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Variations in plasma choline and metabolite concentrations in healthy adults

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ABSTRACT

Background: Plasma concentrations of choline and its metabolites might serve as biomarkers for the health outcomes of several pathological states such as cardiovascular disease and cancer. However, information about the reliability of biomarkers of choline status is limited. We investigated biological variations in repeated measures of choline and metabolites in healthy adults to assess them as biomarkers.

Methods: Blood samples were collected after an overnight fast at three-time points 12 days apart from 40 adults (mean age, 33 y; male, $n = 21$). A subset ($n = 19$; [male, $n = 8$]) provided one additional sample after a breakfast meal. Plasma free choline, betaine and dimethylglycine were measured using liquid chromatography-tandem mass spectrometry, and plasma phosphatidylcholine, sphingomyelin and lysophosphatidylcholine were measured using high-performance liquid chromatography.

Results: The biological variations observed for choline and metabolites were ≤ 13% for adult fasting samples. This corresponded to intra-class correlations (ICC) that ranged from 0.593 to 0.770 for fasting values for choline and metabolites. A similar ICC range was also obtained between fasting and post-prandial states. Although most post-prandial concentrations of choline and metabolites were significantly higher ($P < .05$) than fasting, all fell within a calculated reference interval. The participants were correctly classified in tertiles for fasting and post-prandial states for choline (68%) and metabolites (range = 32% phosphatidylcholine and 79% for sphingomyelin).

Conclusions: These findings indicate that biological variations of choline and metabolites are low in healthy adults and values from a single blood sample can be used as a biomarker. However, choosing phosphatidylcholine as a biomarker is less reliable.

1. Introduction

Choline is essential for diverse functions including neurotransmission and lipid metabolism as the precursor of acetylcholine [1] and phospholipids such as phosphatidylcholine and sphingomyelin [2]. Choline can also be oxidized to betaine, an important osmolyte and methyl group donor for homocysteine re-methylation and thus the synthesis of methionine and dimethylglycine [3]. Circulating concentrations of choline and its metabolites are associated with clinical outcomes in humans such as fatty liver disease, cardiovascular disease and cancer [4–8]. Thus, circulating choline and its metabolite concentrations have emerged as biomarkers of risk for various diseases.

Circulating concentrations of biomarkers are often used to monitor nutrient status and assess risk for adverse health outcomes [9]. Typically, only one blood sample is collected per person in clinical and epidemiological studies and to determine the status of choline and its metabolites [6–8]. An important consideration is the reliability of a specific biomarker, including inherent biological variation [10]; that is, possible misclassification based on assessment of a biomarker at a single time point might affect its applicability. Another important factor

Abbreviations: BMI, body mass index; CV, coefficient of variation; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; ICC, intraclass correlation coefficient; LC-MS/MS, liquid chromatography-tandem mass spectrometry; USDA, United States Department of Agriculture.

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is whether blood samples are obtained after fasting or after consuming meals that contain choline [11]. Most protocols used to analyze nutrition-related biomarkers in adults require blood sample collection after an overnight fast. However, this might not be feasible or ethical, particularly for infants, children and pregnant women.

Only a few published studies have described variations in plasma free choline [12–15], however, circulating choline is predominantly present in the form of phosphatidylcholine. Variations in biomarker concentrations over time are crucial to know and can be impacted by various activities associated with sample handling or processing, the analytical process, or the extent of biological variation within individuals [16–18]. Therefore, the principal aim of the present study was to determine variations in plasma concentrations of choline and its associated metabolites derived from blood samples obtained after repeated fasting in healthy adults. The secondary aim was to compare variations between plasma samples obtained after an 8-h fast (fasting) and 4-h after a breakfast meal (post-prandial). This aim was viewed important because free choline concentrations increase in plasma after food intake [19,20].

2. Materials and methods

2.1. Study design and participants

A repeated measures design included three clinical blood collections at 12-day intervals. We recruited 40 healthy adults who understood and spoke English (male, $n = 21$, female = 19; mean age [± SD], 33 ± 12 y; European ethnicity, $n = 24$ [60%]; BMI, 24.9 ± 4.9 kg/m^2) from the general population in Vancouver, Canada. A subset of the participants ($n = 19$, male = 8; mean age [± SD], 34 ± 12 y; European ethnicity, $n = 11$ [58%]; BMI, 25.5 ± 5.0 kg/m^2) were used to complete a protocol that compared fasting and post-prandial plasma concentrations of choline and its metabolites. The exclusion criteria comprised a history of chronic disease, use of prescribed medication, use of dietary supplements containing choline or betaine, consuming a vegan diet, and being a pregnant or lactating woman.

For the first objective, blood samples were drawn during early morning after each of three overnight fasts (> 8 h) at 12-day intervals. Only water was allowed before each of the three blood collections. A power analysis calculation indicated a minimum sample size of 32 participants were required to provide 90% power to detect an intra-class correlation coefficient (ICC) of ICC ≥ 0.75 using three repeated observations per subject with significance at the 5% (two-tailed).

For the second objective, participants provided a post-prandial blood sample 4 h after choosing a breakfast from a cafeteria menu, on a day when a fasting sample had been provided. The composition of the breakfast was not controlled, as the intent was to reproduce a real-life scenario where dietary intake is random. A power analysis calculation indicated a minimum of 19 participants was required to provide 90% power to detect a large effect size (d = 0.80) in group means when comparing two repeated samples, with significance at the 5% (two-tailed).

The University of British Columbia/ Children's & Women's Health Centre of British Columbia Research Ethics Board (UBC C&W REB) reviewed and approved protocols which were in accordance with the guidelines of the Declaration of Helsinki. All recruits provided written informed consent to participation in the study.

2.2. Biochemical analyses

A phlebotomist collected venous blood at each clinical visit into vacutainers coated with EDTA that were immediately placed on ice and transported to the laboratory. Plasma was separated by centrifugation at 2000 × g (15 min; 4 °C) and stored frozen at −80 °C in cryostat tubes. All samples were processed within 20 min from the time of blood collection.

Plasma concentrations of free choline, betaine and dimethylglycine were quantified by isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described [17,21], using a Waters I-class ACQUITY UPLC system connected to a Quattro Micro tandem MS configured with an electrospray source (Waters Corporation, Milford, MA, USA). The LC was equipped with a pre-column (2.1 × 12.1 mm) and a column (2.1 × 150 mm), both Zorbax Rx-SIL with a particle size of 5 µm (Agilent Technologies, Santa Clara, CA, USA). For analysis, aliquots of 50 µL of plasma were transferred to Eppendorf tubes (1.5 mL) containing 10 µL of internal choline d-9, betaine d-9, and dimethylglycine d-6 standards (CDN Isotopes Inc., Pointe-Clair, QC, Canada). Plasma protein was precipitated, and supernatant was mixed with the mobile phase consisting of 19% 15 mM ammonium formate containing 0.1% formic acid and 81% acetonitrile. A standard curve including choline (1.0 to 20.0 µmol/L), betaine (5.0 to 100.0 µmol/L), and dimethylglycine (0.5 to 10.0 µmol/L), and an in-house pooled plasma sample were analyzed in every run to ensure quality assurance. Inter- and intra- assay CV were 3.8% and 2.5% for free choline, 3.5% and 2.2% for betaine, and 3.8% and 2.4% for dimethylglycine, respectively.

Plasma phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine concentrations were quantified using high-performance liquid chromatography (HPLC) as described [22]. Briefly, an Alliance HPLC system was equipped with a 2690 Separations Module (Waters), a 2424 evaporative light scattering detector (Waters), an auto-sampler and a column heater. Lipid classes were separated on columns (25 cm × 4.6 mm) containing YMC-Pack Diol-NP 120 with a particle size of 5 µm (YMP Co. Ltd., Kyoto, Japan). Total lipids were extracted from 250 µL of thawed plasma samples before analysis [23,24] and 30 µL of internal standard (1 mg/mL betulin) was added to allow for quantification. Phospholipid/betulin ratios for standard curves created for each lipid class of interest allowed identification and quantification of each phospholipid eluted and shown on the chromatogram. Inter- and intra- assay CV were 6.5% and 5.0% for phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine, respectively.

2.3. Dietary intake assessment

Dietary choline and betaine intake was estimated at breakfast from a subset of the participants who provided blood samples after consuming a breakfast freely selected from a cafeteria menu. The food items consumed by each participant were specified on the receipt and dietary intake of choline and betaine was estimated using Food Processor Nutrient Analysis Software version 10.14.41 (ESHA Research, Salem, OR USA) and the United States Department of Agriculture (USDA) database on the Choline Content of Common Foods (Version 2) [11].

2.4. Statistical analyses

All data were statistically analyzed using SPSS for Windows version 19 (SPSS Inc. Chicago, IL, US) and Reference Intervals were calculated using MedCalc version 17.9.2 (MedCalc Software, Ostend, Belgium). The significance level was set at $P < .05$ for two-sided tests. Normality of the data was assessed using Kolmogorov-Smirnov tests. If distributions were skewed, data were logarithmically transformed to improve normality before further analysis as appropriate. All data are presented as means ± SD, unless otherwise stated.

The analytical coefficient of variation ($CV_{analytical}$; also referred to as analytical imprecision), for each metabolite, was calculated from the same quality-control plasma pool as:

$$CV_{analytical} = (SD/mean) \times 100.$$

To estimate the intra-individual biological variability for plasma concentrations of choline and its associated metabolites, the total

coefficient of variation (CV_{total}) for sets of three fasting samples collected from each participant was calculated as:

$CV_{total} = (SD/mean) \times 100.$

The intra-individual coefficient of variation ($CV_{intra\text{-}individual}$) was then calculated as:

$CV_{intra-individual} = \sqrt{(CV^2_{total} - CV^2_{analytical})}$ [25].

In addition, variation among the three fasting values for each metabolite was assessed using ICC calculated using 2-way mixed model, single rater type, and consistency definition [26]. Cronbach's alpha was also determined in order to compare the results with those obtained in previously published reports.

Fasting and post-prandial concentrations of plasma choline and metabolites were compared using Student's *t*-tests for paired samples, and associations were assessed using ICC. A 95% Reference Interval and 90% Confidence Interval (CI) was calculated for choline and its associated metabolites in plasma using all fasting samples using the percentile method, according to current standards (CLSI C28-A3) [27]. Whether or not participant ranking by each metabolite concentration changed according to food intake was assessed using Crosstabulation to obtain the percentage of participants correctly classified within the same tertile and Cohen's Kappa statistic, based on a comparison between tertiles of fasting and post-prandial states. The association between dietary choline and betaine intake from breakfast and differences in fasting and post-prandial plasma concentrations of each metabolite was assessed using Pearson's coefficient of correlation and Bonferroni correction for multiple comparisons.

3. Results

Fig. 1 illustrates the concentrations of choline and metabolites in plasmas from each of three overnight fasts per participant at 12-day intervals ($n = 40$). There was not difference in range of mean values for repeated fasting samples free choline (7.0 to 7.3 μmol/L); betaine (37.2 to 38.9 μmol/L); dimethylglycine (2.4 to 2.5/L μmol/L); phosphatidylcholine (125 to 139 mg/dL); sphingomyelin (23.4 to 25.0 mg/dL); and lysophosphatidylcholine (15.5 to 16.5 ± 5.1 mg/dL) (Supplementary Table 1).

Table 1 shows the analytical, total and intra-individual coefficients of variation in plasma concentrations for fasting samples. The intra-assay CV was ≤5% for all metabolites. The CV for total variation ranged from 8.8% for sphingomyelin to 13.5% for dimethylglycine. In terms of intra-individual biological variation, the CV was the lowest for sphingomyelin (6.8%), and the highest for dimethylglycine (13.1%). Reliability, calculated as ICC, ranged from moderate for dimethylglycine (0.593) to strong for betaine (0.770) followed by sphingomyelin (0.719).

Fig. 2 illustrates the choline and metabolite concentrations in plasma from blood samples obtained at fasting and post-prandial states ($n = 19$). Mean (± SD) at fasting and post-prandial states were 6.9 ± 1.7 and 8.3 ± 2.4 μmol/L for free choline; 35.7 ± 7.9 and 42.0 ± 9.7 μmol/L for betaine; 2.4 ± 0.6 and 2.8 ± 0.9 μmol/L for dimethylglycine; 129.1 ± 24.4 and 143.4 ± 37.9 mg/dL for phosphatidylcholine; 24.0 ± 4.4 and 25.6 ± 6.4 mg/dL for sphingomyelin; and 16.8 ± 4.3 and 18.0 ± 4.0 mg/dL for lysophosphatidylcholine (Supplementary Table 2). The post-prandial plasma concentrations of choline and metabolites were significantly higher than those after fasting ($P < .05$), with exception to sphingomyelin ($P = .125$) and lysophosphatidylcholine ($P = .061$).

Table 2 shows the reliability and tertile cross-classification in plasma samples comparing fasting and post-prandial states. For all metabolites, the ICC ranged from moderate to strong, the lowest for phosphatidylcholine (0.655) and the highest for betaine (0.792). The percentage of participants classified correctly (in the same tertile) was 68% for free choline, 79% for betaine, 68% for dimethylglycine, 32%

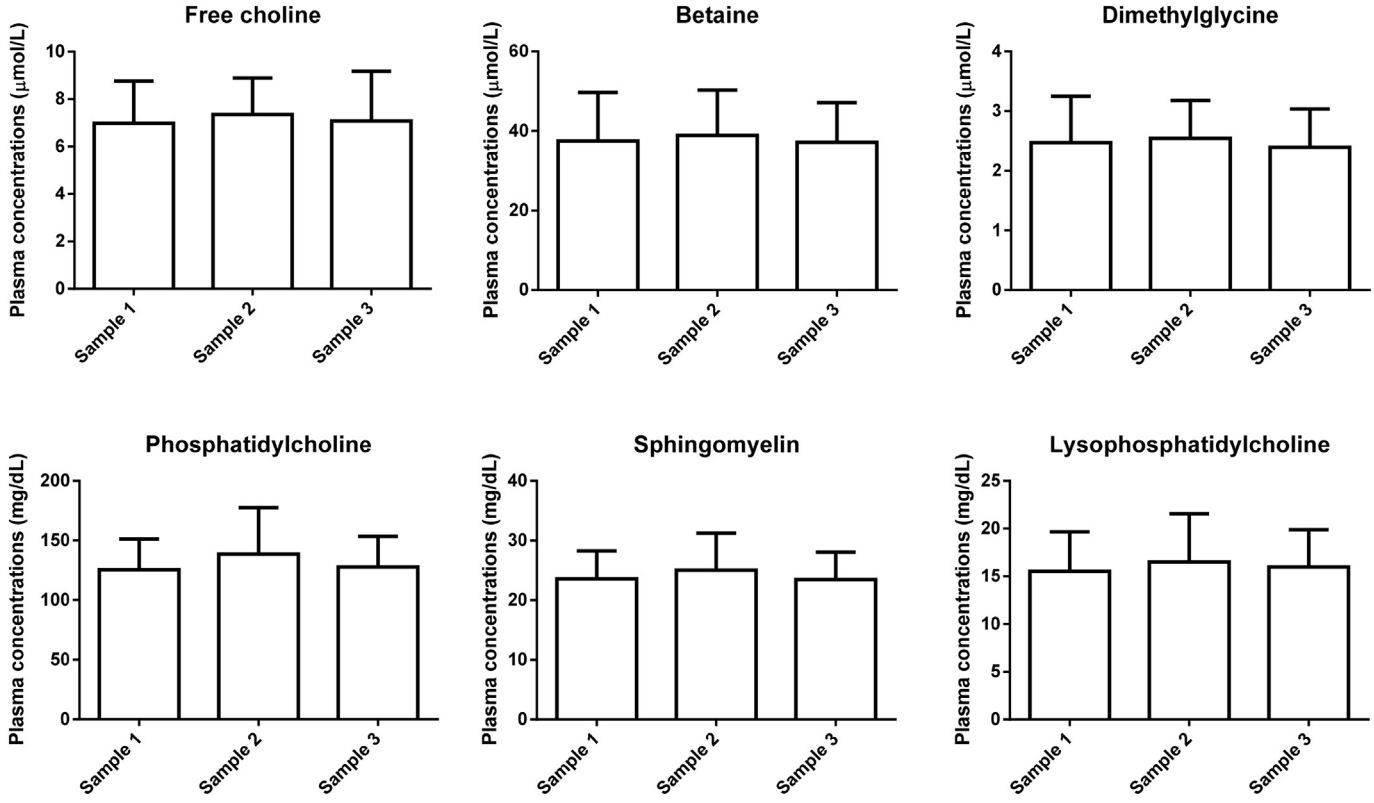

Fig. 1. Concentrations of choline and its metabolites in plasma from repeated fasting samples. Data are presented as mean and SD. Fasting samples (≥ 8 h) from each participant ($n = 40$) were collected three times at 12-day intervals. Concentrations of free choline, betaine, and dimethylglycine were quantified using liquid chromatography-tandem mass spectrometry. Concentrations of phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine were analyzed using high-performance liquid chromatography.

Table 1
Variations in concentrations of choline and its metabolites in plasma from samples collected after fasting for ≥8 h three times at 12-day intervals (n = 40)[a].

	$CV_{analytical}$	CV_{total}	$CV_{intra\text{-}individual}$	Cronbach's alpha	Reliability
Plasma metabolite	(%)	(Mean ± SD, %)	(Mean ± SD, %)		(ICC, 95% CI)
Free choline	2.5	13.3 ± 7.4	12.9 ± 7.6	0.847	0.649 (0.489, 0.780)
Betaine	2.2	12.1 ± 6.9	11.8 ± 7.1	0.910	0.770 (0.649, 0.862)
Dimethylglycine	2.4	13.5 ± 9.4	13.1 ± 9.6	0.814	0.593 (0.420, 0.740)
Phosphatidylcholine	5.0	11.2 ± 9.3	9.2 ± 10.2	0.848	0.650 (0.491, 0.781)
Sphingomyelin	5.0	8.8 ± 8.4	6.8 ± 9.1	0.885	0.719 (0.580, 0.828)
Lysophosphatidylcholine	5.0	12.8 ± 9.6	11.1 ± 10.5	0.870	0.690 (0.541, 0.808)

ICC, intra-class correlation coefficient.
[a] $CV_{analytical}$ was calculated from intra-assay samples, $CV_{intra\text{-}individual}$ was calculated *as* $\sqrt{(CV^2_{total} - CV^2_{analytical})}$.

for phosphatidylcholine, 79% for sphingomyelin, and 53% for lysophosphatidylcholine, and was reflected in the Kappa coefficient obtained (Table 2). Moreover, all post-prandial concentrations were whitin the CI of the lower and upper limits of the Reference Intervals calculated based on the fasting samples (n = 120) (Table 3 and Fig. 3).

Mean dietary intakes of total choline and betaine were 177.7 and 22.5 mg, respectively (Supplementary Table 3). Dietary intakes of total choline consumed at breakfast did not significantly correlate with any of the post-prandial versus fasting differences in plasma concentrations of choline and its associated metabolites (Supplementary Table 4). After adjusting for multiple comparisons none of the correlation between intakes and the difference in plasma concentration remained significant (P > .006).

4. Discussion

We investigated individual variations in concentrations of choline and its metabolites in healthy adults from the general population. Intra-individual variation is defined as random fluctuation around homeostatic set points in individual samples, which indicates the potential usefulness of a biomarker [10]. The reported $CV_{intra\text{-}individual}$ in healthy persons and in diabetic or cardiovascular patients ranges are 12% [14] – 18% [12] for free choline, 14% [12] – 35% [28] for betaine, and 16% [15] – 18% [12] for dimethylglycine. In this study, the $CV_{intra\text{-}individual}$ for plasma free choline, betaine and dimethylglycine based on three samples collected after an overnight fast (≥ 8 h) were 13%, 12%, and 13%, respectively. Thus, our results are in broad agreement with those of previous published studies.

For the fasting samples, reliability, expressed as ICC ranged from moderate-to-strong for plasma free choline, betaine, and dimethylglycine. Our ICC results for free choline are similar to those reported in the general population [14] and also among diabetic patients [13]. In contrast, healthy postmenopausal women [12] and vegans [15] have a

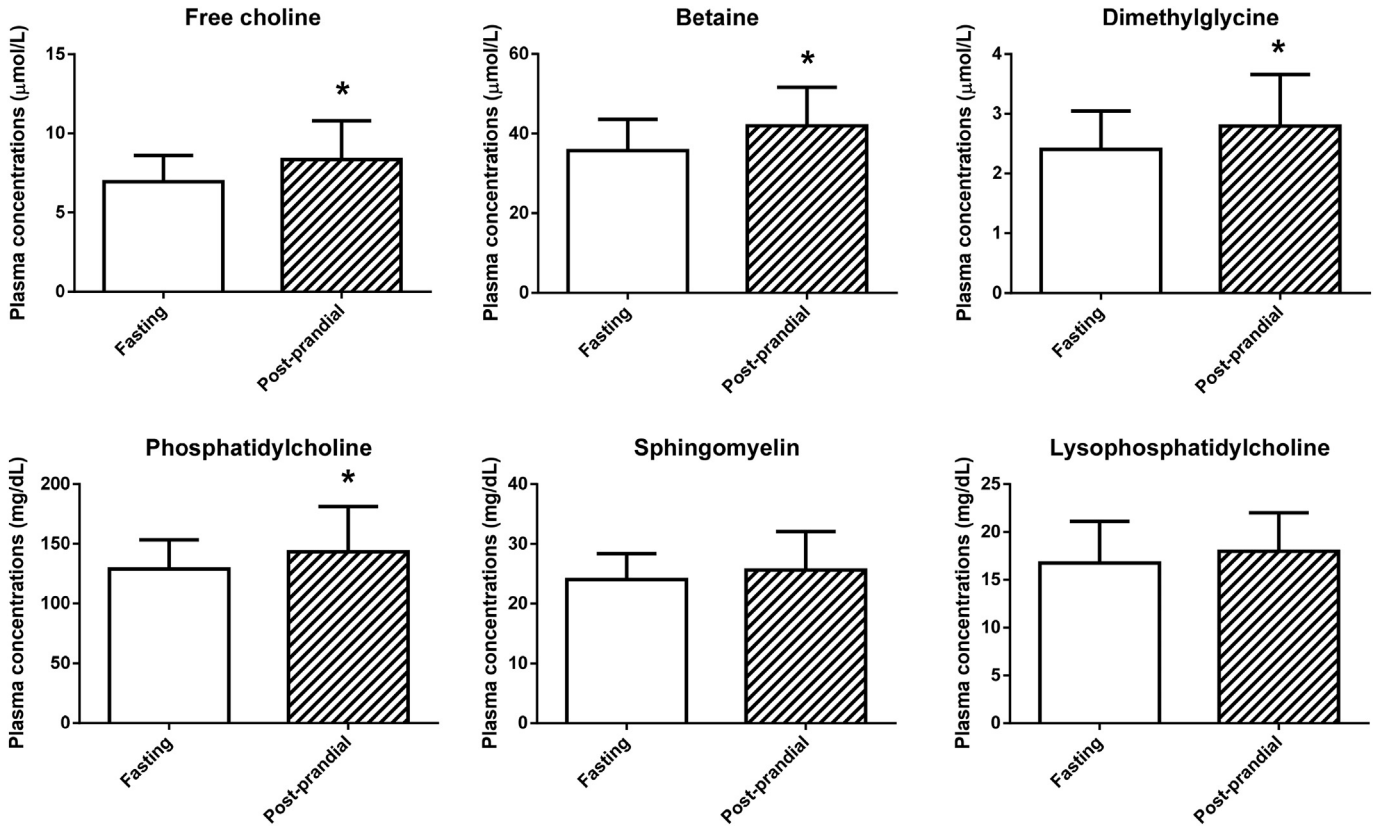

Fig. 2. Concentrations of choline and its metabolites in plasma from fasting and post-prandial samples. Data are presented as mean and SD. Samples were collected in fasting (≥ 8 h) and post-prandial (> 4 h after breakfast) states from a subset of the participants (n = 19). Concentrations of free choline, betaine, and dimethylglycine were quantified using liquid chromatography-tandem mass spectrometry. Concentrations of phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine were analyzed using high-performance liquid chromatography. * Different from fasting concentrations (P < .05).

Table 2
Reliability and tertile cross-classification of choline and its metabolites in plasma samples collected after fasting and post-prandial (n = 19).

	Reliability	Tertile cross-classification (n, %)			Kappa
Plasma metabolite	(ICC, 95% CI)	Same	Adjacent	Opposite	
Free choline	0.739 (0.439, 0.891)	13, 68%	6, 32%	0, 0%	0.525
Betaine	0.792 (0.537, 0.914)	15, 79%	4, 21%	0, 0%	0.683
Dimethylglycine	0.787 (0.528, 0.912)	13, 68%	6, 32%	0, 0%	0.525
Phosphatidylcholine	0.655 (0.298, 0.851)	6, 32%	12, 63%	1, 5%	−0.029
Sphingomyelin	0.696 (0.365, 0.971)	15, 79%	4, 21%	0, 0%	0.683
Lysophosphatidylcholine	0.724 (0.413, 0.884)	10, 53%	9, 42%	1, 5%	0.288

ICC, intra-class correlation.

Table 3
Reference intervals for choline and its metabolites in plasma from samples collected after fasting for ≥8 h (n = 120)[a].

Plasma metabolites	Lower limit (90% CI)	Upper limit (90% CI)
	Concentration (μmol/L)	
Free choline	3.52 (3.30–4.50)	11.99 (10.10–14.50)
Betaine	13.63 (10.70–19.00)	63.19 (57.00–65.00)
Dimethylglycine	1.10 (1.10–1.40)	4.19 (3.60–4.50)
	Concentration (mg/dL)	
Phosphatidylcholine	81.83 (77.90–85.80)	212.34 (178.60–269.60)
Sphingomyelin	15.80 (13.10–16.20)	37.78 (31.90–42.80)
Lysophosphatidylcholine	8.61 (7.40–9.40)	27.07 (24.20–31.00)

[a] Reference Intervals (95%) and 90% Confidence Interval (CI) were calculated using MedCalc, according to current standards (CLSI C28-A3) [27].

much lower ICC value for free choline. The reported ICC for plasma betaine and dimethylglycine [12,15,29] are also similar to our values. The disparity between ICC values for free choline in the present study and those reported from postmenopausal women [12] could be explained in part by the time intervals between blood sample collections. Although fasting samples were collected in both studies, our results are based on three samples obtained over 12-day intervals, compared to only two samples collected between 1 and 2 years apart in the postmenopausal women study [12].

Post-prandial concentrations of choline and metabolites were 7–20% higher at 4 h after breakfast, compared to fasting. Few data are available on how recent food intake can affect plasma concentrations of choline and metabolites. An early study found a small (9%), but significant, increase in plasma free choline one hour after lunch [19], while later studies have shown increases in free choline, betaine, and dimethylglycine concentrations that ranged from 25%–85% in plasma at two and four hours after a meal [17,30–32]. In a controlled feeding study that fed subjects much higher betaine and choline-containing meals [30], reported post-prandial plasma betaine concentrations were much higher compared to our study that was designed to have subjects self-select diets (note that plasma choline was not assessed in the controlled feeding study [30]).

Our findings agree with other studies that have shown a strong positive correlation for betaine concentrations in samples collected

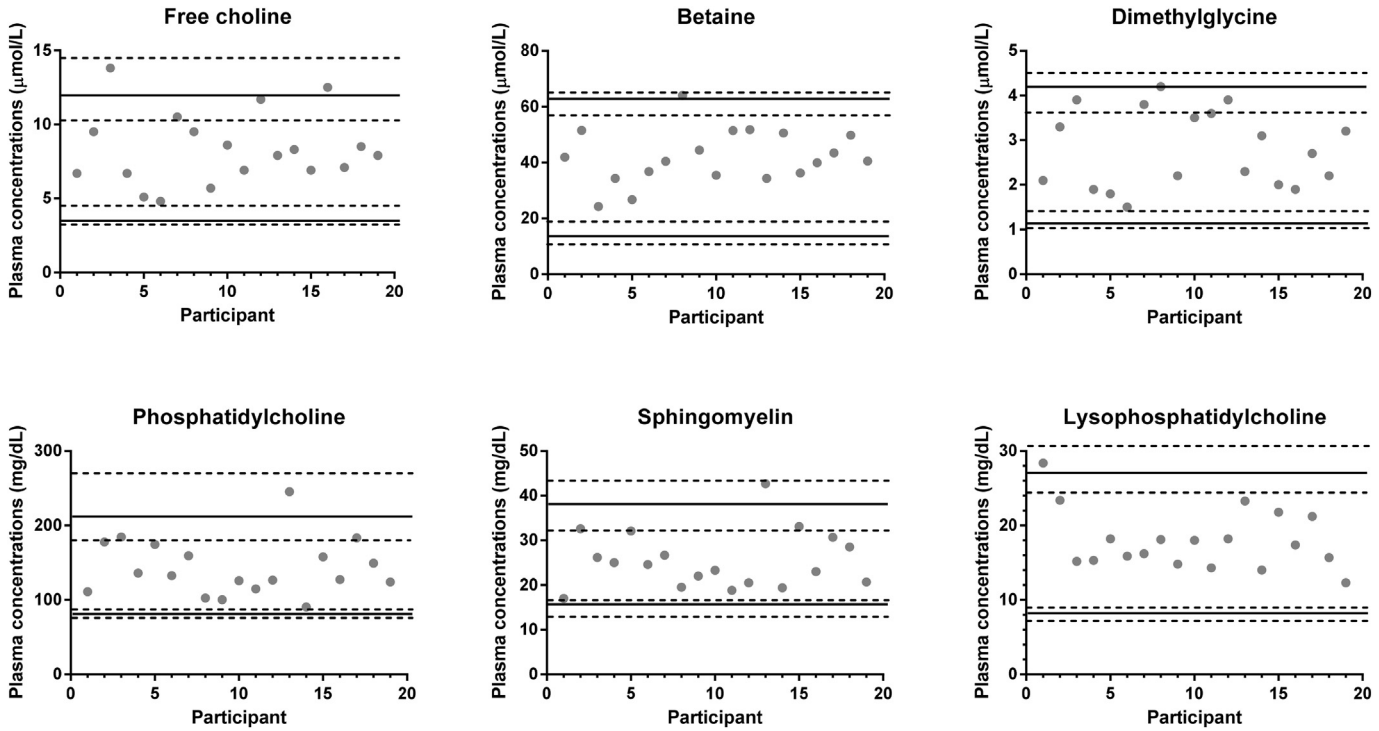

Fig. 3. Concentrations of choline and its metabolites in plasma from post-prandial samples (> 4 h after breakfast) state from a subset of the participants (n = 19) in relation to the Reference Intervals for choline and its metabolites in plasma from samples collected after fasting for ≥8 h (n = 120). Reference Intervals (95%) are presented as solid lines and Confidence Interval (90%) as dash lines were calculated using MedCalc, according to current standards (CLSI C28-A3) [27]. Concentrations of free choline, betaine, and dimethylglycine were quantified using liquid chromatography-tandem mass spectrometry. Concentrations of phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine were analyzed using high-performance liquid chromatography.

after breakfast (2–6 h), compared with those collected after fasting [28]. A positive correlation has also been reported for dimethylglycine concentrations [33]. To our knowledge, there is no information presently available on the intra-individual variability, or reliability of plasma phosphatidylcholine, sphingomyelin, or lysophosphatidylcholine collected in both fasting and post-prandial states.

Although the increases in concentrations of free choline, betaine, dimethylglycine, and phosphatidylcholine after a breakfast meal were significant in this study, the interpretation of our results to establishing estimates of choline status remain to be determined since all of the post-prandial concentrations are within the reference interval calculated for the fasting samples. Moreover, the agreement between fasted and post-prandial states reflects a parallel increase in plasma choline and metabolite concentrations after a recent intake of food. This implies that the tertile classification of these post-prandial concentrations was maintained when compared to fasting values. Moreover, the fact that we report low intra-individual variability, and high ICC values for choline and metabolites suggests the actual cut-off values that define adequate status could be established with further studies that could provide more data on the fasting and post-prandial concentrations from cohorts that vary in ethnicity, age and gender.

The strengths of this study were that we applied standardized protocols to minimize pre-analytical sources of variation such as a consistent time of blood collection, a single phlebotomist, as well as handling and storage of the samples, as these factors can affect accuracy of plasma concentration determinations [18,34,35]. Also, the $CV_{analytical}$ values were low ($\leq$ 5%), as the same analyst quantified plasma concentrations [10]. Moreover, this study is the first to explore the impact of a freely selected breakfast on plasma concentrations of choline and its metabolites, which resembles the reality of free-living, healthy individuals without a controlled dietary intake of choline containing food sources. However, a corresponding limitation is that we did not assess the impact of meals with unusually high intakes of choline and its metabolites. Also, it should be noted that we assessed post-prandial concentrations at only one time point, 4 h after the meal. A final limitation is that we collected blood samples from a relatively small number of self-reported healthy, mostly European individuals. There is some information that intra-individual variation of nutrients could vary according to differences in ethnic groups [36].

5. Conclusions

In summary, analyses of repeated fasting blood samples identified low intra-individual variations in plasma concentrations of choline and its metabolites. Although most post-prandial plasma concentrations of choline and metabolites were higher than with fasting, all values were within the calculated reference interval, and correlations between states were moderate to strong. Therefore, the results presented herein indicate that the status of plasma choline and its associated metabolites can be reliably assessed in healthy adults based on a single blood sample obtained either after an overnight fast (> 8 h) or 4 h post-prandially.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2018.08.002.

Conflicts of interest

None.

Author contributions

AMW and SMI designed the study. AMW and RAD performed the biochemical analyses. AMW, SIB, and DDK interpreted the data and wrote the manuscript. All authors reviewed the manuscript and approved its submission for publication.

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