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Common genetic polymorphisms affect the human requirement for the nutrient choline

Kerry-Ann da Costa[*], **Olga G. Kozyreva**[†], **Jiannan Song**[*], **Joseph A. Galanko**[‡], **Leslie M. Fischer**[*], and **Steven H. Zeisel**[*,1]

[*] *Department of Nutrition, School of Public Health and School of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA;*

† *Gene Therapy Center, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA; and*

‡ *Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA*

Abstract

Humans eating diets deficient in the essential nutrient choline can develop organ dysfunction. We hypothesized that common single nucleotide polymorphisms (SNPs) in genes involved in choline metabolism influence the dietary requirement of this nutrient. Fifty-seven humans were fed a low choline diet until they developed organ dysfunction or for up to 42 days. We tested DNA SNPs for allelic association with susceptibility to developing organ dysfunction associated with choline deficiency. We identified an SNP in the promoter region of the phosphatidylethanolamine N-methyltransferase gene (*PEMT*; −744 G→C; rs12325817) for which 18 of 23 carriers of the C allele (78%) developed organ dysfunction when fed a low choline diet (odds ratio 25, P=0.002). The first of two SNPs in the coding region of the choline dehydrogenase gene (*CHDH*; +318 A→C; rs9001) had a protective effect on susceptibility to choline deficiency, while a second *CHDH* variant (+432 G→T; rs12676) was associated with increased susceptibility to choline deficiency. A SNP in the *PEMT* coding region (+5465 G→A; rs7946) and a betaine:homocysteine methyl-transferase (BHMT) SNP (+742 G→A; rs3733890) were not associated with susceptibility to choline deficiency. Identification of common polymorphisms that affect dietary requirements for choline could enable us to identify individuals for whom we need to assure adequate dietary choline intake.—da Costa, K.-A., Kozyreva, O. G., Song, J., Galanko, J. A., Fischer, L. M., Zeisel, S. H. Common genetic polymorphisms affect the human requirement for the nutrient choline.

Keywords

choline deficiency; phosphatidylethanolamine N-methyltransferase; PEMT; choline dehydrogenase; CHDH; betaine:homocysteine methyltransferase; BHMT; genetic polymorphism

Choline is an essential nutrient needed for structural integrity and signaling functions of cell membranes, methyl group metabolism, and neurotransmitter synthesis (1). Humans eating diets deficient in choline develop fatty liver, liver damage, and muscle damage (2–4). These effects occur, in part, because a specific lack of phosphatidylcholine limits the export of excess triglyceride from liver (5,6) and induces apoptosis and subsequent leakage of enzymes (e.g., AST, ALT, and CPK) from tissues of liver and muscle (3,7,8). Women's dietary requirements for choline are of special interest because deficient maternal dietary intake of choline during

[1] Correspondence: Department of Nutrition, School of Public Health and School of Medicine, University of North Carolina at Chapel Hill, CB# 7461, Chapel Hill, NC 27599, USA. E-mail: steven_zeisel@unc.edu.

pregnancy in humans was associated with a 4-fold increased risk of having a baby with a neural tube defect (9). In addition, offering pregnant rodents diets deficient in choline resulted in perturbed brain development in their fetuses (10–13).

We do not understand all of the factors that influence the dietary requirement for choline in humans, but we know that the requirement is modified by dietary availability of other methyl donors (1) and by endogenous *de novo* biosynthesis of choline moiety (14). (Fig. 1) The methylation of homocysteine can be accomplished by using a methyl group derived from one-carbon metabolism or by using a methyl group derived from choline. When choline is used as a methyl group source, it is first irreversibly oxidized to form betaine by choline dehydrogenase (CHDH) and is no longer available for synthesis of membrane phosphatidylcholine. Betaine, once formed, donates its methyl group to homocysteine via betaine:homocysteine methyltransferase (BHMT) to form methionine (1). Endogenous biosynthesis of choline occurs mainly in the liver, where phosphatidylethanolamine is methylated by phosphatidylethanolamine *N*-methyltransferase (PEMT) to form phosphatidylcholine. *Pemt* −/− mice develop fatty liver and liver damage (15) because they cannot form required amounts of phosphatidylcholine in membranes and they become choline deficient despite eating diets containing recommended amounts of choline (6,8).

Genetic variations exist in these genes in humans, but those SNPs that have functional effects on the above enzymes of choline metabolism have not yet been completely identified. More than 98 polymorphisms exist in the PEMT gene (16), but only 1 SNP is known to be functionally significant (rs7946, +5465 G→A) (17). Two SNPs that produced an amino acid substitution in *CHDH*, rs9001 (318A→C; E40A) and rs12676 (432G→T; L78R) and one in *BHMT*, rs3733890 (742G→A; R239Q) were recently identified (18,19) (Table 1). The latter may play a protective role in the risk of coronary heart disease (20). Nevertheless, there is no information at present about the possible functional effects of these polymorphisms. If decreased availability of methyl groups from choline is responsible for organ dysfunction in choline deficiency, then SNPs in *CHDH* or *BHMT* could alter susceptibility to developing organ dysfunction when fed a low choline diet. Alternatively, if organ damage is due to defective membrane formation, SNPs in *PEMT* could modify *de novo* phosphatidylcholine synthesis and SNPs resulting in decreased CHDH activity could decrease the use of choline as a methyl donor and make more substrate available for phosphatidylcholine synthesis from preexisting choline moiety, thereby altering susceptibility to developing organ dysfunction when fed a low choline diet.

We conducted a study in which humans were fed a diet containing the Adequate Intake concentration for choline for 10 days, then fed a diet containing little choline for up to 6 wk, then fed a diet containing choline. We characterized susceptibility to developing liver and muscle dysfunction when fed the diet low in choline content and determined whether such susceptibility was influenced by common genetic polymorphisms.

MATERIALS AND METHODS

Study design

Healthy males (*n*=31) and females (*n*=35) were recruited by advertising. They ranged in age from 18 to 70 years and had body mass indices between 19 and 33. Informed consent was obtained from all participants after the nature and possible consequences of the study were explained; the criteria for subject selection and all details of the clinical protocol were approved by the Institutional Review Board of the University of North Carolina at Chapel Hill (UNC-CH). The ethnicity of the participants was Caucasians (65%), African-Americans (25%), Asians (5%), Native Americans (3%), and other heritages (2%) reflecting the local population characteristics of the Raleigh-Durham-Chapel Hill area. Inclusion was contingent on age-

typical good state of health as determined by physical examination and standard clinical laboratory tests. Of the originally recruited 66 subjects, 61 completed at least the initial phase and the depletion phase. Of these 61, 1 subject was excluded due to 9 kg wt loss during the study and 3 subjects were excluded because they did not comply with diet restrictions, leaving 57 subjects included in analyses.

The participants were admitted to the UNC-CH General Clinical Research Center for the duration of the study and could leave only for brief periods under the supervision of study staff. The diets, which were composed of 0.8 g/kg high biological value protein, with 30% kcal coming from fat and the remaining kcal from carbohydrate, were prepared in-house to protocol specifications and are described in detail in another publication (21). Total food intake was adjusted to be isocaloric and provided adequate intakes of macro- and micronutrients. Initially, all participants received a diet of normal foods containing 550 mg choline/70 kg body wt/day, the current Adequate Intake (22). This diet contained 50 mg betaine/70 kg body wt/day. After 10 days (Fig. 2) the choline content of the diet was reduced to <50 mg/day (with 6 mg betaine/70 kg body wt/day), as confirmed by analysis of duplicate food portions (23,24). Periodic determinations of urinary choline and betaine concentrations (24) were used to confirm compliance with the dietary restrictions. Subjects remained on this depletion diet until they developed organ dysfunction associated with choline deficiency, or for 42 days if they did not. Subjects were deemed to have organ dysfunction associated with choline deficiency if they had a >5-fold increase of serum creatine phosphokinase (CPK) activity (3) or if they had an increase in liver fat content by >28% while on the choline depletion diet, and if this increased CPK or increased liver fat resolved when choline was returned to the diet. After the depletion study, subjects were repleted by gradually increasing their choline intake to a final concentration of >550 mg per day and maintaining that concentration for at least 3 days. All 57 (54 for *PEMT* rs7946 and *BHMT* rs3733890) individuals were genotyped as described below for the following SNPs (the positions of the *PEMT* SNPs were enumerated with promoter B (25) as a reference): *PEMT* rs7946 (+5465 G→A), rs2278952 (+164 C→T), rs12325817 (−744 G→C) and for two new SNPs of *PEMT* that have not been previously reported at position −314C→T of the PEMT promoter, and +29C→G of exon 2, which adjoins the promoter; *BHMT* rs3733890; *CHDH* rs9001, and rs12676 (Table 1). Plasma collected at the end of the adequate choline intake phase and depletion phase were analyzed for concentrations of betaine, choline, and phosphatidylcholine (24).

Liver fat measurement

Liver fat was measured at the end of the 550 mg choline diet and at 21 and 42 days on the choline-deficient diet. Change in liver fat content was estimated by MRI with a Siemens Vision 41.5T clinical MR system using a modified “In and Out of Phase” procedure (26,27). This approach utilizes the differences in transverse magnetization intensity after an ultra-brief time interval (FLASH; TE=2.2 ms and 4.5 ms, with a flip angle of 80°, and TR=140 ms). Processing of successive FLASH MRI images with software from Siemens Medical Solutions (Malvern, PA, USA) was used to estimate fat content. Organ fat content was derived from measurements across 3–5 liver slices per subject and standardized by relating the results to the fat content of similarly measured slices of spleen.

Laboratory analyses

Fasting blood samples were taken every 3–4 days for blood chemistries (including CPK analysis), after 10 days on the 550 mg/day choline diet (baseline), and at the end of the low choline diet (depletion phase) for choline and genotyping studies. Serum was analyzed using a dry slide colorimetric method for CPK activity by the McClendon Clinical Laboratories at University of North Carolina Hospitals, which is both Clinical Laboratory Improvement Act and College of American Pathologists accredited. Choline and its metabolites were analyzed

and quantified directly by HPLC mass spectrometry (LC/ESI-IDMS) after the addition of internal standards labeled with stable isotopes that were used to correct for recovery (24).

Genotyping

Peripheral lymphocytes were isolated from blood by Ficoll-Hypaque gradient using Vacutainer® CPT™ tubes with sodium citrate (Becton Dickinson, Franklin Lakes, NJ, USA) (28,29) and genomic DNA extracted using PureGene (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. SNP analyses were carried out as described below. Briefly, for the *PEMT* and *CHDH* genes, DNA sequencing was performed on double-stranded DNA templates obtained from genomic DNA by polymerase chain reaction (PCR) amplification. A negative control without DNA and a positive control with human DNA (PROMEGA Inc., Madison, WI, USA) for each PCR set were included. PCR products were purified with QIAquick® PCR Purification Kit 250 (QIAGEN Inc., Valencia, CA, USA) after electrophoresis in 0.8% or 3% agarose depending on the size of the fragment. Sequencing reactions were performed by the University of North Carolina at Chapel Hill Genome Analysis Facility, using a capillary sequencing machine (model 3100, Applied Biosystems, Foster City, CA, USA). Sequence results were interpreted using the programs Sequencher (Gene Code Corp., Ann Arbor, MI, USA). Basic local alignment search tool searches were performed using the National Center for Biotechnology (NCBI) program (http://www.ncbi.nlm.nih.gov/gorf/bl2.html).

Promoter of the PEMT gene

Successful amplification of the 1896 bp DNA fragment of the *PEMT* gene was performed using Takara Ex *Taq* polymerase (Fisher Scientific, Fair Lawn, NJ, USA) with an efficient 3′-5′ exonuclease activity for increased fidelity. Based on the GenBank sequence (accession number NC_000017), we designed a set of primers for amplification, as recommended by the manufacturers, and a set of primers for sequencing the overlapping segments in two directions (Table 2) using the Web primers design program (http://genome-www2.stanford.edu/cgi-bin/SGD/webprimer). The forward and reverse primers were 5′GAGCACGTGAGCTGTCAGTGCCTTTTG3′ and 5′ CCAACCTCCTTCATACAACAGAGGTCC3′, respectively, and a three-step PCR was performed on an Applied Biosystems 2720 Thermal Cycler under the following conditions: 96°C for 2 min; 30 cycles (94°C for 30 s, 60°C for 1 min, 72°C for 2 min); extend 72°C for 7 min, and soak at 4°C. For the sequence determination of PEMT rs12325817, we used an additional primer (PEMT PRO seq-F2; Table 2) to verify the sequence in a region containing Alu repeats and a poly-A tail.

PEMT

The coding region of *PEMT* containing the SNP rs7946 was amplified with the oligonucleotides 5′GGAGCACTTTGCCCCAGAATC3′ and 5′ GACTTGGAGCCTTCAGAGCG3′ as forward and reverse primers, respectively (30). The sequences obtained were compared with ones stored in the NCBI database (http://www.ncbi.nlm.nih.gov/entrez/, accession number AF294467) using ClustalW multiple sequence alignment software http://www.ebi.ac.uk/Tools/sequence.html).

CHDH

Amplification of the 370 bp DNA fragment containing SNPs rs9001 and rs12676 in the *CHDH* gene was performed using BD Advantage GC Genomic PCR Kit (BD Biosciences, Mountain View, CA, USA). Based on the GenBank sequence (accession number NC_000003), we designed the following primers for amplification and sequencing: 5′ AGTCATCTCATTCCCCTCCGTGGATCAGA3′ (forward) and 5′

TAGCACCAGTTGTACCTGTCGTCGCACA3′ (reverse). A two-step PCR was done with the following conditions: 94°C for 1 min; 30 cycles (94°C for 30 s, 68°C for 3 min); extend 70°C for 5 min and soak at 15°C. The SNPs were numbered with the mRNA location (318 and 432, respectively; NT_018397).

BHMT

For the variant rs3733890 in exon 6 of *BHMT* (GenBank accession number NT_006713), the targeted DNA sequence: 5′GCCACTTTGACCCCACCATTAGT3′ and 5′ TGGGAATTCTGGGAGATCGATG3′ as forward and reverse primers, respectively, were amplified by multiplex PCR, purified, then analyzed with matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (31). Samples were analyzed in duplicate.

Statistical analysis

Data for continuous variables are expressed as mean ± SE, and the statistical significance of differences between subjects on the two different diets were assessed using paired *t* test. A two-sample *t* test based on the differences between choline and metabolite concentrations in subjects on the baseline and depletion diets was used to compare the depleted with organ dysfunction and depleted without organ dysfunction groups. Genotype differences associated with organ dysfunction associated with choline deficiency were calculated using Fisher's Exact Test to determine statistical significance (32). For $P < 0.05$, odds ratios and 95% confidence intervals were calculated as the odds of showing signs of deficiency for subjects with the risk allele divided by the odds of showing signs of deficiency for subjects without the risk allele. The Kruskal-Wallis test was used to compare differences in continuous variables by genotype (33).

RESULTS

Of the 57 participants, 68% developed organ dysfunction when fed the low choline diet and this resolved when choline was added back to their diets. Plasma betaine concentrations decreased almost 50% in response to the low choline diet (from 60±3 to 32±2 nmol/ml; $P<0.001$) and choline concentrations decreased almost 30% (from 9.8±0.3 to 7.1±0.2 nmol/ml; $P<0.001$). These decreases were irrespective of whether or not subjects developed organ dysfunction on the low choline diet. Plasma phosphatidylcholine concentrations were 9% lower when subjects were fed the low choline diet (1691±41 vs. 1868±45 nmol/ml than when on baseline diet; $P<0.001$); those subjects who developed organ dysfunction on the low choline diet had a 3-fold greater decrease in phosphatidylcholine (−228±47 nmol/ml) than those who did not (−64±31 nmol/ml; $P<0.029$ by *t* test). Plasma choline and phosphatidylcholine concentrations did not differ from baseline after subjects were repleted with choline-containing diets. We did not detect significant changes in choline, betaine, or phosphatidylcholine concentrations in plasma between genotypes (note that there was no significant change in plasma betaine concentrations associated with the BHMT genotype).

Gender was an important modifier of susceptibility to developing organ dysfunction when fed a low choline diet. When deprived of dietary choline, 77% of men and 80% of postmenopausal women developed fatty liver or muscle damage, whereas only 44% of premenopausal women developed such signs of organ dysfunction associated with choline deficiency (Fischer *et al.*, *Am. J. Clin. Nutr.*, under review). Note that within each gender grouping, a significant number of subjects were resistant to developing organ dysfunction, suggesting that other factors, such as genetic polymorphisms, may contribute to susceptibility to developing organ dysfunction when fed a low choline diet.

For each SNP, we tested for allelic association with susceptibility to developing organ dysfunction associated with eating a low choline diet. Although five tests were performed, no Bonferroni correction was done since small sample size made only very large effect sizes detectable. We identified two SNPs in the *PEMT* promoter region: rs12325817 (−774 G→C) and a new SNP that had not been previously reported at position −314 C→T. We found a SNP in exon 4 (+5465 G→A; rs7946) and two SNPs in exon 2, which adjoined the promoter: rs2278952 (+164, C→T) and + 29 C→G. The latter is a new genetic variant that has not been reported before. For rs12325817 (−774 G→C), the variant C allele was relatively common in our study population, where 18% were CC, 56% GC, and 26% GG genotype. The new SNPs at position −314 in the *PEMT* promoter and + 29 of exon 2 were rare, each occurring as a heterozygous allele in one subject (0.032 frequency); neither developed organ dysfunction when on the low choline diet. The rs2278952 SNP (+164 C→T) occurred at a frequency of 0.25 in women; it, too, was a heterozygous allele and was not associated with changes in susceptibility to developing organ dysfunction when on the low choline diet.

In all women, 18 of the 23 (78%) carriers of the *PEMT*–744C allele (rs12325817) developed organ dysfunction when fed a low choline diet (odds ratio 25, P=0.002; Table 3). In postmenopausal women, 11 of 12 (92%) of the allele carriers developed organ dysfunction when fed a low choline diet, and the 2 women without this allele did not. In the eight premenopausal women who were heterozygous for the allele (GC genotype), half developed organ dysfunction and half did not when fed a low choline diet, while the two premenopausal women who were homozygous for the allele developed organ dysfunction. Overall, the three women homozygous for this allele (CC genotype) developed organ dysfunction when fed a low choline diet, and seven of the eight females without this allele (GG genotype) did not (Table 3). There was no effect in men.

The first of two SNPs in the coding region of the *CHDH* gene (rs9001; +318 A→C) had a protective effect on susceptibility to developing organ dysfunction when fed a low choline diet in all subjects who carried the C allele (Table 4). We found no significant differences when the participants were grouped by gender or menopausal status. Although the second *CHDH* variant (rs12676; +432 G→T) was within 115 base pairs of SNP rs9001 (+318 A→C), it was formed independently (no difference in Hardy-Weinberg expected distribution for the population). Among all subjects, this SNP was not associated with susceptibility to developing organ dysfunction associated with choline deficiency. However, among premenopausal women, 5 of the 6 (83%) who were heterozygous for this variant developed organ dysfunction on a low choline diet compared with 2 of 10 (20%) who did so without this risk allele (odds ratio 20, P=0.04; Table 4).

There was no association between the SNP in exon 4 of the *PEMT* gene (rs7946, +5465 G→A) and susceptibility to choline deficiency (Table 5), nor was the *BHMT* variant (rs3733890; +742G→A) associated with changes in susceptibility to choline deficiency (Table 5).

DISCUSSION

Common genetic polymorphisms have been reported to influence human requirements for nutrients. For example, a common SNP in the methyltetrahydrofolate reductase gene increases dietary requirements for the vitamin folic acid (34). However, these SNPs usually have very modest effects on nutrient needs. We recently reported that individuals who were carriers of the very common 5,10-methylenetetrahydrofolate dehydrogenase-1958A gene allele were more likely than noncarriers to develop signs of choline deficiency (35). We now report that we have identified common genetic variations in the *PEMT* and *CHDH* genes that may be associated with developing organ dysfunction when choline is removed from the diet of humans. While a larger study is needed to confirm these findings, it does appear that these

polymorphisms influence the susceptibility to developing organ dysfunction when fed a low choline diet, and thus they increase the dietary requirement for choline needed to sustain optimal health.

In particular, women with a common variant in the promoter region of the *PEMT* gene rs12325817 (−774 G→C) were at significantly increased risk of developing organ dysfunction when dietary intake of choline is insufficient. PEMT activity is responsible for endogenous biosynthesis of choline moiety (1), and this activity is increased by estrogen treatment (36). We suggest that the promoter region of this gene is likely to have an estrogen response element (ERE). Indeed, the rs12325817 (−774 G→C) SNP is located within 50 bp of a putative ERE, which contains a perfect half-site consensus sequence (TGACC), but four of five bases differ in the other half-site (CGAAC vs. GGTCA). Given the sexually dimorphic effect of *PEMT* rs12325817 (−774 G→C), it is possible that this SNP alters the estrogen responsiveness of the promoter. Studies are under way in our laboratory to confirm this. We suggest that premenopausal women who are heterozygous for the *PEMT* rs12325817 (−774 G→C) C allele have sufficient estrogen to overcome the effects on estrogen-mediated transcription factor of the single allele, whereas post-menopausal women with lower estrogen levels are sensitive to the SNP. Men, with little estrogen, would be unaffected by an SNP that altered estrogen receptor complex binding.

The protective effect of the SNP in the *CHDH* gene (rs9001; 318 A→C) was more modest, and the frequency of this allele was relatively low (0.23). We observed a significant decrease in susceptibility to developing organ dysfunction on a low choline diet in all subjects, but a larger study would be needed to examine whether there are gender differences. More studies are also needed for the *CHDH* rs12676 (+432 G→T) SNP; first to confirm whether this SNP is associated with increased susceptibility to choline deficiency (we only achieved statistical significance in the subgroup of premenopausal women), then to explain this divergent response. It is not known whether these two SNPs have opposite effects on the activity of the CHDH enzyme.

The lack of effect of the SNP in exon 4 of the *PEMT* gene (rs7946, +5465 G→A) was unexpected, because we previously reported that this is a loss of function SNP and that persons with the variant A allele have increased risk of nonalcoholic fatty liver disease (30). Perhaps the modest decrease (30%) in activity of *PEMT* associated with this SNP was overshadowed by compensatory induction of the enzyme that is associated with choline deficiency in males (37,38) and by estrogen-mediated activation of PEMT in females. The BHMT SNP effect was not surprising because, although this SNP has been reported to be protective against the risk of cardiovascular disease (20), the protein product of the gene variant did not differ in either catalytic activity or betaine binding when compared to the enzyme which did not contain the polymorphism (20,39).

The SNPs we identified that increased susceptibility to developing organ dysfunction in humans fed low choline diets are likely to be of clinical importance. Humans fed intravenously (total parenteral nutrition) with solutions that deliver less choline than the adequate intake concentration often develop liver dysfunction that sometimes resolves when a choline source is added to their feeding solution (40). We suggest that humans with the identified SNPs are the ones most likely to be susceptible to this complication of parenteral nutrition. These SNPs, combined with poor dietary intake of choline, could contribute to adverse outcomes during pregnancy, a time when choline demand is high (9,41). As noted earlier, deficient maternal dietary intake of choline during pregnancy in humans was associated with a 4-fold increased risk of having a baby with a neural tube defect (9). In rodent models, maternal dietary choline intake influenced brain development. More choline (~4× dietary levels) during days 11–17 of gestation in the rodent increased hippocampal progenitor cell proliferation (10,11), decreased

apoptosis in these cells (10,11), enhanced long-term potentiation (LTP) in the offspring when they were adult animals (12,42,43), and enhanced visuospatial and auditory memory by as much as 30% in the adult animals through out their lifetime (13,44–49). Mothers fed choline-deficient diets during late pregnancy have offspring with diminished progenitor cell proliferation and increased apoptosis in fetal hippocampus (10,11), insensitivity to LTP when they were adult animals (12), and decremented visuospatial and auditory memory (13). For these reasons, identification of common polymorphisms that increase dietary requirements for choline during pregnancy could enable us to identify women for whom we need to assure adequate dietary choline intake. Further work with a larger sample size is warranted to replicate these important findings and to explore the mechanisms involved.

In summary, we report for the first time that SNPs in the phosphatidylethanolamine N-methyltransferase (*PEMT*) and choline dehydrogenase (*CHDH*) genes are associated with altered susceptibility to developing organ dysfunction on a low choline diet, and they likely affect dietary requirements for the nutrient choline. These SNPs are extremely common, and their effects should be considered when setting dietary reference intake levels. Studies determining the prevalence of these genetic polymorphisms in human populations of diverse composition should be conducted to facilitate such recommendations. In addition, since the genes of interest have many more polymorphisms than we tested, we cannot rule out the possibility that unmeasured but causal genetic variation is in linkage disequilibrium with the SNPs we genotyped.

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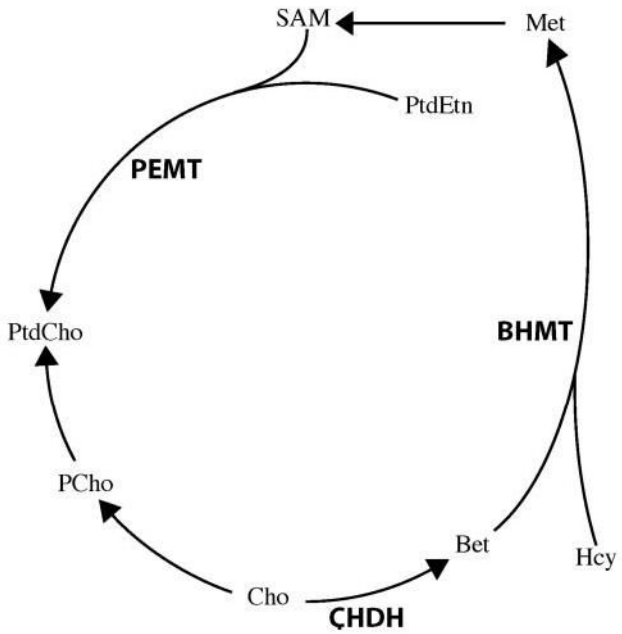

Figure 1.

Three important genes in choline metabolism for which we identified single nucleotide polymorphisms. PEMT = phosphatidylethanolamine N-methyltransferase, which catalyzes the reaction to make phosphatidylcholine (PtdCho) from phosphatidylethanolamine (PtdEtn) using S-adenosylmethionine (SAM) to donate methyl groups; CHDH = choline dehydrogenase, which along with betaine aldehyde dehydrogenase irreversibly oxidizes choline (Cho) to form betaine (Bet); BHMT = betaine:homocysteine methyltransferase, which donates its methyl group to homocysteine (Hcy) to form methionine (Met); PCho = phosphocholine.

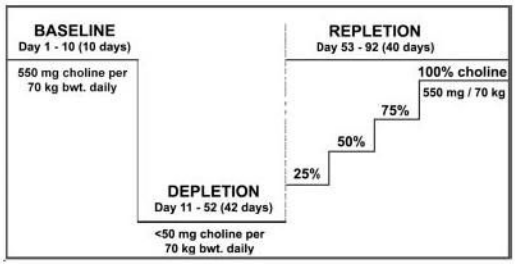

Figure 2.

Study design. Healthy men and women were fed a baseline diet containing the choline Adequate Intake concentration for 10 days. They were then switched to a low choline diet (<50 mg choline) until they developed signs of organ dysfunction associated with choline deficiency or for up to 42 days. Subjects who developed signs of organ dysfunction were repleted with graded amounts of choline at 10 day intervals until their symptoms disappeared; those without signs of organ dysfunction were fed the 100% choline diet for at least 3 days before being discharged from the study. Some subjects were given a folic acid supplement (400 μg per day) during the depletion and repletion phases, but this did not affect their susceptibility to choline deficiency (from da Costa et al., *Am J. Clinc. Nutr*., in press).

TABLE 1

SNPs studied[a]

Gene	rs number	Base pair and sequence change
MTHFD1	rs2236225	+1958 G → A
PEMT	rs12325817	−744 G → C[b]
PEMT	rs2278952	+164 C → T[b]
PEMT	rs7946	+5465 G → A[b]
PEMT	not yet designated	−314 C→T[b]
PEMT	not yet designated	+29 C→G[b]
CHDH	rs9001	+318 A → C
CHDH	rs12676	+432 G → T
BHMT	rs3733890	+742 G → A

[a]Each SNP is mapped to the genome and assigned a RefSNP accession ID (rs number). Base pair and sequence changes, also listed, are subject to revision when genes are resequenced.

[b]PEMT SNP base pair numbers are numbered from transcription start site (25).

TABLE 2

Primers for sequencing the PEMT promoter region[a]

Position of primer (bp)	Primer name	Sequence of primer 5′→3′
7728 - 7757	*PEMT* PRO - F3	GGAGTTATGGATCTAGGGAACTGGAGCAGC
7711 - 7730	*PEMT* PROseq - F1	ATTTCACCCTCCTGAAAGGA
8102 - 8082	*PEMT* PROseq - R1	TGACCAATCTAAGCCCAGGTT
8092 - 8112	*PEMT* PROseq - F2	**TAGATTGGTCATGGGAGGCTT**
8493 - 8474	*PEMT* PROseq - R2	ACAACATGGTGACACTCCGT
8503 - 8522	*PEMT* PROseq - F3	TCTCGAACTCCTGACCATCA
8878 - 8860	*PEMT* PROseq - R3	CCCGTAATCCCAGCACTTT
8915 - 8935	*PEMT* PROseq - F4	GAGGAAAAAGACTCTGGCACA
9300 - 9280	*PEMT* PROseq - R4	TTTACTCCATTGAGGGGTGCA
9084 - 9101	*PEMT* PROseq - F5	TGATGGATCCCAGGAGGA
9510 - 9490	*PEMT* PROseq - R5	GGCTTTCTGCTACCCAGTAAT
9525 - 9498	*PEMT* PRO - R1	ACAACAGAGGTCCCGGCTTTCTGCTAC
8438 - 8458	*PEMT* PROseqMid - F3	ACAACAGAGGTCCCGGCTTTCTGCTAC
8824 - 8802	*PEMT* PROseqMid - R3	TCAGAGATCAGCCTGGCCAATAT
8461 - 8480	*PEMT* PROseqMid - F4	ATTTTTAGTAGAGACGGAGT
8840 - 8821	*PEMT* PROseqMid - R4	ATCACAAGGCCAGGAGTCAG
8374 - 8394	*PEMT* PRO SNP1- F	ACTTCCTGGGTTGAAGCGATT
8597 - 8579	*PEMT* PRO SNP1- R	TTTATTCTCTGGCCGTGCC

[a]Primers were used to sequence the PEMT gene as described in Materials and Methods.

TABLE 3

Effect of PEMT promoter SNP rs12325817 (−744 G→C) on susceptibility to organ dysfunction in humans eating a low choline diet[a]

	Signs of choline deficiency	GG	GC	CC	*P* value OR (95% CI)
All subjects (57)	Yes	7	25	7	0.10
	No	8	7	3	
Men (26)	Yes	6	10	4	0.49
	No	1	2	3	
All women (31)	Yes	1	15	3	**0.002**
	No	7	5	0	**25** (2, 256)
Pre menopausal (16)	Yes	1	4	2	0.10
	No	5	4	0	
Postmenopausal (15)	Yes	0	11	1	**0.03**
	No	2	1	0	**42** (1, 1348)[b]

[a] Subjects were fed a diet low in choline, and some developed signs of organ dysfunction (liver or muscle) that were reversed when choline was added back to their diets. Numbers of subjects are indicated for each genotype. Two-sided *P* values were calculated with a 2 × 3 Fisher exact test. For $P < 0.05$, odds ratios (OR) and 95% confidence intervals (CI) were calculated as the odds of showing signs of deficiency for subjects with the C allele divided by the odds of showing signs of deficiency for subjects without the C allele.

[b] For postmenopausal and premenopausal women (where some cells were 0; see above), the odds ratio and 95% confidence intervals were computed after adding 0.5 to each cell, so these values underestimate the true values.

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TABLE 4

Effect of choline dehydrogenase (CHDH) genotypes on susceptibility to organ dysfunction in humans eating a low choline diet[a]

		***CHDH* rs9001, +318 A → C**				***CHDH* rs12676, +432 G → T**			
	Signs of choline deficiency	**AA**	**AC**	**CC**	***P* value OR (95% CI)**	**GG**	**GT**	**TT**	***P* value OR (95% CI)**
All subjects (57)	Yes	34	4	1	**0.03**	19	19	1	0.23
	No	10	6	2	**0.2** (0.05, 0.7)	13	5	0	
Men (26)	Yes	18	2	0	0.22	10	9	1	1.00
	No	4	2	0		3	3	0	
All women (31)	Yes	16	2	1	0.13	9	10	0	0.07
	No	6	4	2		10	2	0	
Pre menopausal (16)	Yes	6	1	0	0.39	2	5	0	**0.04**
	No	4	3	2		8	1	0	20 (1, 282)
Postmenopausal (15)	Yes	10	1	1	0.52	7	5	0	1.00
	No	2	1	0		2	1	0	

[a] Subjects were fed a diet low in choline and some developed signs of organ dysfunction (liver or muscle), which reversed when choline was added back to their diets. Numbers of subjects are indicated for each genotype. Two-sided *P* values were calculated with a 2 × 3 Fisher exact test. For $P < 0.05$, odds ratios (OR) and 95% confidence intervals (CI) were calculated as the odds of showing signs of deficiency for subjects with the C allele (T allele for *CHDH* 432) divided by the odds of showing signs of deficiency for subjects without the C allele (T allele for *CHDH* 432).

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TABLE 5

PEMT rs7946 (+5465 G→A) and BHMT rs3733890 (+742 G→A) genotypes were not associated with changes in susceptibility to organ dysfunction in humans eating a low choline diet[a]

		***PEMT* rs7946, +5465 G → A**				***BHMT* rs3733890, +742 G → A**			
	Signs of choline deficiency	**GG**	**GA**	**AA**	***P* value**	**GG**	**GA**	**AA**	***P* value**
All subjects (54)	Yes	5	16	15	0.86	21	11	4	0.84
	No	3	9	6		9	7	2	
Men (26)	Yes	4	8	8	1.00	12	6	2	1.00
	No	1	3	2		4	1	1	
All women (28)	Yes	1	8	7	0.75	9	5	2	0.65
	No	2	6	4		5	6	1	
Pre menopausal (16)	Yes	0	5	2	0.63	4	2	1	0.79
	No	2	4	3		3	5	1	
Postmenopausal (12)	Yes	1	3	5	0.66	5	3	1	1.00
	No	0	2	1		2	1	0	

[a]Subjects were fed a diet low in choline and some developed signs of organ dysfunction (liver or muscle) that reversed when choline was added back to their diets. Numbers of subjects are indicated for each genotype. Two-sided *P* values were calculated with a 2 × 3 Fisher exact test. PEMT = phosphatidylethanolamine N-methyltransferase; BHMT = betaine:homocysteine methyltransferase.