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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

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