Amino Acids[5]			
Methionine (*uM/mL*)		25 ± 4.2	
Cysteine (*uM/mL*)		296 ± 33	
Homocysteine (*uM/mL*)		6.2 ± 1	
Glycine (*uM/mL*)		348 ± 76	
Sarcosine (*uM/mL*)		1.2 ± 0.3	
Cystathionine (*nM/mL*)		157 ± 96	
Urinary Amino Acid Excretion[5]			
Methionine (g*/d*)		1.5 ± 0.9	
Cysteine (*g/d*)		28 ± 8.8	
Homocysteine (*g/d*)		0.4 ± 0.1	
Glycine (*g/d*)		95 ± 54	
Sarcosine (*g/d*)		0.32 ± 0.2	
Cystathionine (*mg/d*)		2.2 ± 1.6	

[1] Continuous variables were analyzed using 2-factor ANOVA (i.e., choline and lactating state as independent factors) or 1-factor ANOVA (i.e., choline intake in lactating women as the independent factor); chi-square or Fishers exact test was used to analyze categorical variables. Sample size was n=21-27 per reproductive group (unless noted otherwise) and n=10-14 per

choline intake group. No differences were detected between choline intake groups within a reproductive category (data not shown) with the exception of plasma choline which was lower (p=0.024) in the 930 (6.3 ± 1.5 nM/mL) vs. 480 (7.3 ± 1.7 nM/mL) mg/d group. Data are not stratified by choline intake because reproductive state and choline intake did not interact to affect the dependent variables.

[2] Mean, (range in parenthesis), or spread of data

[3] Arithmetic mean fold mRNA abundance ± standard deviation; n=16-27 per reproductive group. mRNA abundance calculated as fold change relative to control women.

[4] Arithmetic mean fold mRNA abundance ± standard deviation. mRNA abundance calculated as fold change relative to housekeeping gene, *GAPDH*.

[5] Arithmetic means ± standard deviation.

Effect of Reproductive State on Biomarkers of Choline Metabolism

Blood leukocyte choline metabolic gene expression

At study-end (wk10), lactating vs. control women exhibited lower *ACHE* (p=0.0001) and *CHRM4* (p=0.04) expression and tended to have higher (p=0.01) *CHDH* expression (see **Table 2**). Expression of *PEMT* and *PCYT1A* was unchanged. As there were no significant interactions between choline intake and lactation, reproductive groups were not stratified by choline intake and reanalyzed.

TABLE 2. *Effect of reproductive state on mRNA abundance at study-end (wk10).* [1-3]

Genes	Control Women	Lactating Women	P value
ACHE	1 ± 1.3	0.2 ± 0.3	0.0001
PEMT	1 ± 1.9	0.3 ± 0.3	0.1
PCYT1A	1 ± 1.2	0.5 ± 0.2	0.4
CHDH	1 ± 0.6	1.4 ± 0.7	0.1
CHRM4	1 ± 1.4	0.4 ± 0.2	0.04

[1] Arthimetic mean ± standard deviation; n=16 for control women, n=24-27 for lactating women

[2] Data are presented as fold change using control women as the reference (e.g. lactating women average value / control women average value) and were analyzed by 2-factor ANOVA. Data are not stratified by choline intake because reproductive state and choline intake did not interact to affect the dependent variables.

Plasma choline metabolite concentrations

Throughout the study period, lactating (versus control) women exhibited higher plasma concentrations of choline (p=0.001) and betaine (p=0.03); whereas plasma concentrations of dimethylglycine, phosphatidylcholine, and sphingomyelin were not influenced by reproductive state (see **Table 3**). In addition, no interactions were detected between reproductive state and choline intake (P≥0.1) or time (P≥0.3).

TABLE 3. *Effect of reproductive state on plasma choline metabolite concentrations across the study period.* [1,2]

Metabolite	**Control Women**	**Lactating Women**	**P value**
DMG (µmol/L)	2.5 (2.2, 3.0)	2.4 (2.0, 2.8)	0.5
Choline (µmol/L)	6.3 (5.9, 6.7)	7.6 (7.1, 8.1)	0.001
Betaine (µmol/L)	26 (23, 30)	33 (28, 37)	0.03
PC (µmol/L)	1683 (1592, 1782)	1629 (1545, 1722)	0.4
SM (µmol/L)	511 (486, 537)	502 (479, 526)	0.6

[1] All values are back transformed predicted means (95% CIs) across the 10 wk study. Data were derived by using mixed linear models that tested the effect of reproductive state on the dependent variables as described in the main text. Sample size is n=21 for control women; n=27 for lactating women. Data are not stratified by choline intake because reproductive state and choline intake did not interact to affect the dependent variables. DMG, dimethylglycine; PC, phosphatidylcholine; SM, sphingomyelin.

Urinary choline metabolite excretion

Throughout the study period, lactating (versus control) women exhibited decreased urinary loss of DMG (p=0.0001) and betaine (p=0.0001) throughout the study period; whereas urinary loss of choline was not influenced by reproductive state. Reproductive state did not interact with choline intake (P≥0.2) or time (P≥0.3) (see **Table 4**).

TABLE 4. *Effect of reproductive state on urinary choline metabolite across the study period.* [1,2]

Metabolite	Control Women	Lactating Women	P value
DMG (mg/d)	3.9 (3.2, 4.8)	1.6 (1.4, 2)	0.0001
Choline (mg/d)	2.4 (2.1, 2.7)	2.3 (2.1, 2.7)	0.9
Betaine (mg/d)	6.8 (5.8, 8)	3.8 (3.2, 4.4)	0.0001

[1] All values are back transformed predicted means (95% CIs) throughout the 10 wk study. Data were derived by using mixed linear models that tested the effect of reproductive state on the dependent variables as described in the main text. Sample size is n=21 for control women; n=27 for lactating women. No significant interactions (P>0.05) between reproductive state and choline intake or reproductive state and time were detected. DMG, dimethylglycine.

Enrichment of plasma choline metabolites

At study-end (wk10), labeled choline metabolites were detected at acceptable signal-to-noise ratios in blood samples including d3-choline, d9-choline, d3-betaine, d9-betaine, d3-DMG, d6-DMG, d3-sarcosine, d3-methionine, d3-PC, d6-PC, d9-PC, and d9-SM. Lactating women had lower d3-betaine (p=0.05) enrichment than control women (see **Table 5**). Lactating women also had a lower circulating ratio of d3-betaine: d3-choline (p=0.02) versus control women. There was no significant interaction between reproductive state and choline intake in any analysis undertaken, therefore, women were not stratified by choline intake and reanalyzed.

TABLE 5. *Effect of reproductive state on the isotopic enrichment of choline metabolites at study-end (wk10).*[1,2]

Metabolite	**Control Women**	**Lactating Women**	**P value**
Blood Enrichment			
d9-choline	6.6 ± 0.2	6.6 ± 0.2	0.9
d6-DMG	30 ± 0.8	30 ± 0.8	0.8
d3-choline	2.0 ± 0.1	1.9 ± 0.1	0.3
d3-betaine	1.4 ± 0.05	1.3 ± 0.1	0.05
d3-PC	2.9 ± 0.1	2.7 ± 0.1	0.4
d6-PC	0.2 ± 0.01	0.2 ± 0.02	0.9
d9-PC	7.0 ± 0.1	7.0 ± 0.2	0.7
d9-SM	7.3 ± 0.2	6.9 ± 0.2	0.3

d9-betaine	9.5 ± 0.4	8.7 ± 0.5	0.3
Blood Enrichment Ratios			
d9-betaine : d9-choline	1.3 ± 0.04	1.3 ± 0.04	1
d9-PC : d9-choline	1.1 ± 0.03	1.0 ± 0.04	0.1
d9-SM : d9-PC	1 ± 0.009	1 ± 0.01	0.7
d6-DMG : d9-betaine	3.5 ± 0.1	3.5 ± 0.1	0.8
d3-sarcosine : d6-DMG	8.4 ± 0.02	0.8 ± 0.02	0.2
d3+d6-PC : d9-PC	0.4 ± 0.02	0.4 ± 0.02	0.9
d3-choline : d3-PC	0.7 ± 0.03	0.7 ± 0.03	0.5
d3-betaine : d3-choline	0.7 ± 0.009	0.7 ± 0.01	0.02
d9-betaine : d9-PC	1.2 ± 0.05	1.2 ± 0.05	0.3
d9-betaine:d3-methionine	16 ± 1.5	20 ± 1.5	0.1
d3-betaine:d3-methionine	2.6 ± 0.2	2.9 ± 0.2	0.6
d3-sarcosine:d3+d6-PC	8.6 ± 0.3	8.5 ± 0.3	0.8
d3-methionine:d3+d9-betaine	0.06 ± 0.004	0.05 ± 0.004	0.1

[1] Data are presented as estimated marginal means± SEM; n=21 for control women, n=23 for lactating women.

[2] Data were derived by using two factor ANOVA models that tested the effect of reproductive state on the dependent variables as described in the main text. DMG, dimethylglycine; PC, phosphatidylcholine; SM, sphingomyelin.

Effect of Choline Intake Among Lactating Women on Biomarkers of Choline Metabolism

Blood leukocyte choline metabolic gene expression

At study-end (wk10), choline intake did not influence the expression of *ACHE*, *PEMT*, *PCYT1A*, *CHDH*, and *CHRM4* among lactating women (see **Table 6**).

TABLE 6. *Effect of choline intake on mRNA abundance in lactating women randomized to 480 or 930 mg/d choline intake at study-end (wk10).*[1,2]

Genes	480 mg choline/d	930 mg choline/d	P value
ACHE	1 ± 1.3	1.2 ± 2	0.3
PEMT	1 ± 1.2	1.5 ± 1.8	0.5
PCYT1A	1 ± 0.5	1 ± 0.5	0.7
CHDH	1 ± 0.6	1 ± 0.3	0.6
CHRM4	1 ± 0.6	1.3 ± 0.9	0.1

[1] Arithmetic mean ± standard deviation; n=12-14 per choline intake group

[2] Data are presented as fold change using the 480 mg choline/d as the reference (e.g. 930 average value / 480 average value) and were analyzed by 1-factor ANOVA.

Mammary epithelium choline metabolic gene expression

At study-end (wk10), choline intake did not influence the transcript abundance of the mammary epithelium genes among lactating women (see **Table 7**).

TABLE 7. *Effect of choline intake on mRNA abundance in lactating women randomized to 480 or 930 mg/d choline intake at study-end (wk10).*[1,2]

Genes	**480mg choline/d**	**930mg choline/d**	**P value**
PEMT	1 ± 2.1	0.5 ± 0.4	0.7
PCYT1A	1 ± 0.5	1.2 ± 0.9	0.6
CHDH	1 ± 1.0	1.5 ± 1.9	0.6

[1] Arithmetic mean ± standard deviation; n = 13 per choline intake group

[2] Data are presented as fold change using the 480 mg choline/d as the reference (e.g. 930 average value / 480 average value) and were analyzed by 1-factor ANOVA.

Plasma choline metabolite concentrations

Throughout the 10 wk study, time did not influence circulating concentrations of choline metabolites among lactating women. However, consumption of 930 (vs. 480) mg choline/d yielded higher plasma concentrations of DMG (p=0.02) and betaine (P=0.05). Choline (p=0.3), phosphatidylcholine (p=0.5), and sphingomyelin (p=0.8) were unaltered by choline intake (see **Figure 2**).

Urinary choline metabolite excretion

Throughout the 10 wk study, neither time or choline intake influenced circulating concentrations of the choline metabolites among lactating women (see **Figure 3**).

Choline-related plasma amino acid concentrations

Throughout the 10 wk study, lactating women consuming 930 (vs. 480_ mg choline/d exhibited decreased plasma cystathionine (p=0.03) and increased plasma sarcosine (p=0.004) across time. Choline intake did not affect plasma glycine (p=0.7), methionine (p=0.5), cysteine (p=0.9), or homocysteine (p=0.7) (see **Figure 4**). Time was not a significant factor although borderline decreases were observed in cystathionine (p = 0.07).

Urinary choline-related amino acid excretion

Throughout the 10 wk study, lactating women consuming 930 (vs. 480) mg choline/d did not exhibit changes in urinary loss of glycine (p=0.9); cystathionine (p=0.7), methionine (p=0.4), cysteine (p=0.7), homocysteine (p=0.6), or sarcosine (p=0.8) (see **Figure 5**).

Enrichment of plasma choline metabolites

At study-end (wk10), lactating women consuming 930 (vs. 480) mg choline/d had higher plasma enrichment of d9-choline (p=0.04); d6-DMG (p=0.03); d3-choline (p=0.001); d3-betaine (p=0.0001); d3-sarcosine (p=0.0001); d3-methionine (p=0.01); d3-PC (p=0.001); d6-PC (p=0.001); and d9-betaine (p=0.0001) but trended towards lower plasma enrichment of d9-SM (p=0.07) (see **Table 8**). In addition, lactating women consuming 930 (vs. 480) mg choline/d had higher circulating ratios of d9-betaine: d9-choline (p=0.05) and d3+d6-PC:d9-PC (p=0.04) but lower circulating ratios of d6-DMG:d9-betaine (p=0.001) and d3-sarcosine: d3+d6-PC (p=0.05).

TABLE 8. Effect of choline intake on blood enrichments, and the enrichment ratios, of choline metabolites at study-end (wk10) among lactating women consuming 22% of 480 or 930 mg choline/d as *methyl*-d9-choline from study wk-6 to wk 10[1,2]

Metabolite	**480 mg choline/d**	**930 mg choline/d**	**P value**
Blood Enrichment			
d9-choline	6 ± 0.2	6.9 ± 0.3	0.04
d6-DMG	27 ± 1.4	32 ± 1.9	0.03
d3-choline	1.6 ± 0.1	2.2 ± 0.1	0.001
d3-betaine	1.1 ± 0.04	1.4 ± 0.05	0.0001
d3-sarcosine	20 ± 0.8	27 ± 1.1	0.0001
d3-methionine	0.4 ± 0.04	0.6 ± 0.05	0.01
d3-PC	2.2 ± 0.1	3.2 ± 0.2	0.001
d6-PC	0.1 ± 0.02	0.2 ± 0.02	0.001
d9-PC	7 ± 0.1	6.6 ± 0.2	0.1

d9-SM	7.1 ± 0.2	6.3 ± 0.3	0.07
d9-betaine	7.4 ± 0.4	11 ± 0.5	0.0001
Blood Enrichment Ratios			
d9-betaine : d9-choline	1.2 ± 0.04	1.4 ± 0.05	0.05
d9-PC : d9-choline	1.1 ± 0.06	1 ± 0.08	0.2
d9-SM : d9-PC	1 ± 0.02	1 ± 0.02	0.5
d6-DMG : d9-betaine	3.7 ± 0.1	3.2 ± 0.1	0.001
d3-sarcosine : d6-DMG	0.8 ± 0.03	0.8 ± 0.04	0.3
d3+d6-PC : d9-PC	0.4 ± 0.03	0.5 ± 0.04	0.04
d3-choline : d3-PC	0.7 ± 0.04	0.7 ± 0.05	0.7
d3-betaine : d3-choline	0.7 ± 0.01	0.7 ± 0.02	1
d9-betaine : d9-PC	1.2 ± 0.08	1.3 ± 0.1	0.8
d9-betaine : d3-methionine	21 ± 1.7	19 ± 2.4	0.6
d3-betaine : d3-methionine	2.8 ± 0.3	3.3 ± 0.5	0.5
d3-sarcosine : d3+d6-PC	9 ± 0.3	7.8 ± 0.4	0.05
d3-methionine:d3+d9-betaine	0.05 ± 0.004	0.05 ± 0.005	0.5
d6-DMG:d9-choline	4.5 (0.2)	4.4 (0.2)	0.8

[1] Data are presented as estimated marginal means± SEM; n=14 for 480 mg choline/d, n=9 for 930 mg choline/d

[2] Data were derived by using one factor ANOVA models that tested the effect of choline intake on the dependent variables as described in the main text. DMG, dimethylglycine; PC, phosphatidylcholine; SM, sphingomyelin.

Enrichment of urinary choline metabolites

At study-end (wk10), labeled choline metabolites were detected at acceptable signal-to-noise ratios in urine samples including d3-choline, d9-choline, d3-betaine, d9-betaine, d3-DMG, d6-DMG, d3-sarcosine, d3-methionine, and d3-SAM. Lactating women consuming 930 vs. 480 mg choline/d had greater urine enrichment of all metabolites, with significantly greater enrichment of d6-DMG (p=0.0001); d3-choline (p=0.0001); d3-betaine (p=0.0001); d3-DMG (p=0.04); and d3-SAM (p=0.0001). Lactating women consuming 930 vs. 480 mg choline/d had a lower urinary ratio of d6-DMG:d9-betaine (p= 0.02) and trended towards a higher circulating ratio of d3-betaine:d3-choline (p= 0.06), (see **Table 9**).

TABLE 9. Effect of choline intake on urinary enrichments of choline metabolites at study-end (wk10) among lactating women consuming 22% of 480 or 930 mg choline/d as *methyl*-d9-choline from study wk-6 to wk 10[1,2]

Metabolite	480 mg choline/d	930 mg choline/d	P value
d9-choline	10 ± 0.7	12 ± 1	0.4
d9-betaine	9.5 ± 0.5	11 ± 0.7	0.1
d6-DMG	30 ± 0.9	37 ± 1.2	0.0001
d3-choline	1.6 ± 0.03	2 ± 0.04	0.0001
d3-betaine	1.1 ± 0.03	1.4 ± 0.04	0.0001
d3-DMG	1 ± 0.05	1.2 ± 0.07	0.04
d3-sarcosine	17± 1.5	20± 2.2	0.3
d3-methionine	0.4 ± 0.1	0.4 ± 0.1	0.7

d3-SAM	0.5 ± 0.02	0.7 ± 0.03	0.0001
Urinary Enrichment Ratios			
d9-betaine : d9-choline	0.9 ± 0.03	0.9 ± 0.03	0.7
d3-betaine : d3-choline	0.7 ± 0.009	0.7 ± 0.01	0.06
d6-DMG : d9-betaine	3.5 ± 0.1	3.1 ± 0.1	0.02
d3-DMG : d3-betaine	0.9 ± 0.03	0.8 ± 0.04	0.2
d3-methionine : d3+d9-betaine	0.03 ± 0.003	0.03 ± 0.003	0.1
d3-sarcosine : d3+d6 DMG	0.5 ± 0.04	0.6 ± 0.06	0.2
d3-SAM: d3-methionine	1.7 ± 0.2	1.6 ± 0.3	0.7
d3-methionine: d9-betaine	0.03 ± 0.003	0.04 ± 0.004	0.1
d3-methionine:d3-betaine	0.3 ± 0.04	0.3 ± 0.05	0.9

[1] Data are presented as estimated marginal means± SEM; n=14 for 480 mg choline/d, n=9 for 930 mg choline/d

[2] Data were derived by using one factor ANOVA models that tested the effect of choline intake on the dependent variables as described in the main text. DMG, dimethylglycine.

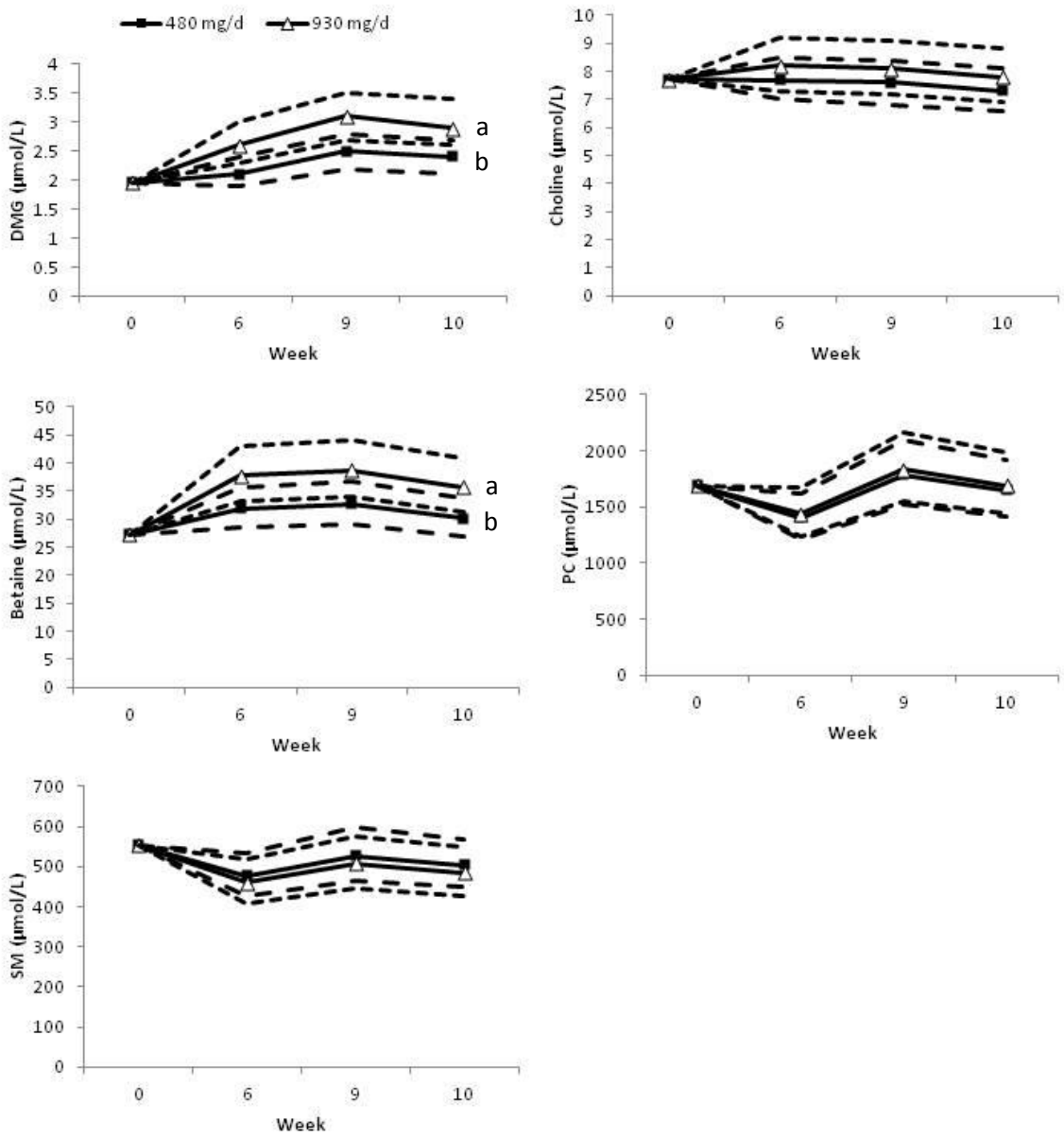

FIGURE2. Back transformed predicted means (95% CIs) of plasma free choline, betaine, dimethylglycine (DMG), phosphatidylcholine (PC), and sphingomyelin (SM) in lactating women consuming 480 mg choline/d (n=14) or 930 mg choline/d (n=13) for 10 wk. The plotted data were estimated by using mixed linear regression models. Different superscript letters indicate that the choline metabolite differed (P≤0.05) between choline-intake groups.

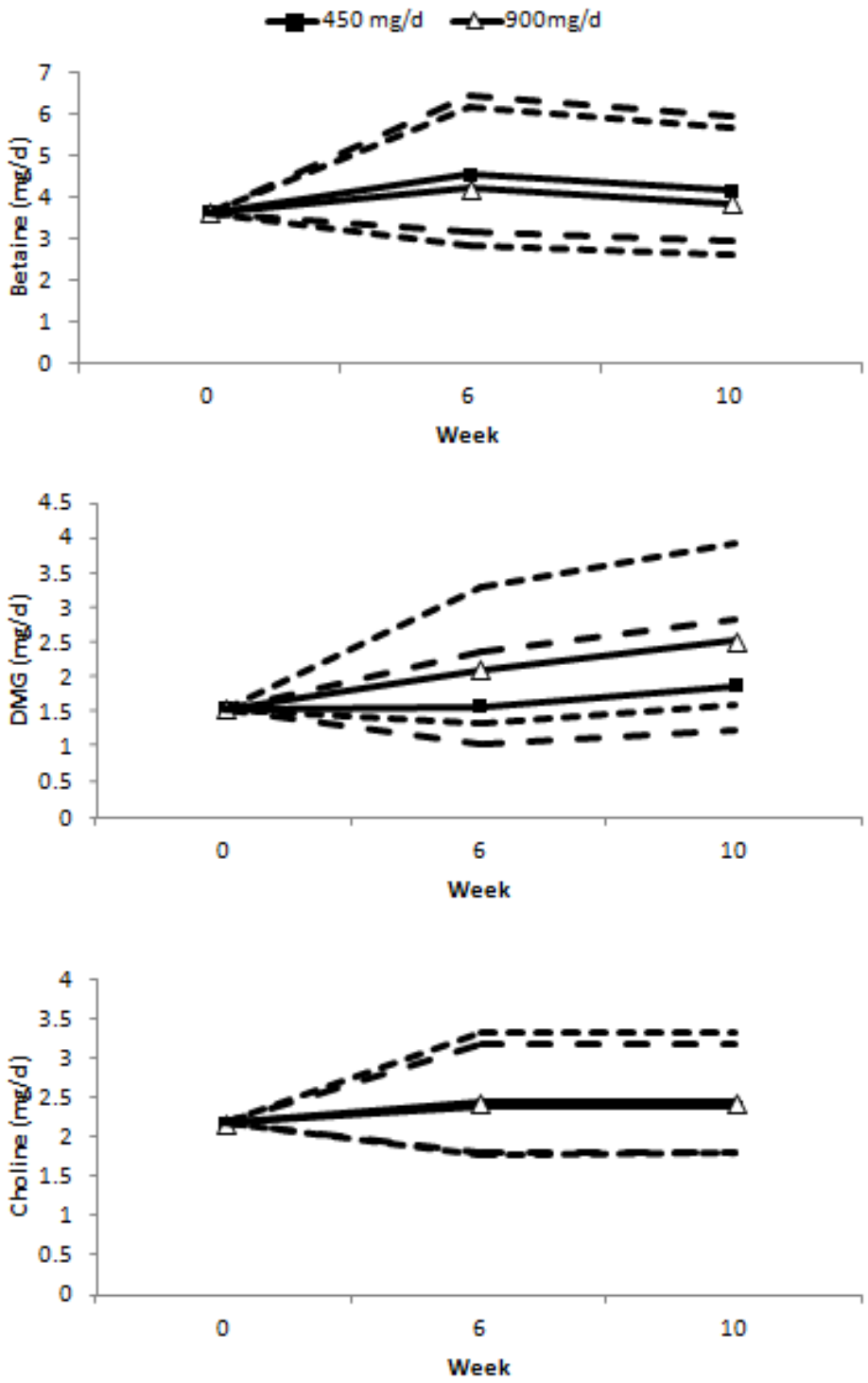

FIGURE 3. Back transformed predicted means (95% CIs) of urinary choline, betaine, and dimethylglycine (DMG) in lactating women consuming 480 mg choline/d (n=14) or 930 mg choline/d (n=13) for 10 wk. The plotted data were estimated by using mixed linear regression models. No metabolites were significantly affected by choline intake among lactating women.

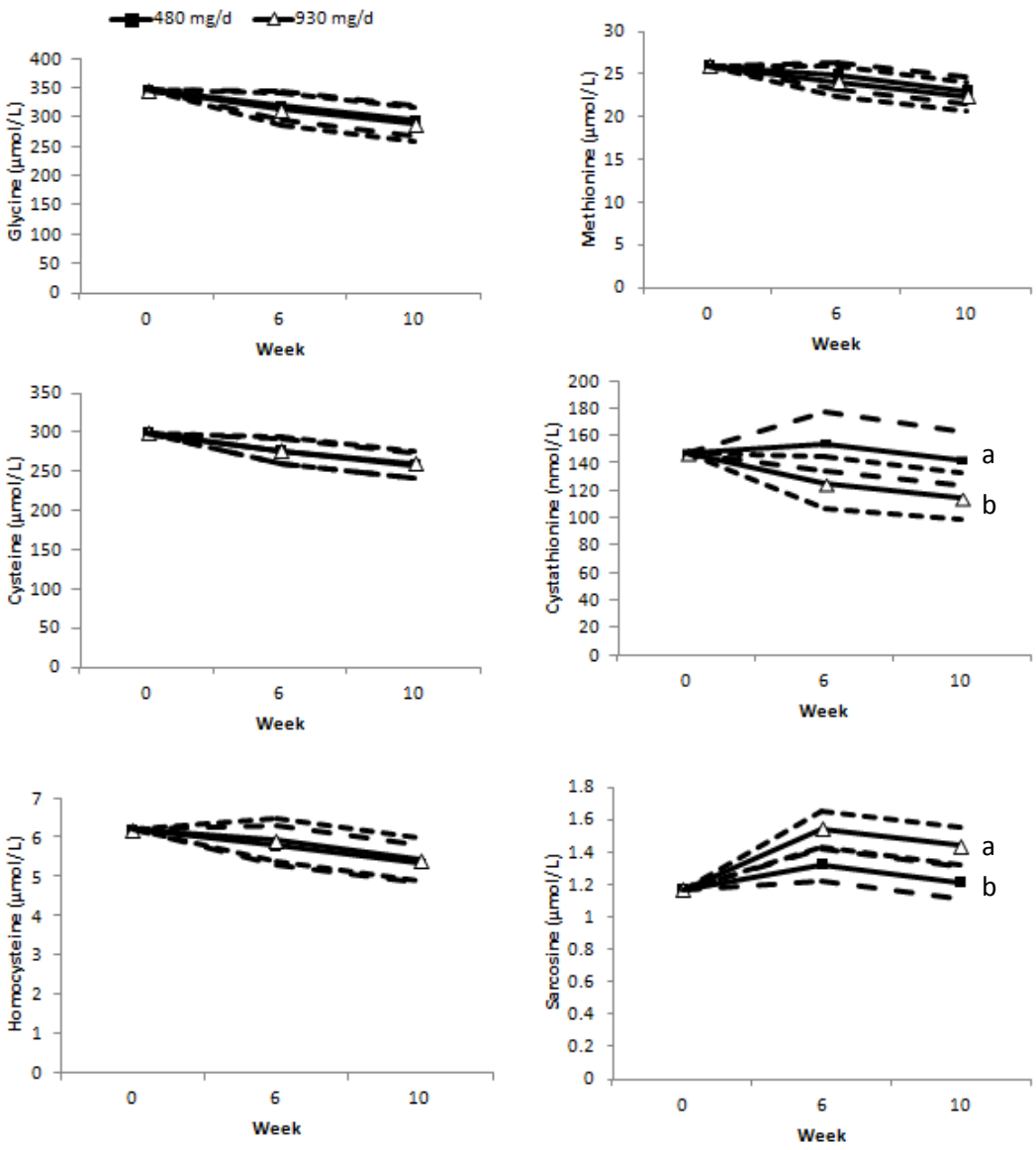

FIGURE 4. Back transformed predicted means (95% CIs) of plasma glycine, methionine, cystiene, cystathionine, homocysteine, and sarcosine in lactating women consuming 480 mg choline/d (n=14) or 930 mg choline/d (n=13) for 10 wk. The plotted data were estimated by using mixed linear regression models. Different superscript letters indicate that the choline metabolite differed (P≤0.05) between choline-intake groups.

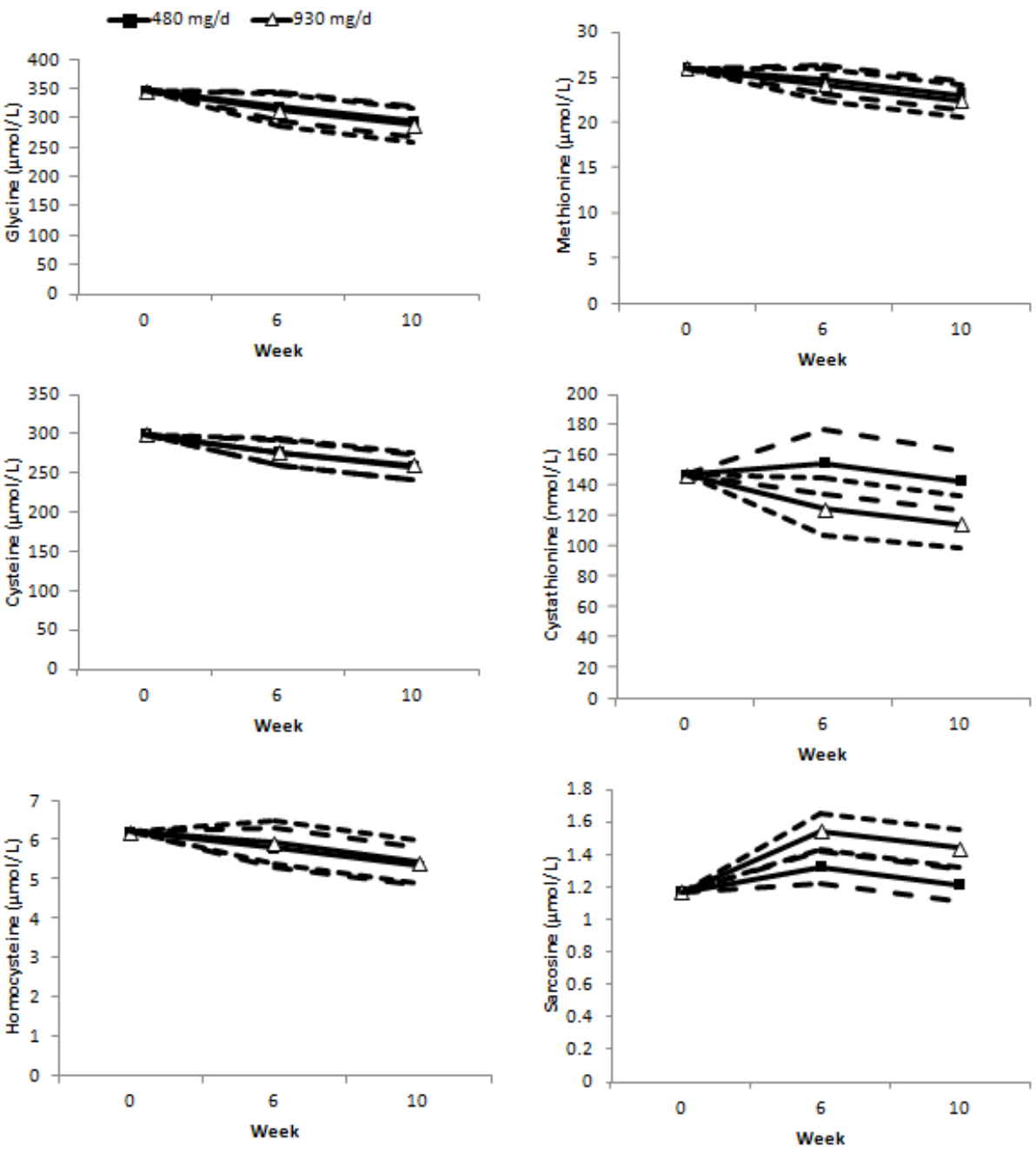

FIGURE 5. Back transformed predicted means (95% CIs) of urinary glycine, methionine, cystiene, cystathionine, homocysteine, and methylglycine in lactating women consuming 480 mg choline/d (n=14) or 930 mg choline/d (n=12) for 10 wk. The plotted data were estimated by using mixed linear regression models. No results were significant.

Correlations between mammary epithelium genes and blood leukocyte genes

At study-end (wk10), mammary epithelium *PEMT* (Pearson correlation = 0.371; p=0.082)*, CHDH* (Pearson correlation = -0.084; p=0.684), and *PCYT1A* (Pearson correlation = -0.004; p=0.986) expression were not significantly correlated with expression of the same gene in plasma leukocytes.

DISCUSSION

This highly controlled feeding study sought to quantify the effects of reproductive state and choline intake during lactation on biomarkers of choline metabolism. The following main findings emerged: 1) lactation alters biomarkers of choline metabolism and elevates plasma betaine and choline; 2) a higher choline intake during lactation increases betaine supply and subsequent synthesis of DMG, sarcosine, and PEMT-PC from betaine; and 3) blood leukocyte gene expression is not a proxy for mammary epithelium gene expression.

Lactating women exhibit elevations in plasma betaine and choline

To the best of our knowledge, this is the first study to report elevated concentrations of plasma betaine during human lactation. Our finding of 27% higher plasma betaine in lactating (vs. control) women diverges from the ~50% lower plasma betaine observed during the latter half of pregnancy (Yan et al. 2012; Velzing-Aarts et al., 2005; Friesen et al., 2007). Whereas pregnancy increases the use of choline as a methyl donor and leads to diminished concentrations of plasma betaine (Yan et al. 2012), lactation appears to conserve betaine perhaps because of betaine's role as an osmolyte (Lever and Slow, 2010; Garrett et al., 2013, Rudolf et al., 2007; Suzuki et al., 1993). Betaine plays a central role in maintaining osmotic balance and is elevated in mouse lactating mammary gland (Rudolf et al. 2007); is released from rat liver upon hypoosmotic stress (Wettstein et al., 1998); and is accumulated in the kidney to counter hyperosmotic stress (Rosas-Rodriguez et al., 2010). Thus, the observed elevations in maternal circulating betaine in the present study may be a mechanism for maintaining osmotic balance amidst large effluxes of water, proteins and metabolites from

maternal circulation to breastmilk (Dewey, 2004). Betaine is particularly well suited as an osmolyte during lactation because it is not a major constituent of breastmilk (Sakamoto et al., 2001) and is normally lost at very low rates in urine, allowing it to remain in plasma and tissues (Lever and Slow, 2010).

The elevations in plasma betaine during lactation may be attributed in part to betaine conservation by the kidney. Lactating (vs. control) women exhibited a 44% decrease in the already low rate of urinary betaine loss which diverges from enhanced loss of urinary betaine during pregnancy (Yan et al. 2012). Alterations in the abundance of choline metabolizing enzymes during lactation may also contribute. In the present study, lactating (vs. control) women exhibited diminished amounts of blood leukocyte mRNA for *ACHE* (80%) and *CHRM4* (60%), but trended towards having elevated mRNA abundance of *CHDH* (40%), encoding the enzyme required for synthesizing betaine from choline. As increases in mRNA abundance of *CHDH* are associated with greater enzymatic activity (Slow and Garrow, 2006), the observed elevation in *CHDH* transcript levels indicates a preference for betaine synthesis and has been reported to occur under conditions of altered osmotic balance (Hoffman et al. 2012).

As previously reported (Ilcol et al., 2005), lactating women exhibited higher circulating plasma choline (21%) than control women. Greater circulating plasma choline is also observed during the second half of pregnancy and appears to arise from enhanced *de novo* choline synthesis through the PEMT pathway (Yan et al., unpublished data), when estrogen is elevated (Resseguie et al., 2007). However, estrogen levels drop after parturition through the antagonistic activation of prolactin secretion to initiate and sustain lactogenesis (Freeman et al., 2000). Therefore, in lactation, *de novo* synthesis may be less significant for meeting choline

demand than in pregnancy. Indeed, d3-labeling of choline metabolites (i.e., produced by the PEMT pathway) did not differ between lactating and control women in the present study, indicating a lack of effect of reproductive state on PEMT activity.

Lactation influences flux through choline metabolic pathways

The larger pool sizes of choline (21%) and betaine (27%) in the lactating (vs. control) women would be expected to dilute the isotopically labeled choline metabolites. Therefore, if flux through these pathways was similar among lactating and control women, we would expect to observe diminished labeling of the choline and betaine pools. However, with the exception of the 1% lower circulating plasma d3-betaine: d3-choline, no differences were detected in the isotopic labeling of the plasma choline metabolites. These data suggest that dietary choline is replacing unlabeled choline and betaine at a faster rate in lactating (vs. control) women thereby contributing to the larger pool sizes of these metabolites in this reproductive group.

Higher consumption of choline during lactation elevates betaine, DMG, and sarcosine

Consumption of 930 vs. 480 mg choline/d by lactating women increased several of the oxidative derivatives of choline including plasma betaine (16%), DMG (26%) and sarcosine (15%). Higher circulating concentrations of DMG and sarcosine are indicators of increased methyl donor activity and were similarly observed in our pregnant cohort in response to choline intakes of 930 vs. 480 mg choline/d (Yan et al., 2012). Notably, higher choline intake did not further increase the already elevated concentrations of plasma choline, as no differences were detected in this biomarker between the choline intake groups.

Increases in the oxidative products of choline metabolism during lactation may have biological implications that extend beyond choline's role as a methyl donor. Elevations in betaine during lactation may enhance the body's ability to handle fluid fluctuations due to its role as an osmolyte. Therefore, betaine may aid lactating women in providing sufficient water for breastmilk synthesis, avoiding the production of high osmolarity breastmilk, which has been proposed to contribute to infant morbidity and mortality (Pearson et al., 2013; Mercier et al., 1986). In addition, dimethylglycine and its metabolite sarcosine can be a source of free glycine for breastmilk synthesis. Indeed, breastmilk free glycine was 29% higher ($p=0.03$) in the 930 (vs. 480) mg choline/d intake group (based on relative peak intensities as measured by Metabolon Inc; Durham, NC). The improvement in breastmilk glycine supply as a result of greater maternal choline intake may be functionally important as free glycine is required for the production of glutathione in infants (Friesen et al., 2007; Jackson et al., 1997). Greater glutathione synthesis would be expected to improve infant redox status, with downstream beneficial effects on health (Ahola et al., 2004, Ahola et al., 2004, Kermorvant-Duchemin et al., 2006; Back et al., 2005; Zhou et al., 2006), especially in premature infants (Friel et al., 2011).

Choline intake during lactation markedly alters flux through choline metabolic pathways

Consumption of 930 vs. 480 mg choline/d during lactation increased the ratio of d9-betaine:d9-choline which is consistent with enhanced partitioning of dietary choline toward betaine synthesis in the higher choline intake group. In addition, the ratio of d3+d6-PC:d9-PC was elevated in the 930 vs. 480 mg choline/d intake group. Because PC derivatives with d3 or d6 labeling are obtained from the de novo PEMT pathway, and d9-PC is derived from the CDP-

choline pathway, elevations in the d3+d6-PC:d9-PC ratio implies that extra choline enhances de novo synthesis of the PC molecule. This finding, which was also observed in our pregnant cohort (West et al. 2013; Yan et al., unpublished data), may be functionally important as PEMT-PC is enriched in DHA relative to CDP-PC (DeLong et al., 1999). Increased synthesis of PEMT-PC may act to provide DHA for breastmilk synthesis and infant consumption. Greater consumption of DHA by infants is associated with enhanced brain development (Tanaka et al, 2009), and superior cognitive performance (Lassek and Gaulin, 2013). Finally, we show a decline in plasma d6-DMG:d9-betaine at 930 vs 480 mg choline/d that may be indicative of unequal changes in the rate of flux between steps in choline metabolism in response to higher choline intakes. To a lesser extent, a dilution effect from a greater increase in the DMG pool (by 26%) at higher choline intakes versus the betaine pool (by 16%) may also be contributing to the decline in d6-DMG:d9-betaine. Overall, our product to precursor ratios support increased use of choline for methyl donation and the production of metabolites (i.e., DHA-PC and glycine) with important roles in infant development.

Peripheral blood leukocytes are not a proxy for mammary epithelium gene expression

Peripheral blood leukocyte mRNA abundance is a commonly excepted non-invasive proxy for average gene expression within less readily accessible tissues in the body (Crescenti et al., 2013; Clelland et al., 2013; Fraenkl et al., 2013). However, we found that expression of the choline metabolism genes *PEMT*, *PCYT1A*, and *CHDH* were not correlated between blood leukocytes and mammary epithelium. Therefore, the leukocyte does not appear to be a good

proxy for mammary epithelium choline metabolic gene expression, perhaps because the mammary epithelium is a unique compartment within the lactating woman.

Conclusion

Lactation is a unique physiologic state which alters choline metabolism in a manner that diverges from that of pregnant and control women. In particular, lactating women exhibit higher circulating concentrations of betaine perhaps because of its role as an osmolyte. A higher choline intake during lactation increases betaine and downstream oxidative products which may improve osmotic balance, increase methyl group donation, and enhance breastmilk glycine supply.

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