Two methods for assessment of choline status in a randomized crossover study with varying dietary choline intake in people: isotope dilution MS of plasma and in vivo single-voxel magnetic resonance spectroscopy of liver

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ABSTRACT

Background: Choline deficiency has numerous negative health consequences; although the preponderance of the US population consumes less than the recommended Adequate Intake (AI), clinical assessment of choline status is difficult. Further, several pathways involved in primary metabolism of choline are estrogen-sensitive and the AI for premenopausal women is lower than that for men.

Objectives: We sought to determine whether in vivo magnetic resonance spectroscopy (MRS) of liver and/or isotope-dilution MS of plasma could identify biomarkers reflective of choline intake (preregistered primary outcomes 1 and 2, secondary outcome 1). Determination of whether biomarker concentrations showed sex dependence was a post hoc outcome. This substudy is a component of a larger project to identify a clinically useful biomarker panel for assessment of choline status.

Methods: In a double-blind, randomized, crossover trial, people consumed 3 diets, representative of ∼100%, ∼50%, and ∼25% of the choline AI, for 2-wk periods. We measured the concentrations of choline and several metabolites using ^{1}H single-voxel MRS of liver in vivo and using ^{2}H-labeled isotope dilution MS of several choline metabolites in extracted plasma.

Results: Plasma concentrations of $^{2}H_9$-choline, unlabeled betaine, and $^{2}H_9$-betaine, and the isotopic enrichment ratio (IER) of betaine showed highly significant between-diet effects ($q < 0.0001$), with unlabeled betaine concentration decreasing 32% from highest to lowest choline intake. Phosphatidylcholine IER was marginally significant ($q = 0.03$). Unlabeled phosphatidylcholine plasma concentrations did not show between-diet effects ($q = 0.34$). $^{2}H_9$ (trimethyl)-phosphatidylcholine plasma concentrations ($q = 0.07$) and MRS-measured total soluble choline species liver concentrations ($q = 0.07$) showed evidence of between-diet effects but this was not statistically significant.

Conclusions: Although MRS is a more direct measure of choline status, variable spectral quality limited interpretation. MS analysis of plasma showed clear correlation of plasma betaine concentration, but not plasma phosphatidylcholine concentration, with dietary choline intake. Plasma betaine concentrations also correlate with sex status (premenopausal women, postmenopausal women, men). This trial was registered at clinicaltrials.gov as NCT03726671. *Am J Clin Nutr* 2021;113:1670–1678.

Keywords: nutritional status biomarkers, choline, betaine, sex differences, magnetic resonance spectroscopy, mass spectrometry

Introduction

Choline is an essential nutrient that is not synthesized in amounts in the human body sufficient to meet its needs (1). It is a precursor to acetylcholine, betaine, and the phospholipid backbone glycerophosphocholine. Via its metabolite phosphatidylcholine (PtdCho), choline is essential for the export of lipids from the liver (2, 3) because it is needed for the membranes used in exporting triglycerides as VLDLs (3–6). Diets low in choline result in fatty liver in people (7–9) as well

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Supplemental Figures 1 and 2 and Supplemental Tables 1–5 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: AI, Adequate Intake; CHDH, choline dehydrogenase; ChoK, choline kinase; d_0, $\mathbf{1}H_9$-(methyl) labeled; d_9, $\mathbf{2}H_9$-(methyl) labeled; FC, fold-change; FDR, false discovery rate; HC, high-choline diet; IER, isotopic enrichment ratio; LC, low-choline diet; LC-SID-MRM/MS, liquid chromatography-stable isotope dilution-multiple reaction monitoring mass spectrometry; MC, medium-choline diet; MRS, magnetic resonance spectroscopy; PEMT, phosphatidylethanolamine methyltransferase; PtdCho, phosphatidylcholine; SAM, *S*-adenosylmethionine; SST, serum-separating tube; tCho, total soluble choline species; TE, spin echo time; TR, recycle time.

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as alterations in other aspects of fatty acid metabolism (10, 11), muscle damage (8, 12), and altered fetal brain development (13). As a precursor to betaine, choline also contributes to cellular methylation potential and the *S*-adenosylmethionine (SAM):*S*-adenosylhomocysteine ratio which, in turn, is a metabolic control point that regulates DNA and histone methylation/epigenetic state (14–17) and flux through the sulfur amino acid pathway.

Although the US Institute of Medicine (now the National Academy of Medicine) established a recommended Adequate Intake (AI) in 1998 (18), dietary intake of choline varies widely. Data from the 2009–2012 NHANES suggest that >90% of adult Americans eat less than the AI, with roughly one-quarter of the US adult population consuming <50% of the AI (19). Clinical determination of choline deficiency, however, is difficult. Dietary choline is carried through the portal vein to the liver, where it is metabolized; circulating plasma concentrations are dependent on numerous processes and regulatory steps, and thus plasma choline rarely drops below ~5 μM, even in severely depleted individuals (7, 20). We are developing a clinically useful panel of biomarkers that can identify choline status before the onset of explicit liver (or other) disease. As a component of this effort, we are examining the correlation of several proxies of choline status. This article presents the results of a planned interim analysis to test several secondary outcomes. Specifically, we compare the efficacy of measurement of *1*) isotope dilution MS of several choline metabolites in plasma with *2*) ^{1}H single-voxel magnetic resonance spectroscopy (MRS) of the liver as methods to assess changes in the metabolically available choline pool size in humans consuming diets differing in total daily choline intake.

Methods

Study design

The study design was a longitudinal, double-blind, randomized, crossover dietary intervention (NCT03726671). Choline content was based on the National Institute of Medicine–defined AI for adult men (550 mg choline/d). Subjects were provided with all foods and drink for each of three 2-wk controlled feeding arms. These diets were identical except for choline content, which was adjusted by changing the choline (as choline chloride) added to the 3 bread rolls consumed per day. In random order these diet arms delivered 5.1 mmol choline/d (531 mg, 97% AI), 2.5 mmol choline/d (47% AI), or 1.3 mmol choline/d (25% AI) and they are hereafter referred to as the high-choline diet (HC), medium-choline diet (MC), and low-choline diet (LC), respectively. All diets contained a mean of 147 mg betaine/d. The amount of choline in each diet was not adjusted for subject sex or BMI; for premenopausal women, the AI is 425 mg/d. There was a minimum of 2 wk of washout between arms during which the participants returned to their usual diet. On day 12 of each dietary arm, in addition to the food supplied, subjects consumed a single bolus of 2.2 mmol trimethyl-$^{2}H_9$-(methyl) labeled (d_9)-choline (as 98% D, methyl-perdeuterated choline chloride; Cambridge Isotope Laboratories). This dose is based on that used by Pynn et al. (21), with an additional ~25% to account for differential absorption between intravenous and oral delivery. We confirmed the total choline and betaine content of diets using duplicate food portions analyzed by liquid chromatography-stable isotope dilution-multiple reaction monitoring mass spectrometry (LC-SID-MRM/MS) as previously described (22) (see **Supplemental Tables 1** and **2** for a nutrient breakdown of the diets, including choline and betaine, and ingredients of the bread rolls). Choline content and stability during storage of choline in the bread rolls were separately determined for each batch produced. Riboflavin (30 mg/d) was added to the bread rolls and was used as a compliance biomarker via 24-h urine collection measures (23–25). Noncompliance for a given dietary arm was defined as day-15 riboflavin 24-h excretion <20% of day-5 riboflavin concentration (all participants exhibited elevated riboflavin at day 5 of each arm). This cutoff resulted in the removal of 6 of the 120 arms from the data. Blood was collected on day 1, and at 6 h, 24 h, 48 h, and 72 h after the day 12 d_9-choline bolus. MRS data were collected 72 h postbolus (day 15), after an overnight fast. The protocol was approved by the Institutional Review Board at the University of North Carolina at Chapel Hill and informed consent was obtained.

Subject recruitment

Participants were screened and required to be of good health status as determined by self-report, assessment by the study physician, and standard clinical laboratory tests including a comprehensive metabolic panel. Inclusion criteria were age within the range of 17–70 y and BMI (in kg/m^2) within the range of 20–30. Exclusion criteria included use of drugs or medications known to be damaging to liver or muscle or that have the potential to alter choline metabolism; a history of hepatic, renal, or other chronic systemic disease; being a substance abuser, current smoker, or consuming >2 alcoholic beverages per day (14/wk); using choline-containing dietary supplements; regularly consuming a diet that might interfere with the study; or allergies to soy proteins or any foods in the required diet. Women who were breastfeeding, pregnant, or planning to become pregnant were excluded owing to the potential risk to the fetus/infant of a low-choline diet. Subjects who performed intense exercise of >1 h every day or other intense muscle-building exercise (such as weightlifting beyond low-weight maintenance repetitions) were also excluded. Ultimately, 40 healthy people (21 premenopausal women, 8 postmenopausal women, 1 perimenopausal woman, 10 men) were included in this planned interim analysis (**Table 1**). A Consolidated Standards of Reporting Trials diagram is included as **Supplemental Figure 1**. Power calculations ([*F* test, between-factors/repeated-measures ANOVA, calculated with G∗Power version 3.1.9.7 (26)] showed that a sample size of 30 would be sufficient to yield a power of 0.8 given 3 groups, $\rho = 0.5$, and an effect size of 0.5. This effect size is readily observed in our data.

Sample collection

Whole blood was collected in serum-separating tubes (SSTs) for clinical laboratory measurements and in lithium heparin–coated tubes for plasma metabolite analysis. Tubes were gently inverted to mix. Lithium heparin–coated tubes were immediately put on wet ice and centrifuged at 2200 × *g* at 4°C for 10 min, then returned to the ice bath. Plasma samples were pooled, divided into aliquots, and frozen at −80°C within 1 h of collection time. SSTs were allowed to clot at room temperature then centrifuged at 2200 × *g* at 4°C for 10 min. SSTs were

TABLE 1 Participant data (beginning of the study)[1]

	Premenopausal women	Postmenopausal women	Perimenopausal women	Men
n	21	8	1	10
Age, y	34 ± 7	54 ± 6	49	36 ± 10
Height, cm	165 ± 6	166 ± 7	161	175 ± 4
Weight, kg	70.8 ± 7.8	71.4 ± 7.0	74.1	71.9 ± 6.5
BMI, kg/m^2	26.0 ± 3.3	26.1 ± 2.5	28.6	23.5 ± 2.6
Ethnicity, white/black/Hispanic	14/4/3	5/3/0	1/0/0	9/0/1
FSH, mIU/mL	4.7–21.5	25.8–134.8	21.5–25.8	

[1] Values are mean ± SD or ranges unless otherwise indicated. FSH concentrations as measured by LabCorp were used to classify menopausal status.
FSH, follicle-stimulating hormone.

sent for a comprehensive metabolic panel and creatine kinase testing to the clinical laboratories at Lab Corporation of America, Burlington, NC, which is both Clinical Laboratory Improvement Act (ID 34D0655059) and College of American Pathologists (no. 1396901) accredited. At enrollment, follicle-stimulating hormone and estrogen were also measured in female participants. Urine was collected by participants into a commode specimen collector and immediately poured into a 3-L plastic jug that contained 3 mL acetic acid. The plastic jug was kept cool with ice packs in an insulated cooler during the 24-h collection. On completion, the total volume and collection times were noted. Aliquots of 1 mL were created with and without formic acid (10 μL) and stored at −80°C.

MS

Plasma (choline metabolites) and urine (riboflavin) samples were spiked with stable isotope–labeled internal standards and extracted using a modified Bligh and Dyer extraction as previously described (27). Briefly, samples were extracted with 4 volumes of methanol:chloroform (2:1, vol:vol), mixed on a vortex, held at −20°C for 2–24 h, and mixed on a vortex. Supernatants were collected and pellets re-extracted with methanol:chloroform:water (2:1:0.8, by vol.). Combined supernatants were treated with water and chloroform to induce phase separation. The aqueous phase (choline and betaine) was transferred to HPLC vials for analysis. The organic phase (phosphatidylcholine and sphingomyelin) was diluted with acetonitrile and transferred to HPLC vials for analysis. A series of standards of known concentrations and containing the corresponding internal standards were prepared and treated identically to samples.

Analytes were quantified using LC-SID-MRM/MS (aqueous) or liquid chromatography-stable isotope dilution-mass spectrometry (organic) as previously described (22, 27). Chromatographic separations were performed on an Acquity HILIC 1.7 μm 2.1 × 50 mm column (Waters Corp) using a Waters ACQUITY UPLC system (0.37 mL/min at 40°C). Mobile phases for aqueous analytes were A: 100% water with 0.125% formic acid, and B: 90% acetonitrile/10% water with 10 mM ammonium formate and 0.125% formic acid. Mobile phase A for organic analytes was 10% acetonitrile/90% water and 0.125% formic acid and B was the same as for aqueous metabolites. As reported elsewhere (27), phosphatidylcholine undergoes in-source fragmentation and is detected as phosphocholine. The analytes and their corresponding isotopes were monitored on a Waters TQ detector using characteristic precursor–product ion transitions (**Supplemental Table 3**). Concentrations of each analyte in the samples were determined using the peak area ratio of the analyte to its isotope (response) and read off a standard curve of response values against standard concentrations for each analyte. The isotopic enrichment ratio (IER) was calculated as the fractional amount of labeled metabolite compared with the total (labeled + unlabeled) metabolite. Given a known quantity of isotopically labeled compound, the IER provides insight into the metabolically available precursor pool (28).

MRS

Magnetic resonance images and spectra were acquired at the Wake Forest School of Medicine Clinical Imaging Core using a 3.0 T scanner (Magnetom Skyra, Siemens Healthcare) with an 18-channel torso coil. Torso images were acquired (T_2-weighted 2D half-Fourier acquisition single-shot turbo spin echo imaging) and used for localization of single-voxel MRS. Regions for metabolite measurement were localized to the middle of the right hepatic lobe and adjusted to avoid large vessels and fatty tissue. Single-voxel (3 × 3 × 3 cm^3) spectra were collected using point resolved spin-echo spectroscopy with a 1200-Hz spectral width, a recycle time (TR) = 2 s, a spin echo time (TE) = 33 ms, and number of scans = 80. Data collection used 2D Prospective Acquisition Correction navigated acquisition (29) to synchronize acquisition with breathing motions.

Single-voxel spectra were processed using jMRUI version 6.0b (30) with the Amares plugin (31) and manually inspected to adjust phase if necessary and to ensure there were no artifactual integrations. Choline concentration in the selected voxel was determined by comparing the integral of the peak at 3.2 ppm from the water-suppressed spectrum [total soluble choline species (tCho): choline, phosphocholine, glycerophosphocholine] with the water peak from the unsuppressed spectrum using the relation: tCho = (choline_signal/water_signal) × scale. The scale term accounts for differential choline and water T_1 (water, 990 ms; tCho, 842 ms) and T_2 (water, 30 ms; tCho, 50 ms) relaxation times (32), TR, TE, the number of protons giving rise to the signals, and the fractional water content of liver (72% of 55.5 M) (33).

TABLE 2 Concentrations of choline metabolites measured by MRS and MS[1]

Metabolite	Diet	Concentration			
		All[2]	Premenopausal female	Postmenopausal female	Male
MRS-tCho, mM	LC	7.80 ± 4.20	8.83 ± 4.46	8.10 ± 3.80	5.36 ± 3.25
	MC	7.99 ± 5.02	8.78 ± 5.30	7.81 ± 4.78	6.66 ± 4.97
	HC	9.83 ± 4.61	10.42 ± 2.92	12.15 ± 6.45	6.38 ± 3.17
d_0-choline at 6 h, μM	LC	8.12 ± 2.07	7.40 ± 1.61	8.77 ± 2.53	9.09 ± 2.33
	MC	9.26 ± 2.69	8.21 ± 2.29	10.70 ± 3.09	10.21 ± 2.42
	HC	10.41 ± 2.84	9.30 ± 1.79	11.85 ± 3.22	11.26 ± 3.70
d_9-choline at 6 h, μM	LC	0.08 ± 0.13	0.06 ± 0.13	0.11 ± 0.16	0.08 ± 0.09
	MC	0.09 ± 0.13	0.08 ± 0.13	0.12 ± 0.15	0.09 ± 0.10
	HC	0.12 ± 0.14	0.10 ± 0.14	0.14 ± 0.17	0.11 ± 0.11
IER-choline at 6 h	LC	0.0497 ± 0.0175	0.0520 ± 0.0202	0.0454 ± 0.0133	0.0472 ± 0.0152
	MC	0.0513 ± 0.0168	0.0536 ± 0.0195	0.0487 ± 0.0127	0.0488 ± 0.0144
	HC	0.0509 ± 0.0152	0.0545 ± 0.0175	0.0464 ± 0.0127	0.0476 ± 0.0121
d_0-betaine at 24 h, μM	LC	38.14 ± 11.77	32.83 ± 11.23	39.29 ± 6.85	49.30 ± 7.81
	MC	43.90 ± 13.69	37.08 ± 12.70	45.49 ± 6.53	56.25 ± 11.05
	HC	56.89 ± 18.75	50.59 ± 19.67	57.44 ± 13.13	71.25 ± 14.51
d_9-betaine at 24 h, μM	LC	1.34 ± 0.45	1.29 ± 0.51	1.27 ± 0.37	1.52 ± 0.35
	MC	1.44 ± 0.43	1.35 ± 0.49	1.44 ± 0.30	1.60 ± 0.35
	HC	1.71 ± 0.55	1.72 ± 0.60	1.57 ± 0.45	1.87 ± 0.50
IER-betaine at 24 h	LC	0.0343 ± 0.0075	0.0375 ± 0.0078	0.0311 ± 0.0068	0.0299 ± 0.0041
	MC	0.0324 ± 0.0069	0.0354 ± 0.0069	0.0310 ± 0.0069	0.0277 ± 0.0033
	HC	0.0299 ± 0.0065	0.0337 ± 0.0058	0.0264 ± 0.0048	0.0256 ± 0.0045
d_0-PtdCho at 24 h, μM	LC	2175 ± 354	2138 ± 347	2390 ± 329	2099 ± 378
	MC	2228 ± 471	2082 ± 356	2615 ± 529	2209 ± 497
	HC	2228 ± 493	2104 ± 419	2612 ± 532	2152 ± 510
d_9-PtdCho at 24 h, μM	LC	28.25 ± 8.19	27.79 ± 8.22	30.40 ± 12.69	27.82 ± 4.62
	MC	27.42 ± 8.88	24.94 ± 8.05	32.92 ± 10.95	27.98 ± 7.30
	HC	25.67 ± 5.64	25.43 ± 5.28	28.94 ± 5.43	23.43 ± 6.14
IER-PtdCho at 24 h, μM	LC	0.0129 ± 0.0032	0.0129 ± 0.0035	0.0125 ± 0.0044	0.0132 ± 0.0017
	MC	0.0122 ± 0.0030	0.0119 ± 0.0030	0.0126 ± 0.0040	0.0126 ± 0.0023
	HC	0.0115 ± 0.0021	0.0121 ± 0.0022	0.0112 ± 0.0020	0.0108 ± 0.0018

[1]Values are mean ± SD. Metabolites were measured by single-voxel MRS (liver; soluble choline species at 72 h after the d_9-choline bolus) or by MS (plasma; choline at 6 h postbolus, betaine and PtdCho at 24 h postbolus), from study participants on LCs, MCs, or HCs. d_0, $^1H_9$-(methyl) labeled; d_9, $^2H_9$-(methyl) labeled; HC, high-choline diet; IER, isotopic enrichment ratio; LC, low-choline diet; MC, medium-choline diet; MRS, magnetic resonance spectroscopy; PtdCho, phosphatidylcholine; tCho, total soluble choline species.

[2]For MRS: *n* (all) = 37 (LC), 34 (MC), 32 (HC); *n* (premenopausal female) = 21 (LC), 17 (MC), 17 (HC); *n* (postmenopausal female) = 6 (LC), 8 (MC), 8 (HC); *n* (male) = 9 (LC), 9 (MC), 6 (HC). For MS: *n* (all) = 39 (LC), 38 (MC), 37 (HC); *n* (premenopausal female) = 21 (LC), 20 (MC), 19 (HC); *n* (postmenopausal female) = 7 (LC), 8 (MC), 8 (HC); *n* (male) = 10 (LC), 10 (MC), 9 (HC). Data for the single perimenopausal female are included in "All" but not listed separately.

Statistics

Repeated-measures mixed-effect analyses and 1-sample *t* tests were calculated using GraphPad Prism 9.0 and adjusted for multiple comparisons using the 2-stage linear step-up method of Benjamini, Krieger, and Yekutieli (34) with a false discovery rate (FDR) of 5%. The mixed-effect model was chosen to take advantage of the randomized crossover experimental design while being more tolerant of missing values than a repeated-measures ANOVA. One- and 2-factor ANOVAs were calculated using GraphPad Prism 9.0 and adjusted for multiple comparisons using Tukey's test. Data met normality criteria as judged by D'Agostino and Pearson tests and Shapiro–Wilk tests in GraphPad Prism 9.0.

Results

Dietary choline is absorbed from the gut via the portal vein into the liver, where it is phosphorylated to form phosphocholine or oxidized to form betaine. Although circulating plasma concentrations of choline do reflect dietary choline, they are subject to regulation and, as noted, generally do not dip below ~5 μM, even at very low dietary intake amounts (7, 20). Hence, we sought to identify other biomarkers of choline status.

Metabolite concentrations as a function of choline intake

Liver tCho concentration measured by MRS 72 h after the d_9-choline bolus, plasma concentrations of choline [$^1H_9$-(methyl) labeled (d_0), d_9, IER] measured by MS 6 h postbolus, and betaine (d_0, d_9, IER) and PtdCho (d_0, d_9, IER) measured by MS 24 h postbolus, as a function of diet, are given in **Table 2**. Mixed-effect analysis found highly significant [(FDR-adjusted *P*) $q < 0.0001$] between-diet effects for d_0-choline, d_9-choline, d_0-betaine, d_9-betaine, and IER-betaine, whereas IER-PtdCho was marginally significant ($q = 0.03$). IER-choline ($q = 0.25$), d_0-PtdCho ($q = 0.34$), and d_9-PtdCho ($q = 0.08$) were not significant, nor was MRS-measured tCho ($q = 0.08$). Of the

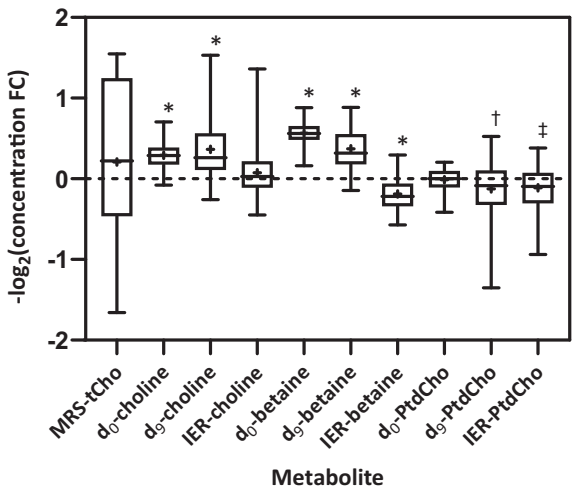

FIGURE 1 Choline metabolites as a function of dietary choline intake. Box and whiskers plot of -$\log_2$(concentration FC) of MRS-derived tCho ($n = 30$) and MS-derived d_0, d_9, and IER for choline, betaine, and PtdCho ($n = 36$). Choline was measured at 6 h after the d_9-choline bolus, betaine and PtdCho at 24 h postbolus. MRS scans were performed on day 15 (72 h postbolus). Positive values of -$\log_2$(FC) indicate higher concentrations for HCs than for LCs. For each metabolite, the box spans the IQR, the horizontal line is the median, + marks the mean, and whiskers span the full data range. Adjusted P using FDR (5%): $*q < 0.0001$; $\dagger q = 0.006$; $\ddagger q = 0.019$; no symbol: $q > 0.1$. d_0, $^1H_9$-(methyl) labeled; d_9, $^2H_9$-(methyl) labeled; FC, fold-change; HC, high-choline diet; IER, isotopic enrichment ratio; LC, low-choline diet; MRS, magnetic resonance spectroscopy; PtdCho, phosphatidylcholine; tCho, total soluble choline species.

time points sampled, d_9-PtdCho was maximal at 24 h. Although plasma d_9-betaine concentrations were higher at 6 h postbolus than at 12 h, we have observed in this study (**Supplemental Table 4**) and pilot studies (data not shown) that buildup rates of plasma concentrations of d_9-betaine show substantial interindividual variability and that more consistent data (i.e., smaller relative SD) are obtained at 24 h postbolus. Supplemental Table 4 gives metabolite concentrations measured by MS at 6, 24, 48, and 72 h after the d_9-choline bolus.

Fold-change analysis of choline metabolites

Although the observed metabolite concentrations at the MC dietary intake amount were consistently in between the concentrations for the HC and LC diets, a given metabolite concentration is not, in general, a linear function of intake of a metabolic precursor. In addition, the MC diet represents a fairly mild metabolic challenge. Consequently, our fold-change (FC) analysis focuses on relative changes in metabolite concentrations between the HC and LC diets.

We calculated FC for each metabolite using (concentration on the LC diet)/(concentration on the HC diet) at the 6 h time point (choline), the 24 h time point (betaine, PtdCho) or the single 72 h measurement point (MRS-tCho). Calculated across all participants, d_0-betaine (FC = 0.68, $q = 4.2e^{-15}$), d_9-betaine (FC = 0.77, $q = 1.6e^{-10}$), d_9-choline (FC = 0.78, $q = 5.1e^{-6}$), and d_0-choline (FC = 0.82, $q = 3.15e^{-11}$) concentrations decreased noticeably going from the HC to the LC diet, consistent with the mixed-effect analysis of raw metabolite concentrations. **Figure 1** shows -$\log_2$(FC) for each metabolite.

The IER, expressed as d_9-metabolite/(d_9-metabolite + d_0-metabolite), is a measure of newly formed deuterium-labeled metabolite (derived from the isotopic d_9-choline bolus on day 12) compared with the pool of unlabeled metabolite present in the tissue. Whereas d_0-betaine was sensitive to and positively correlated with dietary choline intake (FC = 0.68, $q = 4.2e^{-15}$), betaine IER was considerably less responsive to choline intake (FC = 1.14, $q = 1.2e^{-6}$). Under the dietary conditions used in this study, we expected that the plasma concentration of d_0-betaine would be sensitive to d_0-choline amounts in the HC/MC/LC, but would not be affected by the single d_9-choline bolus. Surprisingly, we observed that concentrations of both d_0- and d_9-betaine increased after the d_9-choline bolus. Consequently, the betaine IER is less sensitive because both numerator and denominator change together in the same direction. The origin of this effect is unclear but may reflect the different kinetic properties (K_M, V_{max}) of choline dehydrogenase (CHDH) and choline kinase (ChoK): at low concentrations in the liver, choline is preferentially phosphorylated by ChoK; at higher concentrations choline is predominantly oxidized to betaine by CHDH (35). Alternatively, the observed effect could be caused by upregulation of CHDH protein at higher concentrations of choline. However, hepatic *CHDH* expression is not affected by choline availability (36). There also may be other substrate-inducible processes. Regardless of mechanism, the observed dependence of hepatic choline metabolic fate (i.e., oxidation or phosphorylation) on hepatic choline concentration may be of relevance to studies that use bolus dosing of choline.

Betaine and PtdCho are the major choline metabolites released by the liver into the circulation. Relative to betaine, plasma PtdCho concentration is considerably less sensitive to diet. Over the choline diet range used in this study, d_0-PtdCho was invariant (FC = 1.01, $q = 0.27$), whereas d_9-PtdCho (FC = 1.09, $q = 0.025$) and PtdCho IER (FC = 1.08, $q = 0.019$) were slightly negatively correlated with dietary choline content. Lower concentrations of d_9-PtdCho (and smaller IERs) at higher amounts of dietary (d_0) choline are expected. Under HC conditions, the hepatic choline pool is higher in d_0-choline concentration, and also enriched in d_0-choline relative to d_9-choline, compared with under LC conditions. The utility of the IER is to identify relative concentrations of newly formed metabolites; given the large (~2 mM) plasma pool of extant unlabeled PtdCho (Table 2), this approach is limited in its sensitivity.

Single-voxel MRS measurement, although a direct measure of liver total choline, was not found to be a reliable indicator of dietary choline intake across the diet variation studied. Although the observed mean FC is similar to that of plasma betaine and choline concentrations (0.87), there was substantial variability among subjects (see the Discussion), and the FC was not statistically significant ($q = 0.1$) (Figure 1). Of the 30 MRS comparisons, 17 showed decreased tCho and 13 increased tCho as choline intake decreased from the HC to the LC diet. In contrast, 36 of 36 subjects exhibited decreased plasma betaine concentrations, and 35 of 36 subjects exhibited decreased plasma choline concentrations, under the same dietary conditions (**Supplemental Table 5**).

Plasma concentrations of betaine were stratified by sex

We observed sex-dependent differences in plasma concentrations of betaine and choline as a function of dietary choline

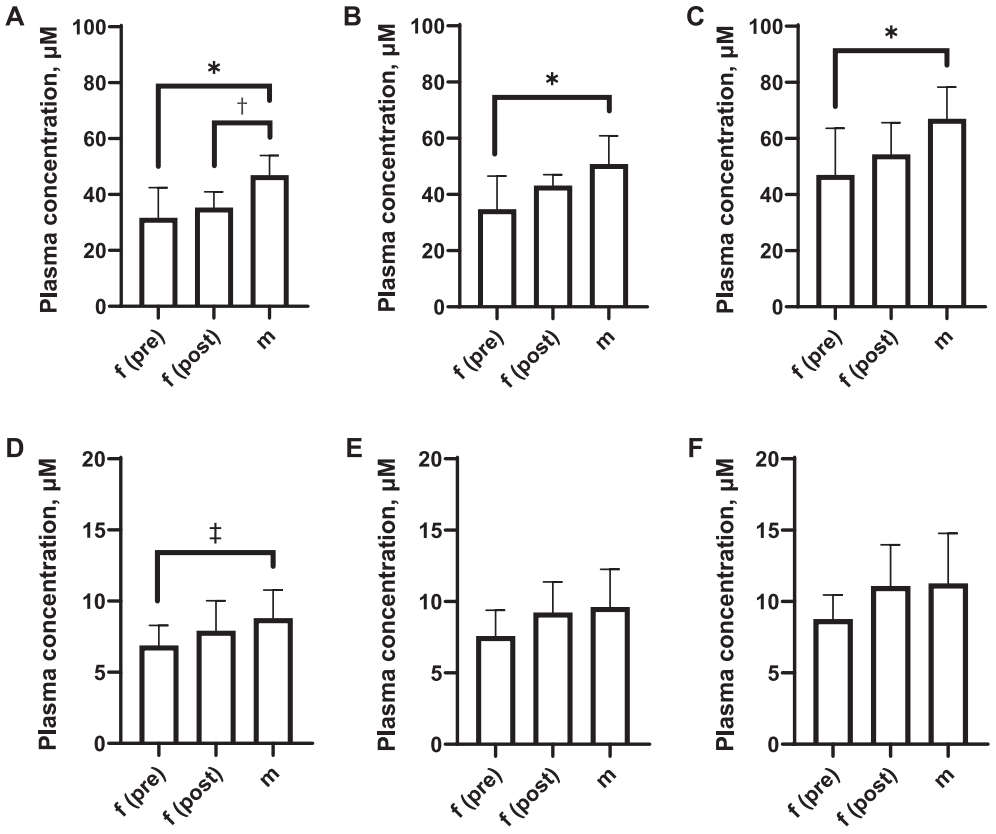

FIGURE 2 Plasma choline and betaine concentrations as a function of sex status and dietary choline intake amount. Between-sex comparisons of plasma (d_0-)betaine concentration at the end of each arm for (A) LC, (B) MC, and (C) HC and plasma (d_0-)choline concentration at the end of each arm for (D) LC, (E) MC, and (F) HC. Error bars show SDs. Adjusted *P* using Tukey's test: ∗*P*(adjusted) < 0.005; †*P*(adjusted) < 0.05; ‡*P*(adjusted) < 0.02. d_0, $^1H_9$-(methyl) labeled; f(post), postmenopausal female; f(pre), premenopausal female; HC, high-choline diet; LC, low-choline diet; m, male; MC, medium-choline diet.

intake. At a given dietary intake amount, males had significantly higher betaine concentrations than did premenopausal females (**Figure 2**A–C), consistent with previous reports (37). Postmenopausal females had plasma betaine concentrations intermediate to those of males and premenopausal females. Males also had higher plasma choline concentrations than did premenopausal females across all diets, but statistical significance was only observed at the lower choline intake amounts (Figure 2D–F). Repeated-measures 2-factor ANOVA of plasma choline:betaine ratios showed significant between-diet (LC compared with HC, MC compared with HC) but not between-sex effects (**Supplemental Figure 2**).

Daily choline intake was not adjusted for sex (intake is relative to the AI for adult males) or plasma volume (we note that the daily choline recommended AI is also not adjusted for BMI). However, because of the repeated-measures nature of this study, and the fact that there were no substantial changes in weight throughout the entirety of this trial, the conclusions were not affected by use of plasma concentration as opposed to total plasma amount.

Discussion

Of the metabolites investigated, and over the dietary choline range used in this study, plasma betaine concentrations were the most responsive biomarker of recent dietary choline intake, with plasma choline concentrations also somewhat responsive. This is consistent with previous findings (38), which also found that plasma choline, but not PtdCho, reflected dietary choline intake.

Bolus compared with steady-state administration of d_9-choline

Previous studies (38, 39) used extended d_9-choline intake (3 wk, 15% total dietary intake of choline as d_9). Such dosing can establish an isotopic steady state which is necessary to allow interpretations of d_3- and d_6-PtdCho concentrations in addition to d_9-metabolite concentrations. This is not practical in a clinical setting and it requires a considerable amount of d_9-choline: 15% of daily AI is 1.58 mmol choline, equivalent to 221 mg d_9-choline chloride. Over 3 wk, this amounts to 4.64 g d_9-choline chloride per person per experimental arm. Administering d_9-choline chloride as a single bolus, as in this study, decreases the required d_9-choline chloride to 307 mg (2.2 mmol) per person, but we lose the ability to trace secondary metabolic products. In addition, although we retain the ability to calculate IER, we cannot relate IER as easily to pool size: for PtdCho, extant d_0-PtdCho is too high; for betaine, biosynthesis and secretion are fast and would require sampling at additional time points in the first 24 h; for choline, the plasma concentrations of d_9-choline are too low for a reliable calculation of IER.

Results from both our study and a previous study suggest that regulation of betaine metabolism may be more complex than

commonly appreciated. Our study differed from Yan et al. (39) in *1*) using a bolus as opposed to long-term administration of d_9-choline and *2*) using a fixed amount of dietary d_9-choline (2.2 mmol) and a variable amount of d_0-choline (1.319, 2.637, or 5.275 mmol/d) as opposed to a fixed ratio of d_9- to d_0-choline with a variable total amount (0.79 mmol d_9/d and 4.48 mmol d_0/d or 1.72 mmol d_9/d and 8.97 mmol d_0/d). Comparing these 2 approaches, we expected to see a substantially decreasing IER with increasing dietary choline intake, whereas Yan et al. expected to see no change in IER with increasing dietary choline intake. Instead, we observed a muted decrease in betaine IER corresponding to a dietary choline increase which can be traced to the increase in d_0-betaine after ingestion of the d_9-choline bolus at each dietary choline amount. Yan et al., similarly, saw an increase in IER (choline, betaine, PtdCho) as dietary (d_0-) choline increased (men only, $n = 23$). Yan et al. concluded that their:

> observed increase in isotope enrichment from precursor d_9-choline to several products in blood and urine indicates that pools are in flux and that more than one precursor pool exists for derivation of subsequent products. The high enrichment of betaine, relative to that of choline, may also reflect an increased release of more enriched products as a result of gut-level oxidation of choline to methylamines.

We note that the HC, MC, and LC used in this study contained the same amount of betaine, so differences in plasma d_0-betaine concentration were not caused by differences in ingestion of betaine.

Single-voxel ^{1}H MRS as a monitor of systemic choline availability

Analysis of localized choline concentration by ^{1}H MRS has been reported as a promising measure of hepatocellular carcinoma (40), although whether choline is elevated or depressed in/near tumors is unclear (33). In this study, we found that although the mean FC [and mean -$\log_2$(FC)] were suggestive of responsiveness to diet, the overall variability drove significance >0.1. In part this discrepancy reflects the functional difficulty in data collection (magnetic resonance hardware, technician skill, subject compliance, voxel location). Spectra for comparative analysis of tumor and normal tissue are typically acquired in the same imaging session for a given patient. In our case we compared spectra acquired $\geq$4 wk apart for each subject (each diet condition), and across 40 persons over a period of multiple years. Spectral quality (line widths, water suppression) varied within and especially between subjects. This leads to another confounder of data analysis (accurate measurement of signal in the face of widely variable data quality). Similar variability across and within subjects has been reported (41). In addition, the MRS signal at 3.2 ppm may include contributions from phosphoethanolamine and other phosphomonoesters (40). We also have not accounted for differences in liver fat, because we assumed a uniform 72% water content to standardize the unsuppressed MRS spectra (see the Methods). However, the effects of differential liver fat are minimized by our use of a crossover design, because only within-subject/between-diet changes will affect FC. Overall, therefore, our results suggest that MRS may reflect hepatic choline concentrations, because the mean -$\log_2$(FC) was >0. However, the variability in our particular data set makes its utility marginal.

Implications

Plasma PtdCho concentrations were not significantly decreased after 2 wk of LC compared with HC. Although Zeisel et al. (8) observed decreased plasma PtdCho in response to 3 wk consuming a choline-deficient diet, that study used 0.125 mmol choline/d ($\sim$2.5% AI) for the low-choline arm, whereas the lowest amount used here was 1.3 mmol/d. Plasma choline concentrations do reflect dietary intake and decrease as choline decreases from 5.1 to 1.3 mmol/d, but they do not drop below $\sim$5 μM even at very low amounts (0.5 mmol/d) of choline intake (7, 20). Rather, the primary effect observed is in circulating betaine concentrations, which decreased $\sim$30% as dietary choline decreased from 5.1 to 1.3 mmol/d.

Premenopausal females have lower circulating betaine concentrations than do males or postmenopausal females. One possible contributor to this lower circulating betaine is greater utilization of hepatic betaine as a methyl-donor to form SAM, which is used by phosphatidylethanolamine methyltransferase (PEMT) to methylate phosphatidylethanolamine and produce PtdCho. PEMT activity is induced by estrogen, and this pathway is one of the major consumers of hepatic SAM (17). Whether females with functional polymorphisms in the *PEMT* gene have higher plasma concentrations of betaine is currently unknown. Betaine, unlike choline, is stored in cells, and plasma concentration is not necessarily representative of tissue concentration (42). That said, higher plasma betaine concentrations are correlated with increased fibroblast growth factor 21 concentrations and correlate closely with insulin sensitivity (43). In addition, plasma betaine concentration is inversely correlated with plasma total homocysteine (44, 45), which is implicated in certain cardiovascular diseases (46).

Conclusions

In summary, we report measurements of several choline metabolites as a function of dietary choline intake. Across the intake range studied (1.3–5.1 mmol choline/d, $\sim$25%–100% adult male AI), plasma choline and betaine concentrations were strongly correlated with choline intake amounts, whereas liver tCho measured by single-voxel MRS was weakly correlated. Plasma PtdCho concentrations were not correlated. In light of a study that found plasma PtdCho decreased in response to very low (0.5 mmol/d) choline intake (8), decreased PtdCho biosynthesis may become of clinical concern as choline intake drops to $<$25% of the AI. We also observed stratification by sex status of plasma betaine concentration (premenopausal women $<$ postmenopausal women $<$ men) across the choline intake range studied. Consequently, the health consequences of a choline-deficient diet may depend, in part, on sex status.

The authors' responsibilities were as follows—SHZ: designed the research and had primary responsibility for the final content; JMS, SH, WBF, and DRK: conducted the research; DAH: analyzed the data and wrote the manuscript; JMS, SH, and SHZ: helped edit the manuscript; and all authors:

read and approved the final manuscript. SHZ has an equity interest in SNP Therapeutics, a company that identifies people with certain genetic polymorphisms that are associated with health problems and develops medical foods to treat these problems. He has had grant funding from Balchem Company, a company that makes choline for diet supplements and animal feed, and is on advisory boards for ProTara, ByHeart, and Ingenuity Foods, all companies with an interest in choline relative to their products. All the other authors report no conflicts of interest.

Data Availability

Data described in the article will be made publicly and freely available pending completion of the full study.

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