Dietary choline requirements of women: effects of estrogen and genetic variation[1–3]

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ABSTRACT

Background: Choline is obtained from the diet and from the biosynthesis of phosphatidylcholine. Phosphatidylcholine is catalyzed by the enzyme phosphatidylethanolamine-*N*-methyltransferase (PEMT), which is induced by estrogen. Because they have lower estrogen concentrations, postmenopausal women are more susceptible to the risk of organ dysfunction in response to a low-choline diet. A common genetic polymorphism (rs12325817) in the *PEMT* gene can also increase this risk.

Objective: The objective was to determine whether the risk of low choline–related organ dysfunction increases with the number of alleles of rs12325817 in premenopausal women and whether postmenopausal women (with or without rs12325817) treated with estrogen are more resistant to developing such symptoms.

Design: Premenopausal women ($n = 27$) consumed a choline-sufficient diet followed by a very-low-choline diet until they developed organ dysfunction (or for 42 d), which was followed by a high-choline diet. Postmenopausal women ($n = 22$) were placed on the same diets but were first randomly assigned to receive estrogen or a placebo. The women were monitored for organ dysfunction and plasma choline metabolites and were genotyped for rs12325817.

Results: A dose-response effect of rs12325817 on the risk of choline-related organ dysfunction was observed in premenopausal women: 80%, 43%, and 13% of women with 2, 1, or 0 alleles, respectively, developed organ dysfunction. Among postmenopausal women, 73% who received placebo but only 18% who received estrogen developed organ dysfunction during the low-choline diet.

Conclusions: Because of their lower estrogen concentrations, postmenopausal women have a higher dietary requirement for choline than do premenopausal women. Choline requirements for both groups of women are further increased by rs12325817. This trial was registered at clinicaltrials.gov as NCT00065546. *Am J Clin Nutr* 2010;92:1113–9.

INTRODUCTION

Choline or its metabolites ensures the structural integrity and signaling functions of cell membranes, is the major source of methyl groups in the diet (one of choline's metabolites, betaine, participates in the methylation of homocysteine to form methionine), and is important for cholinergic neurotransmission and lipid transport from the liver (1). The recommended Adequate Intake of choline by the Institute of Medicine of the National Academy of Sciences is 550 mg/d for men and 425 mg/d for women (2). Choline is found in a wide variety of foods that contain membranes, such as eggs and organ meats (3, 4). The only source of choline other than the diet is from the de novo biosynthesis of phosphatidylcholine, which is catalyzed by phosphatidylethanolamine-*N*-methyltransferase (PEMT) (1). The *PEMT* gene has several estrogen response elements in its promoter region, and the gene is induced by estrogen (5). Female mice produce more phosphatidylcholine via the PEMT pathway than do male mice (6) and estradiol-treated castrated rats have more hepatic PEMT activity than do untreated controls (7). Estrogen likewise is a mediator of increased PEMT activity in humans (5). When deprived of dietary choline, almost 80% of men and postmenopausal women developed liver or muscle damage, whereas only 43% of premenopausal women developed such signs of organ dysfunction (8). It makes sense, from an evolutionary perspective, that young women can supply more of their choline requirement from endogenous biosynthesis because pregnancy and lactation are times when the demand for choline is especially high, particularly to support the developing nervous system (9, 10).

Susceptibility to choline-related organ dysfunction among women is increased by a haplotype characterized by a common single nucleotide polymorphism (SNP) in the *PEMT* gene (rs12325817; almost 75% of the North Carolina population in the United States has one variant allele) (11); having 1 or 2 variant alleles increased the likelihood of developing organ dysfunction when choline was removed from the diet (odds ratio: 21; $P = 9 \times 10^{-6}$); a total of 64 women have now completed the same clinical protocol).

In the current study, we tested the hypothesis that postmenopausal women who are treated with estrogen will have a lower risk of developing organ dysfunction while consuming a low-choline diet than will postmenopausal women not treated with estrogen. We hypothesized that women with at least one

normal allele for PEMT will be estrogen responsive. We also examined whether the PEMT rs12325817 SNP has a dose-response effect in premenopausal women with higher circulating estrogen concentrations.

SUBJECTS AND METHODS

Subjects

Healthy pre- and postmenopausal women (defined as having had their last spontaneous menstrual bleeding >12 mo previously and as having a follicle stimulating hormone concentration >30 IU/L) were recruited for a protocol approved by the Institutional Review Board at the University of North Carolina at Chapel Hill. Written informed consent was obtained from all participants before initiating any study activities. Subject enrollment began on 27 June 2007. Of the initially recruited 58 subjects, 49 (27 premenopausal, 22 postmenopausal) participants completed the study per protocol, whereas 9 (2 premenopausal, 7 postmenopausal) voluntarily dropped out or were discharged for various reasons, including claustrophobia or an abnormal liver as determined by magnetic resonance imaging (MRI) at study entry. The 27 premenopausal subjects who completed the study ranged in age from 18 to 49 y and had a body mass index (BMI; in kg/m²) between 18 and 30. The 22 postmenopausal subjects who completed the study ranged in age from 50 to 73 y and had a BMI between 20 and 32. The ethnic heritages of the 27 premenopausal subjects were white (44%), African American (30%), Asian (11%), Hispanic (11%), and Native American (4%), whereas the ethnic distribution of the 22 postmenopausal subjects (**Table 1**) was white (64%), African American (27%), and Hispanic (9%), which reflected the local population characteristics of the Raleigh-Durham-Chapel Hill area.

Inclusion was contingent on being a woman in a good state of health as determined by physical examination and standard clinical laboratory tests, having a normal mammogram in the past 12 mo (for postmenopausal subjects only), and having a BMI between 18 and 34. Subjects were excluded if they were using drugs or medications known to be damaging to liver or muscle at typically prescribed doses or that had the potential to alter choline metabolism (eg, methotrexate); had a history of hepatic, renal, or other chronic systemic disease; bleeding or a blood-clotting disorder; gastric bypass; breast, uterine, or other estrogen-dependent cancer; anemia; positive serology for HIV; or hepatitis B or C. Individuals who were current smokers, who consumed >2 alcoholic beverages/d or >14/wk, who were substance abusers or drug addicted, who were eating unusual diets that would interfere with the study, who had an allergy to soy, or who were using choline-containing dietary supplements during the previous 30 d were likewise ineligible. We also excluded postmenopausal women who were being treated with hormone therapy or premenopausal women who were planning on becoming pregnant in the near future. Finally, individuals with a pacemaker or other device that would make an MRI unsafe (eg, cochlear implants, metal aneurysm clip) were not eligible.

Study design

On entry to the study the participants were admitted to the University of North Carolina at Chapel Hill Clinical and Translational Research Center, where they remained under the constant supervision of study staff for the entire duration of the study. Postmenopausal subjects were randomly assigned to receive estrogen (0.625 mg conjugated equine estrogen/d; Premarin; Wyeth Pharmaceuticals, Philadelphia, PA) or a placebo from day 1 until the end of the study.

The diets administered to the subjects, which were composed of 0.8 g high biologic value protein/kg [current Recommended Dietary Allowance (RDA)], with 30% kcal coming from fat and the remaining kcal from carbohydrate, were prepared in-house to

TABLE 1
Demographic characteristics, estrogen concentrations, and change in liver fat among postmenopausal subjects[1]

	Placebo group (n = 11)	Estrogen-treated group (n = 11)
Age (y)	64 ± 2[2]	59 ± 2
BMI (kg/m²)	25.9 ± 1.3	27.8 ± 0.4
Race [n (%)]		
White	9 (82)	5 (45.5)
African American	1 (9)	5 (45.5)
Hispanic	1 (9)	1 (9)
Plasma estrogen (pg/mL)		
Start of study	<20	<20
End of study	<20	69 ± 21[3]
Change in liver fat (%)		
End of depletion	32 ± 10	8 ± 7[3]
End of study	2.8 ± 0.8	−5.3 ± 4

[1] Subjects were enrolled into the study and randomly assigned to receive either a placebo or estrogen-replacement therapy (0.625 mg conjugated equine estrogen/d; Premarin; Wyeth Pharmaceuticals, Philadelphia, PA) from day 1 until the last day of participation. Demographic information at study entry is presented for both groups. Estrogen was measured from a blood sample collected on study entry and on the last day of study participation. Liver fat (measured at the end of the depletion phase and at the end of the study) was measured as the change in ratio of liver to spleen fat determined by magnetic resonance imaging as described in Subjects and Methods. Continuous and categorical variables were compared between the estrogen and placebo groups by using a t test and Fisher's exact test, respectively.

[2] Mean ± SEM (all such values).

[3] Significantly different from the placebo group, $P < 0.05$.

protocol specifications and are described in detail elsewhere (12). Total food intake was adjusted to be isocaloric and to provide adequate intakes of macro- and micronutrients. All diets met or exceeded the Estimated Average Requirement for methionine plus cysteine, and the RDA for vitamins B-12, B-6, and folate. A multivitamin supplement (Kirkland Signature Daily Multi Vitamins and Minerals; Costco, Seattle, WA) provided vitamins and minerals at or above the RDA or Adequate Intake. Subjects were also given a dietary supplement containing the RDA of magnesium and 1.5 times the Adequate Intake of calcium (Calcium Magnesium Complex; Vitamin Shoppe, North Bergen, NJ).

Initially, all subjects consumed a conventional diet of normal foods delivering 550 mg choline/70 kg body weight daily [the current Adequate Intake (2)], and 50 mg betaine/70 kg body weight daily (baseline). After 10 d of this initial diet, the subjects were switched to a low-choline diet containing <50 mg choline/70 kg body weight daily (with 6 mg betaine/70 kg body weight daily), as confirmed by chemical analysis of a sample of duplicate food portions (3, 13). Periodic determinations of urinary betaine concentrations (13) were used to confirm compliance with the dietary restrictions (data not shown). Subjects remained on the depletion diet until they developed organ dysfunction associated with choline deficiency or for 42 d if they did not. The subjects were deemed to have organ dysfunction associated with choline deficiency if they had a >1.5-fold increase in aspartate aminotransferase (AST) or alanine aminotransferase (ALT), a >5-fold increase in creatine phosphokinase (CPK), or an increase in liver fat content of >28% while consuming the choline-depletion diet and if these increased measures resolved when choline was returned to the diet.

After the subjects developed organ dysfunction associated with choline deficiency, or if they did not by the end of the 42-d period, they were transitioned to a choline-repletion diet (550–850 mg/70 kg body weight daily) for up to 10 d. The choline-repletion diet was identical to the baseline diet, except for the amount of choline. For the baseline and repletion phase diets, in addition to the choline contributed by the foods, granulated lecithin was used to add additional defined amounts of choline into the diets. This was achieved by either baking it into a bread or by using it as an ingredient in cookies. Subjects who manifested signs of organ dysfunction that did not resolve after 10 d of the repletion diet ($n = 4$) were sent home for 2 wk and instructed to eat an ad libitum high-choline diet. They were then brought in for repeat measures and a final study day.

Clinical assessment

A complete panel of laboratory tests was performed on each subject at screening, on study entry (day 1), and at the end of each dietary phase (baseline, depletion, and repletion). Blood was drawn by venipuncture and sent to the McClendon Clinical Laboratory at the University of North Carolina at Chapel Hill Hospitals, which is accredited by both the Clinical Laboratory Improvement Act (CLIA) and the College of American Pathologists (CAP). Laboratory markers measured included fasting glucose, insulin, blood urea nitrogen, creatinine, alkaline phosphatase, AST, ALT, CPK, lactate dehydrogenase, total protein, albumin, uric acid, total bilirubin, γ-glutamyl transferase, amylase, lipase, complete blood count with differential (white blood cells, red blood cells, hemoglobin, hematocrit, platelet count,

mean cell volume, mean cell hemoglobin, mean cell hemoglobin adjusted for cell volume, red blood cell distribution width, neutrophils, lymphocytes, monocytes, eosinophils, and basophils), prothrombin time, partial thromboplastin time, total cholesterol, triglycerides, HDL, LDL, estrogen, folate, homocysteine, C-reactive protein, and troponin-T. An abbreviated toxicity panel, which included creatinine, AST, ALT, and CPK was run every 3–4 d throughout the duration of the study to monitor the depletion and repletion status of the subjects. Also, pregnancy tests were performed on all premenopausal subjects every 10–14 d throughout the duration of the study. Additional samples of blood were collected to measure plasma choline and related metabolite concentrations. Urinalysis was conducted at screening and at the end of each dietary phase. The urinalysis included measurements of betaine, specific gravity, pH, protein, glucose, and myoglobin. The urinary betaine concentration was used as a means of assessing noncompliance with the choline-depletion diet (ie, cheating). These concentrations decreased (compared with basal concentrations measured at the end of the baseline diet) and remained low during the depletion phase of the study. They rose when choline was returned to the diet and would also be expected to rise if subjects were to eat high-choline foods during the choline-depletion phase. Each subject also received an electrocardiogram at screening and on the last day of the depletion and repletion diets.

Choline and methyl-group metabolites

Choline, phosphatidylcholine, sphingomyelin, and betaine were extracted from plasma by the method of Bligh and Dyer (14). Aqueous and organic compounds were separated, analyzed, and quantified directly by using liquid chromatography electrospray ionization-isotope dilution mass spectrometry after the addition of internal standards labeled with stable isotopes to correct for recovery (13).

Fatty liver

Liver fat was measured by MRI at the beginning (day 1) and end (day 10) of the baseline (550 mg choline/70 kg body weight daily) diet, after 21 and 42 d of the low-choline diet, and at the end of the repletion period. Liver fat content was estimated by MRI with a Siemens TRIO 3T clinical MR system with a modified Dixon "In and Out of Phase" procedure (15, 16). This approach used the differences in transverse magnetization intensity after an ultrabrief time interval (FLASH; TE = 2.2 and 4.5 ms, flip angle = 80°, and TR = 140 ms). Processing of successive FLASH MRI images with software from Siemens Medical Solutions (Malvern, PA) was used to estimate fat content. Quantification of organ fat content was based on measurements across 5 images (liver slices) per subject and standardized by relating the results to the fat content of similarly measured images of spleen (it was assumed that spleen fat remained constant during the study and could be used to normalize values over time). As described previously, we used a 28% increase in the ratio of liver fat to spleen fat as a predetermined threshold value for indicating liver dysfunction secondary to choline deficiency (8). The 28% increase over baseline that we chose for depletion was an average grade of moderate steatosis from several published studies (17). All liver fat measurements

were conducted within 2 d of the stipulated end of the study period (there was some variation based on availability of MRI).

Genotyping

Blood samples were collected by venipuncture, and peripheral lymphocytes were isolated from blood by Ficoll-Hypaque gradient by using evacuated cell preparation tubes containing sodium citrate (Becton Dickinson, Franklin Lakes, NJ) (18, 19). Genomic DNA was then extracted by using a PureGene kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions. The PEMT rs12325817 SNP occurs in the middle of an Alu repeat and consequently is not detected in the GWAS chip set. Therefore, we do not know whether it is associated with published haplotypes. It is found on chromosome 17 in gb37.1 and is a G to C conversion. Forward primers specific to the rs12325817 allele were designed so that the SNP would be located at the 3′ end of the priming sequence, which allows for specific polymerase chain reaction products to be synthesized only if the primer is 100% complementary to its template DNA (11). Primers were designed by using GeneFisher (http://bibiserv. techfak.uni-bielefeld.de/genefisher) and purchased from Qiagen Operon (Huntsville, AL). The polymerase chain reactions were optimized for each pair of primers, and the products were visualized on a 1.5% agarose gel to determine the genotype.

Statistical methods

In the premenopausal subjects, statistical analyses were performed in 27 subjects. Depletion status and genotype for rs12325817 SNP was obtained for each subject, and a Cochran-Armitage Test of Trend was performed to examine whether depletion was associated with an increased likelihood of having a *C* allele.

In the postmenopausal subjects, the primary statistical analyses were performed by using the data from the 22 subjects (11 in the estrogen-treated group and 11 in the placebo group) who completed the study per protocol. Nine additional subjects enrolled in the study but dropped out or were asked to leave for various reasons. These subjects were not included in these analyses. In evaluating subject responses to the experimental choline deprivation and repletion, the primary outcome variable was the occurrence of organ dysfunction during consumption of a low-choline diet as defined in Subjects and Methods. Descriptive statistics—including means, SDs, frequencies, and percentages—were computed. Comparisons of continuous and categorical variables between the placebo and estrogen-treated groups were performed by using *t* tests and Fisher's exact tests, respectively.

Repeated-measures regression models were fit for each of the 4 continuous responses (choline, phosphatidylcholine, sphingomyelin, and betaine). The continuous response was checked for the normality assumption required in regression models, and the assumption was met in all cases. The predictors were dummy variables created by the 8 possible combinations of diet periods [study entry (day 1), baseline (day 10), depletion, and repletion] and treatments (estrogen and placebo). Levene's Test of Homogeneity (20) was used to test the homogeneity of variance assumption, and in the cases when the assumption was not met (betaine and choline) weighted least squares regression was used with the weights being the reciprocal of the variance of the residuals from the corresponding unweighted model. The correlation between observations on the same subject at the 4 different diet periods was modeled with a compound symmetric variance-covariance matrix, which was found to fit the observed data. Comparisons between any of the diet periods, treatments, or combinations thereof were able to be constructed via contrast statements from the resulting model.

RESULTS

All diets, including the low-choline (depletion) diet, were well tolerated by subjects and caused no side effects other than those associated with the removal of choline (hepatic and muscle dysfunction). Moreover, estrogen treatment was well tolerated and did not cause any adverse events in the postmenopausal participants.

Dose-response effect of PEMT rs12325817 SNP in premenopausal subjects

Premenopausal subjects were genotyped for the rs12325817 SNP in the *PEMT* gene and were fed a low-choline diet as described above. A significant dose-response effect was seen between the number of alleles of the rs12325817 SNP and risk of low choline–related organ dysfunction ($P = 0.02$). Eighty percent of the women who were homozygous for the SNP manifested signs of choline depletion (liver or muscle dysfunction), relative to 43% of subjects carrying one copy of the variant allele and 13% of subjects without the SNP (**Table 2**).

Effect of estrogen treatment on susceptibility to low choline–related organ dysfunction in postmenopausal subjects

We measured estrogen concentrations in all postmenopausal subjects at the beginning and end of their study participation. At the start of the study, circulating estrogen concentrations in both groups were not detectable (<20 pg/mL; Table 1). At the end of

TABLE 2
Risk of organ dysfunction in response to a low-choline diet in premenopausal subjects carrying the phosphatidylethanolamine-*N*-methyltransferase (PEMT) rs12325817 single nucleotide polymorphism[1]

	Number with *PEMT* genotype		
	GG	*GC*	*CC*
Depleted	1	6	4
Not depleted	7	8	1

[1] Premenopausal subjects consumed a baseline diet containing 550 mg choline/70 kg body weight for 10 d and were then advanced to a choline-depletion diet (<50 mg choline/70 kg body weight daily) until they developed signs of organ dysfunction or for 42 d. Finally, subjects received a choline-repletion diet (550–850 mg choline/70 kg body weight daily) for ≤10 d. The numbers of subjects who manifested signs of low choline–related organ dysfunction are shown by genotype: homozygous wild-type (*GG*), heterozygous (*GC*), and homozygous (*CC*) for the PEMT rs12325817 single nucleotide polymorphism. *G* is the major allele, and *C* is the variant allele. A significant ($P = 0.02$) dose-response effect was observed based on the Cochran-Armitage test.

the study, hormone-treated subjects had, on average, significantly higher circulating estrogen concentrations (69 pg/mL) than did untreated subjects, which confirmed compliance with the estrogen-replacement therapy, whereas none of the placebo subjects had detectable concentrations. Note that the mean concentration observed in the estrogen-treated group was beyond the University of North Carolina at Chapel Hill McClendon Clinical Laboratory normal range for postmenopausal women (5–38 pg/mL).

Postmenopausal women receiving placebo were 4 times more likely to develop liver dysfunction when fed the low-choline diet than were those receiving estrogen (**Table 3**; $P = 0.03$). In the placebo group, 7 of 8 women developed fatty liver as measured by MRI when deprived of choline, and 1 subject was deemed choline-depleted based on an elevation in AST concentration (1.6 times the upper limit of normal) at the end of the baseline phase. (Apparently, 550 mg choline/70 kg body weight daily was not sufficient to meet this woman's choline requirement.) Per protocol, this subject skipped the depletion phase and was directly advanced to the repletion phase. In the estrogen-treated group, 2 women manifested organ dysfunction as fatty liver in response to a low-choline diet. The difference in response to the low-choline diet between the 2 groups is also reflected in the mean percentage change in ratio of liver fat to spleen fat observed at the end of the depletion phase (Table 1). In addition, both groups, on average, had baseline levels of liver fat at the end of the study that indicated that the choline in the repletion diet was effective at resolving the observed liver dysfunction.

Influence of PEMT rs12325817 SNP on susceptibility to choline-related organ dysfunction

We previously showed that postmenopausal women with the haplotype characterized by the PEMT rs12325817 SNP have a far greater likelihood of developing organ dysfunction when fed a low-choline diet (11). Consistent with earlier findings, postmenopausal women in the placebo group who carried 1 (*GC*) or 2 (*CC*) copies of the PEMT SNP were significantly more likely to develop organ dysfunction when fed a low-choline diet than were those with no copies of the SNP (*GG*; $P = 0.02$). In the placebo group, one subject who was homozygous for this SNP and 6 subjects who were heterozygous for the SNP developed organ dysfunction. Of the 4 subjects in the placebo group without this SNP, 1 was choline-depleted and 3 were not (Table 3).

Estrogen treatment mitigated this effect of the PEMT SNP. Whereas 100% of women with one or more allele for the SNP developed organ dysfunction in the placebo group, only 40% with one or more allele for the SNP developed organ dysfunction in the estrogen-treated group ($P < 0.05$). In the estrogen-treated group, 5 subjects were heterozygous for the SNP (none were homozygous). Of these subjects, 2 manifested signs of organ dysfunction secondary to choline deficiency, whereas 3 did not. In this group, there were also 6 subjects without the SNP; none developed organ dysfunction after 42 d of the choline-depletion diet (Table 3).

Choline and related metabolite concentrations in placebo- and estrogen-treated subjects

We measured plasma concentrations of choline, phosphatidylcholine, sphingomyelin, and betaine in all postmenopausal subjects at the end of each dietary phase (**Table 4**). None of the metabolites were significantly different between the placebo and estrogen groups at study entry (day 1). At the end of the baseline phase (day 10), the betaine concentration was significantly higher in the placebo group than in the estrogen group ($P = 0.03$). No statistically significant difference in the other 3 metabolites was observed at the end of the baseline phase between the placebo and estrogen groups. At the end of depletion, the betaine concentration was again significantly higher in the placebo group than in the estrogen group ($P = 0.009$). No statistically significant differences in the other 3 metabolites were observed at depletion between the placebo and estrogen groups. The betaine concentration was likewise significantly higher in the placebo group than in the estrogen-treated group at the end of the repletion diet period ($P = 0.003$). No other significant differences in the other metabolites were observed between the treatment groups at any of the diet periods.

When looking at changes over time within groups, all metabolites in the estrogen group changed significantly from study entry (day 1) to day 10, and all metabolites in the placebo group (with the exception of phosphatidylcholine) changed over the same time period. Choline and betaine concentrations decreased significantly from baseline (day 10) to depletion (all P values = 0.0001) in both the estrogen and placebo groups. This

TABLE 3
Frequency of organ dysfunction associated with choline deficiency in the placebo and estrogen-treated groups by phosphatidylethanolamine-*N*-methyltransferase (*PEMT*) genotype[1]

	Placebo group ($n = 11$)				Estrogen-treated group ($n = 11$)			
	All	*GG*	*GC*	*CC*	All	*GG*	*GC*	*CC*
Depleted (*n*)	8	1	6	1	2	0	2	0
Did not deplete (*n*)	3	3	0	0	9	6	3	0
Frequency of depletion (%)	73	25	100	100	18[2]	0	40	—

[1] Postmenopausal subjects were randomly assigned to receive a placebo or estrogen (0.625 mg conjugated equine estrogen/d; Premarin; Wyeth Pharmaceuticals, Philadelphia, PA) from day 1 until the end of their study participation. They consumed a baseline diet containing 550 mg choline/70 kg body weight for 10 d and then advanced to a choline-depletion diet (<50 mg choline/70 kg body weight daily) until they developed signs of organ dysfunction or for 42 d. Finally, subjects received a choline-repletion diet (550–850 mg choline/70 kg body weight daily) for ≤10 d. The numbers of subjects who manifested signs of low choline–related organ dysfunction are shown by genotype. Depletion outcomes are presented for subjects sorted by genotype (*C* = variant allele): homozygous wild-type (*GG*), heterozygous (*GC*), and homozygous (*CC*) for the PEMT rs12325817 single nucleotide polymorphism.

[2] Significantly different from the placebo group (all subjects combined), $P < 0.05$.

TABLE 4
Choline and metabolite concentrations in the placebo- and estrogen-treated postmenopausal women[1]

Time	Choline	PtdCho	SM	Betaine
	nmol/mL	nmol/mL	nmol/mL	nmol/mL
Placebo group				
Study entry	7.7 ± 0.5[2]	1939 ± 117	474 ± 42[2]	48 ± 5[2]
Baseline (day 10)	11.0 ± 0.3	2000 ± 118	392 ± 28	88 ± 11
Depletion	5.4 ± 0.4[2]	1916 ± 140	406 ± 30	41 ± 9[2]
Repletion	11.6 ± 1.1[3]	2067 ± 126	438 ± 38[2]	123 ± 19[2,3]
Estrogen-treated group				
Study entry	8.7 ± 0.6[2]	2073 ± 98[2]	427 ± 49[2]	43 ± 4[2]
Baseline (day 10)	11.9 ± 0.8	2254 ± 115	379 ± 37	61 ± 8[4]
Depletion	5.6 ± 0.4[2]	2168 ± 151	397 ± 43	16 ± 2[2,4]
Repletion	13.4 ± 1.7[3]	2283 ± 119	402 ± 39	49 ± 5[3,4]

[1] All values are means ± SEs. Subjects were randomly assigned to receive either a placebo or estrogen-replacement therapy and consumed a baseline diet containing 550 mg choline/70 kg body weight daily for 10 d. If subjects did not manifest organ dysfunction during this diet, they were advanced to a choline-depletion diet (<50 mg choline/70 kg body weight daily) until they developed signs of organ dysfunction or for 42 d. Subjects then received a choline-repletion diet (550–850 mg choline/70 kg body weight daily) for ≤10 d. Blood was drawn from subjects at the end of each dietary phase. Plasma choline, phosphatidylcholine (PtdCho), sphingomyelin (SM), and betaine concentrations were measured by using liquid chromatography–mass spectrometry. Entries are for each group at the end of the indicated study phase.

[2] Significantly different from baseline value within the same group, $P < 0.05$ (repeated-measures regression models).

[3] Significantly different from depletion value within the same group, $P < 0.05$ (repeated-measures regression models).

[4] Significantly different from corresponding value in the placebo group, $P < 0.05$ (repeated-measures regression models).

also occurred independently of whether subjects developed organ dysfunction (data not shown). None of the other metabolites changed significantly from the end of baseline to depletion within each group. Conversely, mean choline and betaine concentrations increased significantly from depletion to repletion (all P values = 0.0001) in both groups, whereas the other metabolites remained unchanged. Finally, a significant increase in sphingomyelin ($P = 0.02$) and betaine ($P = 0.04$) concentrations from baseline (day 10) to repletion was observed in the placebo group. None of the other combinations of metabolites and time points showed a statistically significant change from baseline to repletion within each group.

DISCUSSION

To our knowledge, this was the first study to show that estrogen treatment in postmenopausal women can decrease the dietary requirement for the nutrient choline; ≈4 times more women in the placebo group than in the estrogen group developed liver dysfunction when fed a low-choline diet.

In this study, choline deficiency was associated with fatty liver. Nonalcoholic fatty liver occurs in ≈25% of the population and can be associated with progression to more severe liver disease (21). Progression to fibrosis is much more common in men and women older than 60 y of age, which suggests that estrogen modulates liver fibrosis (22). It is interesting that individuals with fatty liver have a higher prevalence of an exonic SNP in *PEMT* (23)—the gene that catalyzes phosphatidylcholine synthesis in the liver. In this study, postmenopausal women in the placebo group who had a haplotype characterized by the rs12325817 SNP in *PEMT* developed liver dysfunction when fed a low-choline diet. Estrogen treatment reduced the risk of developing fatty liver in postmenopausal women with this SNP. Although the experimental cohort was of a relatively small size,

we had sufficient statistical power to detect clinically important differences.

Herein we showed that the PEMT rs12325817 SNP has a dose-response effect on risk of choline-related organ dysfunction in premenopausal women. We also confirmed previous results showing that women (both pre- and postmenopausal) carrying 1 or 2 copies of the SNP have a significantly greater risk of developing liver or muscle dysfunction when fed a low-choline diet. We had hoped to recruit more postmenopausal women who were homozygous for this allele. However, despite all of our recruiting efforts, we were only able to identify one postmenopausal subject homozygous for this allele (*CC*) who was eligible to participate. We do not know why this was the case and suspect that there may be an underlying reason to explain why the homozygous genotype occurs at a greater frequency in premenopausal women (and men) who are eligible for our study than in postmenopausal women. We hypothesize that one or more of the rigorous eligibility criteria for the study may co-present with the homozygous genotype in women after menopause and thus exclude such individuals. This topic will be the subject of future studies.

No increases in plasma choline or its related metabolite concentrations due to estrogen treatment were observed. The concentrations of these metabolites in plasma are homeostatically regulated and are not good indicators of intracellular choline concentrations in liver (24). The estrogen-treated group had lower betaine concentrations in plasma; perhaps choline dehydrogenase is inhibited by estrogen. This may be yet another mechanism (in addition to the induction of PEMT) that would result in sparing choline pools in women. It is also possible that the betaine concentration was lower in the estrogen-treated group because of increased use of betaine as a methyl donor (PEMT is a major user of *S*-adenosylmethionine). However animal studies conducted in our laboratory have not been able to confirm this (unpublished findings).

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