RELATIONSHIP OF CHOLINE AND TRIMETHYLAMINE-*N*-OXIDE INTAKE WITH METABOLIC AND HEALTH OUTCOMES IN HUMANS

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This dissertation is focused on dietary choline and its derivative trimethylamine-*N*-oxide (TMAO). Recent discoveries have implicated circulating TMAO as a candidate risk factor for cardiovascular disease (CVD) among patients, but whether TMAO and dietary choline play a causative role in the disease process remains controversial. This dissertation describes 1) the production of TMAO from its dietary precursors (TMAO, choline and L-carnitine) in healthy individuals, 2) its metabolic fate within the body, and 3) the relationship between dietary choline and high blood pressure, a major risk factor for CVD.

In Chapter 1, a randomized crossover feeding study was conducted where 40 healthy men consumed a test meal consisting of either fish (TMAO), eggs (choline), beef (choline and L-carnitine) or a fruit control. Postprandial blood collected for 6 hours after consumption of the test meal revealed that fish consumption yielded the highest increase in plasma TMAO metabolites among the test meals. Furthermore, production of TMAO following consumption of eggs or beef varied among individuals and correlated with the gut microbiome. The overall findings show that TMAO can be temporarily elevated in healthy individuals following consumption of various foods, especially heart-healthy fish. High variation in plasma TMAO in response to the same

meals indicates metabolic differences among individuals that may be due to other factors such as composition of the gut microbiome.

In Chapter 2, the metabolic fate of dietary TMAO was investigated. Participants enrolled in the feeding study consumed 50 mg deuterium-labeled methyl d9-TMAO (d9-TMAO) in the fruit control arm. We found that d9-TMAO entered circulation within 15 min and that 96% of the tracer was excreted in urine within 24 hours. These results demonstrate rapid absorption of intact TMAO along with its efficient elimination from the human body.

In Chapter 3 the hypothesis that increased choline intake may increase CVD risk through elevated blood pressure was investigated. Using cross-sectional 2007-10 National Health and Nutrition Examination Survey, we examined the relationship of choline intake with blood pressure and prevalence odds of hypertension in a general U.S. population. We found a borderline inverse association between choline intake and odds of hypertension in women but not in men. Furthermore, supplemental choline use by both sexes showed a significant inverse association with hypertension. We concluded that choline intake is not associated with blood pressure, a risk factor of CVD, in this population.

Taken together, evidence in this dissertation does not support the hypothesis that dietary choline increases disease risks by elevating baseline circulating TMAO in healthy adults. More epidemiologic and experimental evidence are still needed to further confirm or dispute this hypothesis.

BIOGRAPHICAL SKETCH

Siraphat, who goes by her nickname Fay, was born on June 20, 1990 in Bangkok, Thailand. She spent her comfortable childhood with her parents and a younger sister in Nonthaburi, a Bangkok suburb. Growing up as an avid reader and diligent student, she attended a top-rank high school in Bangkok and eventually earned a scholarship from the Thai government to study abroad from Bachelor's to Doctoral Degrees in the fields of Food and Nutrition.

In 2008, Siraphat commenced her study in the United States at Pomfret School in Connecticut, where she adjusted to American culture and applied to college. She went on to study at University of California, Davis and graduated Summa Cum Laude with a Bachelor of Science in Food Science. During her time at Davis, Siraphat always enjoyed working with human panelists in a product development laboratory. However, she wanted to help improving people's health rather than developing better products for companies so she decided to study Nutrition for her doctorate. Siraphat came to Cornell in 2013. Her interest in Human Nutrition led her to join the Caudill group, where she had an opportunity to conduct a controlled trial presented in this dissertation.

After completing her study, as part of the scholarship agreement, Siraphat will teach at Food Science Department at Chiang Mai University in Thailand.

To Taesuwan and Patoombal families

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LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BMI	Body mass index
BUN	Blood urea nitrogen
Cr	Creatinine
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DHA	Docosahexaenoic acid
DMA	Dimethylamine
EBA	Ethyl bromoacetate
FMO3	Flavin-containing monooxygenase 3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GGT	Gamma-glutamyl transpeptidase
HDL	High-density lipoprotein
HMRU	Human Research Metabolic Unit
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
MA	Methylamine
NHANES	National Health and Nutrition Examination Survey
OTU	Operational taxonomic unit
PC	Phosphatidylcholine
PCoA	Principal coordinate analysis
PEMT	phosphatidylethanolamine *N*-methyltransferas
RBC	Red blood cell
SBP	Systolic blood pressure
SEM	Standard error of the mean
TMA	Trimethylamine
TMAO	Trimethylamine-*N*-oxide
WBC	White blood cell

INTRODUCTION

OVERVIEW

Choline is a quaternary ammonium cation ($C_5H_{14}NO^+$) that is a part of several metabolites including acetylcholine, glycerophosphocholine, phosphocholine phosphatidylcholine (PC) and sphingomyelin, all of which are integral to the body. Acetylcholine is a neurotransmitter synthesized by cholinergic neurons [1]. Glycerophosphocholine and phosphocholine are major storage forms of choline in cytosol, while PC and sphingomyelin maintain membrane structure and integrity [1]. Sphingomyelin is also found in the myelin sheath surrounding nerve fibers. These choline metabolites are found in diet and/or derived from choline of dietary or endogenous origins.

Although choline can be synthesized *de novo*, the body also relies on choline from food sources. The majority of dietary choline is comprised of PC, which is abundant in liver, eggs, meat and seafood [2]. Consumption of choline-deprived diet leads to increased signs of liver damage, the concern that is the basis of the Dietary Reference Intake for choline [3]. The Adequate Intake, established in 1998, recommends 550 mg/day for adult men and 425 mg/day for women [3]. In addition to liver function, consumption of choline has been associated with favorable outcomes in *in utero* development, cognitive health, cardiovascular disease, and neurodegenerative diseases, among others [1,4].

Choline was recently linked to cardiovascular disease (CVD) as a precursor of trimethylamine-*N*-oxide (TMAO). Using metabolomics approach, Wang *et al.* (2011) identified plasma choline and TMAO as predictors of CVD risk among patients undergoing elective cardiac evaluations [5]. The group further showed that atherosclerosis-prone mice (C57BL/6J *Apoe*$^{-/-}$) supplemented with choline or TMAO had increased plasma TMAO concentrations, aortic atherosclerotic plaques and macrophage foam cell formation compared to chow-fed mice. Subsequent studies in rodents indicated that TMAO may promote atherosclerosis via increased cholesterol uptake by macrophage [5], decreased reverse cholesterol transport [6], enhanced platelet response [7] and prolonged hypertension [8].

Whether circulating TMAO is a cause of CVD and whether dietary choline contributes to CVD are currently debated. A systematic review of studies in cardiac and renal patients concluded that elevated TMAO concentrations were associated with increased risks for all-cause mortality and major adverse cardiovascular events [9]. On the other hand, another systematic review of prospective studies in general populations found no association between dietary choline and incident CVD [10]. This inconsistency may be indicative of other unknown factors and considerations in this diet—disease relationship.

First, TMAO production from dietary choline requires other key metabolic factors. Upon consumption, unabsorbed dietary choline is metabolized by gut microbiota to trimethylamine [5], which is then absorbed via enterohepatic circulation and primarily oxidized to TMAO by hepatic flavin-containing monooxygenases (FMOs) [11]. Both FMOs and gut microbiota have been shown to influence pathways leading to insulin

resistance and atherosclerosis independently of TMAO [12,13]; this could account, at least in part, for the metabolic effect of TMAO.

Second, TMAO is found abundantly in fish and seafood, which are recognized for their cardio-protective benefits [14]. In fact, levels of trimethylamine, a product of TMAO degradation in dead fish, is a universal indicator of fish spoilage [15]. It is unknown how fish consumption impacts circulating TMAO. Hypothetically, fish consumption could increase TMAO concentrations and, according to the proposed adverse effects of TMAO, would increase the risk of heart disease. This paradox suggests that TMAO may be a biomarker of other factors that in turn affect CVD risk, especially when most studies used dietary choline as a microbial-dependent source of TMAO.

Third, the dietary choline/TMAO—CVD relationship may depend on the stage of the disease. Epidemiologic studies on TMAO and CVD endpoints were conducted in patients [9], while studies on dietary choline were population-based [10]. Similarly, mechanistic studies showing the contribution of TMAO to atherosclerosis must be conducted in disease-prone animal models due to their characteristics. Metabolic profiles of animals and humans with complications may also differ from those of their generally healthy counterparts. This difference could create an environment for an adverse dietary choline—disease association. Whether this association exists in a generally healthy population is unclear.

In summary, choline is a semi-essential nutrient that is speculated to contribute to CVD through the production of its metabolite TMAO. Current research findings are inconsistent, and key questions in the TMAO pathway remain unanswered.

AIMS

In this dissertation, the impact of dietary TMAO and its precursors (i.e., choline) on circulating TMAO is studied as well as how dietary TMAO is metabolized in healthy adults. We further investigate the relationship of choline intake with blood pressure as a proxy for CVD risk in a U.S. population.

Aim I: (i) To quantify TMAO response to animal source foods among healthy men and (ii) to investigate how this response correlates with their gut microbiota composition.

Aim II: (i) To quantitatively elucidate the metabolic fate of orally consumed TMAO in healthy men and (ii) to examine the effect of a polymorphism (G472A) in *FMO* isoform 3 coding gene on the metabolic fluxes of TMAO metabolites.

Aim III: To assess the relationship of choline intake with both blood pressure and the prevalence odds of hypertension, using a cross-sectional National Health and Nutrition Examination Survey from 2007 to 2010.

REFERENCES

[1] Zeisel SH, da Costa K-A. Choline: an essential nutrient for public health. Nutr Rev 2009;67:615–23. doi:10.1111/j.1753-4887.2009.00246.x.

[2] Patterson KY, Bhagwat S a, Williams JR, Howe JC, Holden JM, Zeisel SH, et al. USDA Database for the Choline Content of Common Foods Release Two. US Dep Agric Agric Res Serv Beltsv Hum Nutr Res Cent Nutr Data Lab 2008:1–37.

[3] Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins and C. 12, Choline. Diet. Ref. Intakes Thiamin, Riboflavin, Niacin, Vitam. B6, Folate, Vitam. B12, Pantothenic Acid, Biot. Choline, Washington, DC: National Academies Press (US); 1998.

[4] Zeisel SH. Choline: critical role during fetal development and dietary requirements in adults. Annu Rev Nutr 2006;26:229–50. doi:10.1146/annurev.nutr.26.061505.111156.

[5] Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 2011;472:57–63. doi:10.1038/nature09922.

[6] Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat Med 2013;19:576–85. doi:10.1038/nm.3145.

[7] Zhu W, Gregory JC, Org E, Buffa JA, Gupta N, Wang Z, et al. Gut Microbial Metabolite TMAO Enhances Platelet Hyperreactivity and Thrombosis Risk. Cell 2016;165:111–24. doi:10.1016/j.cell.2016.02.011.

[8] Ufnal M, Jazwiec R, Dadlez M, Drapala A, Sikora M, Skrzypecki J. Trimethylamine-N-oxide: a carnitine-derived metabolite that prolongs the hypertensive effect of angiotensin II in rats. Can J Cardiol 2014;30:1700–5. doi:10.1016/j.cjca.2014.09.010.

[9] Heianza Y, Ma W, Manson JE, Rexrode KM, Qi L. Gut Microbiota Metabolites and Risk of Major Adverse Cardiovascular Disease Events and Death: A Systematic Review and Meta- Analysis of Prospective Studies. J Am Heart Assoc 2017;6:e004947. doi:10.1161/JAHA.116.004947.

[10] Meyer K, Shea J. Dietary Choline and Betaine and Risk of CVD: A Systematic Review and Meta-Analysis of Prospective Studies. Nutrients 2017;9:711. doi:10.3390/nu9070711.

[11] Krueger SK, Williams DE. Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism. Pharmacol Ther 2005;106:357–87. doi:10.1016/j.pharmthera.2005.01.001.

[12] Tremaroli V, Bäckhed F. Functional interactions between the gut microbiota and host metabolism. Nature 2012;489:242–9. doi:10.1038/nature11552.

[13] Miao J, Ling A V., Manthena P V., Gearing ME, Graham MJ, Crooke RM, et al. Flavin-containing monooxygenase 3 as a potential player in diabetes-associated atherosclerosis. Nat Commun 2015;6:6498. doi:10.1038/ncomms7498.

[14] Kris-Etherton PM. Fish Consumption, Fish Oil, Omega-3 Fatty Acids, and Cardiovascular Disease. Circulation 2002;106:2747–57. doi:10.1161/01.CIR.0000038493.65177.94.

[15] Ghaly a E, Dave D, Budge S, Brooks MS. Fish Spoilage Mechanisms and Preservation Techniques : Review Department of Process Engineering and Applied Science , Dalhousie University Halifax , Nova Scotia , Canada. Am J Appl Sci 2010;7:859–77. doi:10.3844/ajassp.2010.859.877.

CHAPTER 1

TRIMETHYLAMINE-N-OXIDE (TMAO) RESPONSE TO ANIMAL SOURCE FOODS VARIES AMONG HEALTHY YOUNG MEN AND IS INFLUENCED BY THEIR GUT MICROBIOTA COMPOSITION: A RANDOMIZED CONTROLLED TRIAL

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ABSTRACT

Scope: Trimethylamine-*N*-oxide (TMAO), a metabolite linked to the gut microbiota, is associated with excess risk of heart disease. We hypothesized that: (i) TMAO response to animal source foods would vary among healthy men; and that (ii) this response would be modified by their gut microbiome.

Methods and Results: A crossover feeding trial in healthy young men (n=40) was conducted with meals containing TMAO (fish), its dietary precursors, choline (eggs) and carnitine (beef), and a fruit control. Fish yielded higher circulating and urinary concentrations TMAO (46-62-times; P<0.0001), trimethylamine (8-14-times; P<0.0001) and dimethylamine (4-6-times; P<0.0001) than eggs, beef or the fruit control. Circulating TMAO concentrations were increased within 15-min of fish consumption suggesting that dietary TMAO can be absorbed without processing by gut microbes. Analysis of 16S rRNA genes indicated that high-TMAO producers

(≥20% increase in urinary TMAO in response to eggs and beef) had more Firmicutes than Bacteroidetes (P=0.04) and less gut microbiota diversity (P=0.03).

Conclusion: Consumption of fish yielded substantially greater increases in circulating TMAO than eggs or beef. The higher Firmicutes to Bacteroidetes enrichment among men exhibiting a greater response to dietary TMAO precursor intake indicates that TMAO production is a function of individual differences in the gut microbiome.

1.1 Introduction

Trimethylamine-*N*-oxide (TMAO) is a naturally occurring small organic dietary compound that is abundant in fish, or can be generated from other nutrients including choline (abundant in eggs) and carnitine (abundant in beef). Upon consumption of foods containing TMAO or its dietary precursors (choline and carnitine), it is proposed that gut bacteria generate trimethylamine (TMA; volatile, fish-odor compound) [1] and to a lesser extent, dimethylamine (DMA) with subsequent absorption via enterohepatic circulation [2]. The majority of TMA is enzymatically converted to the odorless TMAO metabolite in a reversible reaction catalyzed by vitamin B2-dependent flavin-containing monooxygenase 3 (FMO 3) in the liver. Loss-of-function mutations in this enzyme give rise to the rare genetic disorder known as trimethylaminuria or “the fish odor syndrome” [3]. Alternatively, TMA can be demethylated to DMA and methylamine (MA).

The significance of TMAO in physiologic processes received early attention due to its function as an osmolyte [4]. In addition, farmers recognized the benefits of adding TMAO to animal feed for the purposes of improving growth, carcass quality and nutrient digestibility [5]. More recently, TMAO has emerged as a predictive risk factor for heart disease in cardiac patients [6, 7] and colorectal cancer among post-menopausal women [8]. However, very little is known about the effects of animal source foods on TMAO generation, absorption and elimination in healthy adults. Further, although there is an apparent role of the gut microbes in TMAO production, the gut microbiota composition in relation to TMAO production in humans has not been determined.

Therefore, we aimed to test the hypotheses that: (i) TMAO response to animal source foods would vary among healthy men; and that (ii) this response would be modified by their gut microbiota composition. To achieve these aims, we conducted a crossover feeding trial whereby healthy young men consumed study meals of fish, eggs, meat and a fruit control in random order with one-week washout periods. TMAO biomarker response to the study meals was quantified in plasma and urine, while gut microbiota composition was assessed in feces. Because the *FMO3* G472A genetic variant may adversely influence TMA conversion to TMAO [9], men were genotyped for this polymorphism, which was considered as a covariate in the statistical models.

1.2 Materials and methods

1.2.1 Participants

Healthy men (n = 40) aged 21-50 y with a body mass index (BMI) of 20-29.9 kg/m^2 were recruited by flyers posted around the Cornell University's Ithaca campus and surrounding area from May to June 2014. A sample size of n = 40 was determined from a power analysis of a within-subject design to detect a 10% difference of plasma TMAO concentration at $\alpha < 0.05$ and $\beta = 0.8$. Participants were screened by use of a blood chemistry profile, cell count and health history questionnaire. The exclusion criteria were men of age > 50 y, BMI $\geq$ 30 kg/m^2, women, vegetarians, smokers and individuals with gastrointestinal diseases or complaints, chronic illnesses or other metabolic diseases (including trimethylaminuria), abnormal blood chemistry values indicative of organ dysfunctions, and those taking nutritional supplements, antibiotics or probiotics within 2 months of recruitment. Written informed consent was obtained from all participants, and the protocol was approved by the Institutional Review Board for Human Study Participants at Cornell University (Protocol ID#: 1403004534). This trial was registered at clinicaltrials.gov as NCT02558673.

1.2.2 Study design

A randomized, controlled crossover design with four arms comprised of study meals representing animal sources of TMAO and a fruit control was used in this study. **Figure 1.1A** depicts the participant flow throughout the study and the study design. The study meals were (i) eggs (3 whole hard-boiled; Wegmans); (ii) beef (6 ounces Philly-Gourmet Beef Patties, 100% Pure; Tops); (iii) fish (6 ounces cod fillet; Tops)]

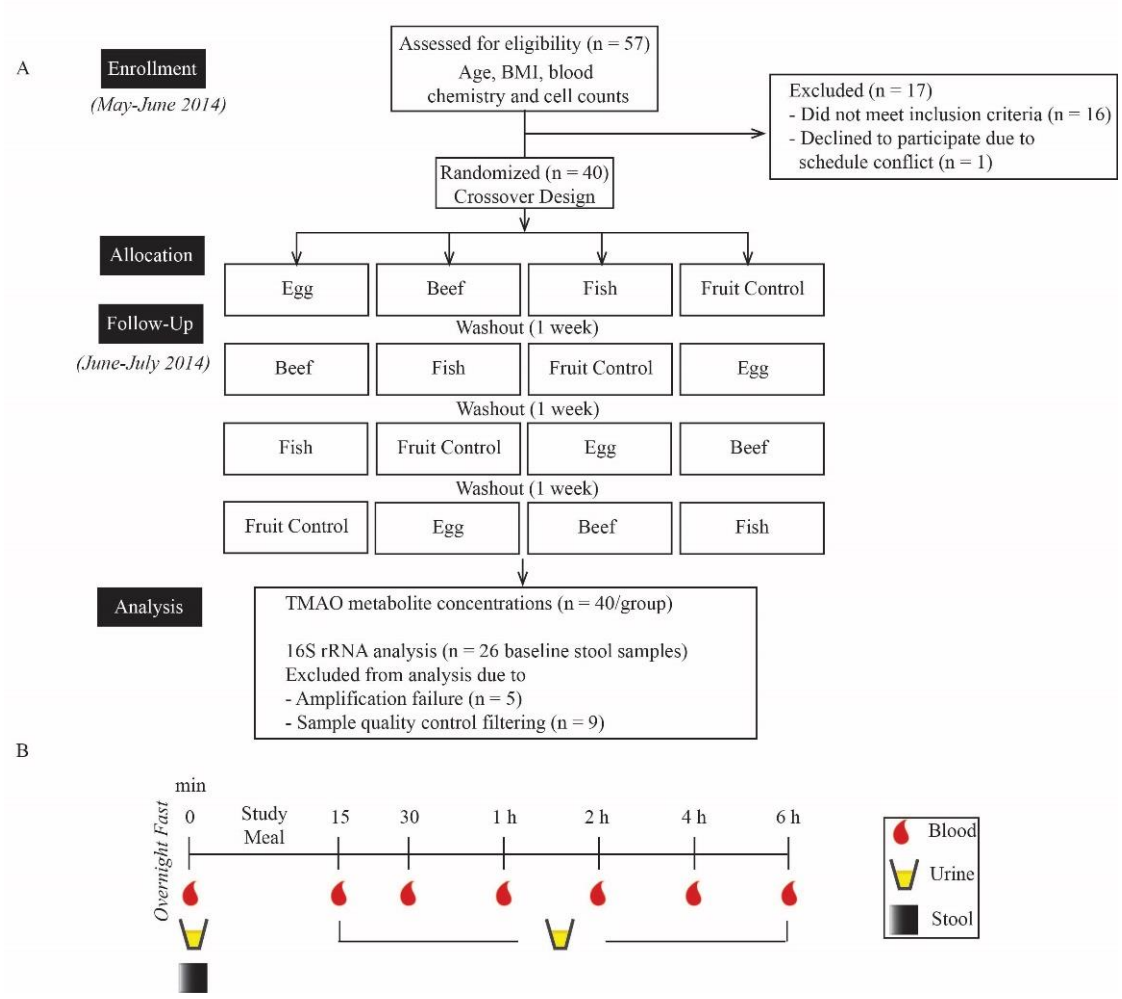

Figure 1.1 A schematic of the participant flow throughout the study and the study design (A) and the study protocol (B)

and (iv) the fruit control (2 single-serve packages of Mott's natural applesauce; Tops), and were administered in commonly consumed servings. The order of study meals for each participant was assigned by a study investigator using random number generator (random.org), and each meal was administered in a single day separated by a 1-week washout period. All meals were prepared in the Human Metabolic Research Unit (HMRU) kitchen at Cornell University on the morning of testing.

1.2.3 Protocol

The study procedure is illustrated in Figure 1.1B. After an overnight fast (10-h), participants arrived at the HMRU between 0700 and 1000 for each of the four visits. The day prior to the study session, participants were advised to avoid consumption of grapefruit juice and indole-containing vegetables (i.e., broccoli, Brussel sprouts, cabbage, cauliflower, kale and bok choy) as these foods can decrease FMO3 enzyme activity and alter TMAO metabolism [2]. Participants were also asked to maintain their normal routine, including exercise, and eat a similar meal the night before each of the four sessions.

At the beginning of each session, participants completed a 24-h dietary recall to assess compliance to the grapefruit juice and indole food restriction. If participants reported consumption of these foods, or significant deviations from their normal routine, the session was rescheduled. A baseline blood sample was obtained by a phlebotomist using a standard venipuncture procedure, and participants collected their baseline urine sample.

Participants then consumed a randomly allocated study meal with one cup of water within a 15-min period. Following the consumption of the study meal, serial blood samples were obtained at 15, 30-min, 1, 2, 4 and 6-h, and each participant collected their urine samples into bottles throughout the 6-h study period which were subsequently pooled. At 4.5-h, participants were provided a fixed snack (i.e., applesauce) and water. Throughout each study session, participants refrained from

eating and drinking foods and beverages (other than water) outside those provided by study personnel.

1.2.4 Sample collection

At the screening visit, fasting serum and whole blood were obtained for blood chemistry profiles and complete cell counts, respectively, using a previously described method [10]. During the feeding trials, plasma and buffy coat were obtained for metabolite measurements [11, 12] and *FMO3* G472A (rs 2266782) genotyping.

Urine was self-collected in wide-mouth specimen containers (120 mL; Thermo Scientific) at study-baseline, and in wide-mouth polyethylene bottles (1 L; Nalgene) during the 6-h period, and kept on ice. Urine volumes were recorded separately for study-baseline and the 6-h study period. In addition, prior to consumption of the fruit control, participants collected stool (complete bowel movement) in a bag provided by study personnel, and delivered to the HMRU in a thermo-insulated bag with ice packs. All samples were de-identified, distributed among several storage vials and stored at -80 °C for further analyses.

1.2.5 Analytical methodology

1.2.5.1 Blood chemistry profiles and complete cell counts

High-density lipoprotein (HDL), low-density lipoprotein (LDL), lipids, protein, creatinine (Cr), bilirubin, alkaline phosphatase (ALP), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT) and blood urea nitrogen (BUN) concentrations were measured

in serum collected at screening by the Dimension Xpand chemistry analyzer (Siemens Healthcare Diagnostic).

White blood cells (WBC), lymphocytes, monocytes, granulocytes and red blood cells (RBC) were measured in whole blood collected at screening by the Act diff 2 hematology analyzer (Beckman Coulter).

1.2.5.2 TMAO metabolite measurements

TMAO, TMA, DMA and MA were measured in plasma, urine and study meals by liquid chromatography (LC)-tandem mass spectrometry MS/MS as described by Johnson *et al.* [13] with modifications to measure all of the methylamine metabolites in a single run. Samples were run in batches with each batch containing all time points and study meals. Study meal total choline (sum of free choline, glycerophosphocholine, phosphocholine, phosphatidylcholine, sphingomyelin), betaine and carnitine contents were quantified by the methods of Koc *et al.* [14] and Holm *et al.* [15] with modifications [16]. All meals were quantified in a single run.

1.2.5.2.1 Sample preparation

For plasma metabolites, 100 µL plasma was mixed with 200 µL acetonitrile (ACN) and 10 µL internal standard, and centrifuged at 20,817 x g for 5 min at room temperature. Stock solutions for the internal standard were prepared by mixing d3-MA hydrochloride (Sigma-Aldrich), d6-DMA hydrochloride (Sigma-Aldrich), *NG*-methyl-*L*-arginine acetate (Sigma-Aldrich), $^{13}C_3$-TMA hydrochloride (Isotec), d3-betaine ammonium chloride (CDN Isotopes), *L*-carnitine-d3 hydrochloride (CDN Isotopes), d13-choline chloride (CDN Isotopes), $^{13}C_3$-TMAO (Toronto Research Chemicals Inc),

methyl-^{13}C *L*-methionine (Cambridge Isotope Laboratories) in methanol:water (1:1). The supernatant was transferred to a vial with a disposable glass insert and 2 µL concentrated NH_4OH and 30 µL ethyl bromoacetate (EBA; 20 mg/mL in ACN) were added. After incubation at room temperature for 45 min, the solution was diluted 1:1 with a mixture of water:ACN:CH_2O_2 (1:1:0.0005) prior to injection into the LC-MS/MS system. The calibration points were prepared from stock solutions to yield the following final concentrations (all in nmol/mL): TMAO (0.04-400), TMA (0.002-0.3), DMA and choline (0.2-30), betaine (0.4-200), carnitine (0.2-200).

For urine metabolites, 50 µL urine was mixed with 10 µL internal standard, 2 µL concentrated NH_4OH and 60 µL EBA (20 mg/mL in ACN). After incubation at room temperature for 45 min, the solution was diluted 7:1 with a mixture of water:ACN:CH_2O_2 (1:1:0.0005) and injected into the LC-MS/MS system. The calibration points were prepared from stock solutions to yield the following final concentrations (all in nmol/mL): TMAO and DMA (40-8000), TMA (0.1-20), MA (2-400), and choline, betaine and carnitine (2-400). Metabolite concentrations in urine were adjusted for Cr, which was measured using the Dimension Xpand chemistry analyzer (Siemens Healthcare Diagnostic) in the Human Nutritional Chemistry Service Laboratory at Cornell University.

For food, 40 mg food homogenate (study meal homogenized in 0.1 M sterilized potassium phosphate buffer) was mixed with 150 µL of 0.1% CH_2O_2 in ACN and 10 µL of internal standard. The mix was centrifuged at 20,817 x g for 10 min at 4 °C followed by transfer of the supernatant into a vial with a disposable glass insert. Concentrated NH_4OH (2 µL) and 30 µL EBA were added to the supernatant and

incubated at room temperature for 45 min. The solution was then diluted 1:1 with a mixture of water:ACN:CH_2O_2 (1:1:0.0005) prior to injection into the LC-MS/MS system. The calibration points were prepared from stock solutions to yield the following final concentrations (all in nmol/extract): TMAO, betaine and carnitine (0.3-80), TMA and DMA (0.15-40), MA (0.75-200) and choline (1.5-400).

1.2.5.2.2 LC-MS/MS Analyses

Metabolites in urine and study meals were measured using the Accela HPLC system TSQ Quantum Access mass spectrometer (ThermoElectron Corp), while those in plasma were measured using the Surveyor HPLC system TSQ Quantum Ultra mass spectrometer. The injection volume was 10 µL, and the column and autosampler temperatures were at 25°C and 5 °C, respectively. Metabolites were separated on a Prevail silica column (150 x 2.1 mm, 5 µm; Grace) with a matching guard column (7.5 x 2.1 mm, 5 µm). The mobile phase consisted of ACN and 0.1% formic acid in 0.15 mmol/L ammonium acetate in water (CH_2O_2-NH_4OAc) with the following gradient: 81% ACN, 19% CH_2O_2-NH_4OAc at 0 min with a flow rate of 200 µL/min, 81% ACN, 19% CH_2O_2-NH_4OAc at 8 min with a flow rate of 500 µL/min, then linear gradient to 65% ACN, 35% CH_2O_2-NH_4OAc at 10 min with a flow rate of 500 µL/min, 65% ACN, 35% CH_2O_2-NH_4OAc at 13 min with a flow rate of 400 µL/min, then from 13 to 15-min linear gradient back to 81% ACN, 19% CH_2O_2-NH_4OAc, and from 15 to 20 min the column was equilibrated to original conditions with a flow rate of 500 µL/min.

Electrospray ionization in the positive ion mode and multiple-reaction monitoring were used to detect the following transitions: m/z 76.3 → 58.4 for TMAO and m/z 162.1 → 60.1 for carnitine. TMA, DMA and MA were derivatized with EBA and the transitions were as follows: m/z 146.1 → 118.1 for TMA-EBA, m/z 132.1 → 58.1 for DMA-EBA and m/z 118.1 → 44.3 for MA-EBA.

Metabolite and food concentrations were determined relative to standard curves which were prepared by mixing various amounts of TMAO, TMA, DMA and MA with water. The intra-assay CV for plasma and urine was 2% and 1% for TMAO, 17% and 4% for TMA, 2% and 2% for DMA, and ND and 8% for MA, whereas the inter-assay CV for plasma and urine was 6% and 5% for TMAO, 38% and 8% for TMA, 6% and 6% for DMA, and ND and 49% for MA based on in-house control duplicates (n = 3 with differing TMAO concentrations for plasma; n = 2 with differing TMAO concentrations for urine). The relatively low precision for TMA and MA represents the volatility and/or low abundance of these compounds.

1.2.5.3 Genotyping

DNA was extracted according to the manufacturer's protocol (DNeasy Tissue kit, Qiagen) from buffy coat. The *FMO3* G472A (rs 2266782) variant [9] was determined using a commercially available fluorescent Taqman probe kit (ThermoFisher Scientific) on the LightCycler® 480 real-time RT-PCR instrument (Roche) as previously described [17].

1.2.5.4 16S rRNA gene sequencing

Genomic DNA was extracted from ~100 mg of stool using the PowerSoil High-Throughput DNA Isolation Kit (MoBio Laboratories) with bead beating according to the manufacturer's protocol. Bacterial 16S rRNA genes were PCR amplified for the V4 hypervariable region using the 515F and 806R primers as previously described [18] and sequences found at the Earth Microbiome Project (http://www.earthmicrobiome.org/emp-standard-protocols/16s/). PCR reactions consisted of Hot Master PCR mix (Five Prime), 10-100 ng DNA template and 10 μM of each primer, with initial denaturation at 94 °C for 3 min followed by 25 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 60 s, extension at 72 °C for 90 s, and final extension at 72 °C for 10 min. Duplicate PCR reactions were performed for each sample, and were combined and purified using magnetic beads (Mag-Bind EZPure, Omega Bio-Tek). Purified PCR amplicons were quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). Amplicons were then combined into a single tube with a final concentration of 11 ng/μL. Of the 40 stool samples, 5 samples failed to amplify, thus 35 samples of DNA were sequenced paired-end on the Illumina MiSeq platform at Cornell Biotechnology Resource Center Genomics Facility.

1.2.6 16S rRNA gene sequence analyses

Matching paired-end sequences were merged using the fastq-join command and demultiplexed in the open-source software package Quantitative Insights Into Microbial Ecology (QIIME) version 1.8.0 [19]. Quality filtering removed sequences containing low-quality reads (Phred quality score ≤ 25), ambiguous bases, primer

mismatches, uncorrectable barcodes and lengths < 200 bp. Of the 35 sequenced samples, 9 samples did not meet the quality control filtering, therefore 26 samples were used for the gut microbiome analyses. De novo operational taxonomic unit (OTU) picking was performed using the UCLUST algorithm. Representative OTU sequences were aligned using PyNAST with 80% identity and were taxonomically classified using Ribosomal Database Project (RDP) classifier at 97% identity. A phylogenetic tree was built using FastTree. Rarefaction was performed at 60,000 sequences per sample to calculate α-diversity (within sample diversity) using Faith's phylogenetic diversity, Chao 1 and Observed Species, as well as jack-knifed β-diversity (between sample diversity) using the unweighted UniFrac distance matrices for principal coordinates analysis (PCoA). A heatmap was created from the log abundance of all genera using the R package.

1.2.7 Statistical analyses

Statistical analyses were conducted in SAS (Version 9.3, SAS Institute). Two-way repeated measures analysis of variance (ANOVA) using the PROC MIXED model procedure determined the effect of study meal, time and study meal-by-time interaction on metabolite concentrations in plasma and urine. The covariates, age, BMI, study session order and genotype (*FMO3* G472A), that did not reach a significance of $P \leq 0.05$ were removed from the model in a stepwise manner. A significant interaction effect was followed by one-way repeated measures ANOVA and Tukey-Kramer post-hoc test. Participants were categorized as high-TMAO producers or low-TMAO producers based on the median excretion of TMAO (20%) following

the consumption of the egg and beef meals. After sample sequencing and data quality filtering, the gut microbiota composition was compared between high-TMAO producers (n = 11) and low-TMAO producers (n = 15) using False Discovery Rate (FDR)-corrected ANOVA and nonparametric anosim statistical method within QIIME. Unpaired t-test was used to assess differences in the baseline characteristics of the TMAO response groups. Significant differences were reported at $P < 0.05$ with a FDR at 95%. All data are expressed as means ± SEM.

1.3 Results

1.3.1 Participant characteristics and baseline metabolite concentrations

Forty healthy men had a mean age of 27.8 ± 1.0 y, BMI of 24.2 ± 0.4 kg/m^2, and serum blood chemistry and blood cell counts within the normal range (Table 1.1). Thirty-five percent of the participants were homozygous wild-type GG genotype for *FMO3* G472A, 55% were heterozygous GA and 10% were homozygous variant AA, which is consistent with the distribution observed in the general population [20]. TMAO and its derivatives did not differ across study meals.

1.3.2 Study meal TMAO content (see Table 1.2)

1.3.2.1 TMAO and its derivatives

The fish meal contained 650 times more TMAO, 200 times more TMA and 1600 times more DMA compared to the egg and beef meals ($P < 0.0001$), whereas the fruit did not show any detectable levels of these metabolites. Food MA did not differ among the study meals. Total choline concentration was 125 times higher in eggs and

Table 1.1 Participant characteristics and baseline measures for all participants (n = 40).

Participant Characteristics	
Age (y)	27.8 ± 1.0
BMI (kg/m^2)	24.2 ± 0.4
Genotype	
GG %	35
GA %	55
AA %	10
Blood Chemistry Concentrations (all serum)	
HDL (mg/dL)	55 ± 2
LDL (mg/dL)	117 ± 5
Cholesterol (mg/dL)	170 ± 6
Triglycerides (mg/dL)	67 ± 5
Total bilirubin (mg/dL)	0.8 ± 0.0
Direct bilirubin (mg/dL)	0.2 ± 0.0
ALP (U/L)	75 ± 3
Cr (mg/dL)	1.1 ± 0.0
CK (U/L)	156 ± 10
LDH (U/L)	157 ± 3
Amylase (U/L)	57 ± 3
Lipase (U/L)	157 ± 8
AST (U/L)	21 ± 1
ALT (U/L)	33 ± 2
GGT (U/L)	32 ± 2
Total protein (g/dL)	7.6 ± 0.1
Albumin (g/dL)	4.3 ± 0.0
BUN (mg/dL)	15 ± 1
Cell Counts	
WBC ($\times 10^3/\mu$L)	5.6 ± 0.2

Lymphocytes ($\times 10^3/\mu L$)	1.9 ± 0.1
Monocytes ($\times 10^3/\mu L$)	0.2 ± 0.0
Granulocytes ($\times 10^3/\mu L$)	3.5 ± 0.2
RBC ($\times 10^3/\mu L$)	5.2 ± 0.1
TMAO (nmol/mL)	3.3 ± 0.2
TMA (pmol/mL)	18.1 ± 0.8
DMA (nmol/mL)	1.9 ± 0.0
MA (nmol/mL)	ND
Urinary Methylamine Excretion	
TMAO (nmol/mmol Cr)	26.6 ± 1.0
TMA (nmol/mmol Cr)	0.1 ± 0.0
DMA (nmol/mmol Cr)	27.0 ± 0.3
MA (nmol/mmol Cr)	4.2 ± 0.4

Abbreviations: BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ALP, alkaline phosphatase; Cr, creatinine; CK, creatine kinase; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; BUN, blood urea nitrogen; WBC, white blood cell; RBC, red blood cell; TMAO, trimethylamine-*N*-oxide; TMA, trimethylamine; DMA, dimethylamine; MA, methylamine. Baseline plasma and urine levels (averaged across all four sessions) of TMAO and methylamine derivatives TMA, DMA and MA. ND denotes not detectable. Urinary concentrations were adjusted by urinary Cr. Values are mean ± SEM, n = 40.

38 times higher in beef and fish ($P < 0.0001$) compared to the fruit control. Food betaine content was 27 times higher in beef and fish compared to egg and fruit meals ($P < 0.0001$). Carnitine was highest ($P < 0.0001$) in beef followed by fish and then eggs, and was not detected in the fruit control.

Table 1.2 Food concentrations of trimethylamine-*N*-oxide (TMAO) and methylamine derivatives trimethylamine (TMA), dimethylamine (DMA) and methylamine (MA) as well as total choline (sum of free choline, glycerophosphocholine, phosphocholine, phosphatidylcholine, sphingomyelin), betaine and carnitine in egg, beef, fish and fruit study meals. ND denotes not detectable. Values within rows with different letter superscripts are significantly different by one-way ANOVA, Tukey-Kramer post-hoc test. NS denotes not significant. Values are mean ± SEM, each meal with 5 replicates.

Food content (mg)	**Fruit**	**Egg**	**Beef**	**Fish**	**P value**
TMAO	-	0.8 ± 0.1^{a}	0.9 ± 0.1^{a}	528.9 ± 9.4^{b}	< 0.0001
TMA	-	0.0 ± 0.0^{a}	0.1 ± 0.0^{a}	5.1 ± 0.7^{b}	< 0.0001
DMA	-	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	58.5 ± 0.2^{b}	< 0.0001
MA	-	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	NS
Total Choline	3.8 ± 0.1^{a}	478.8 ± 15.7^{c}	132.3 ± 7.0^{b}	161.2 ± 0.6^{b}	< 0.0001
Carnitine	-	0.0 ± 0.0^{a}	71.0 ± 3.0^{c}	7.5 ± 0.3^{b}	< 0.0001
Betaine	0.0 ± 0.0^{a}	0.9 ± 0.0^{a}	12.7 ± 0.3^{b}	12.4 ± 0.2^{b}	< 0.0001

1.3.3 TMAO biomarker response to the study meals

1.3.3.1 Circulating concentrations of TMAO and methylamine derivatives

Study meal and time interacted ($P < 0.0001$) to influence plasma concentrations of TMAO and its methylamine derivatives **(Figure 1.2**). As compared to egg, beef and fruit control, fish consumption yielded plasma concentrations that were 48-62 times higher for TMAO ($P < 0.0001$), 8-14 times higher for TMA ($P < 0.0001$) and 4-5 times higher for DMA ($P < 0.0001$), all of which peaked at 2-h and remained elevated until the end of the 6-h study period. MA concentrations were not detectable in plasma. The

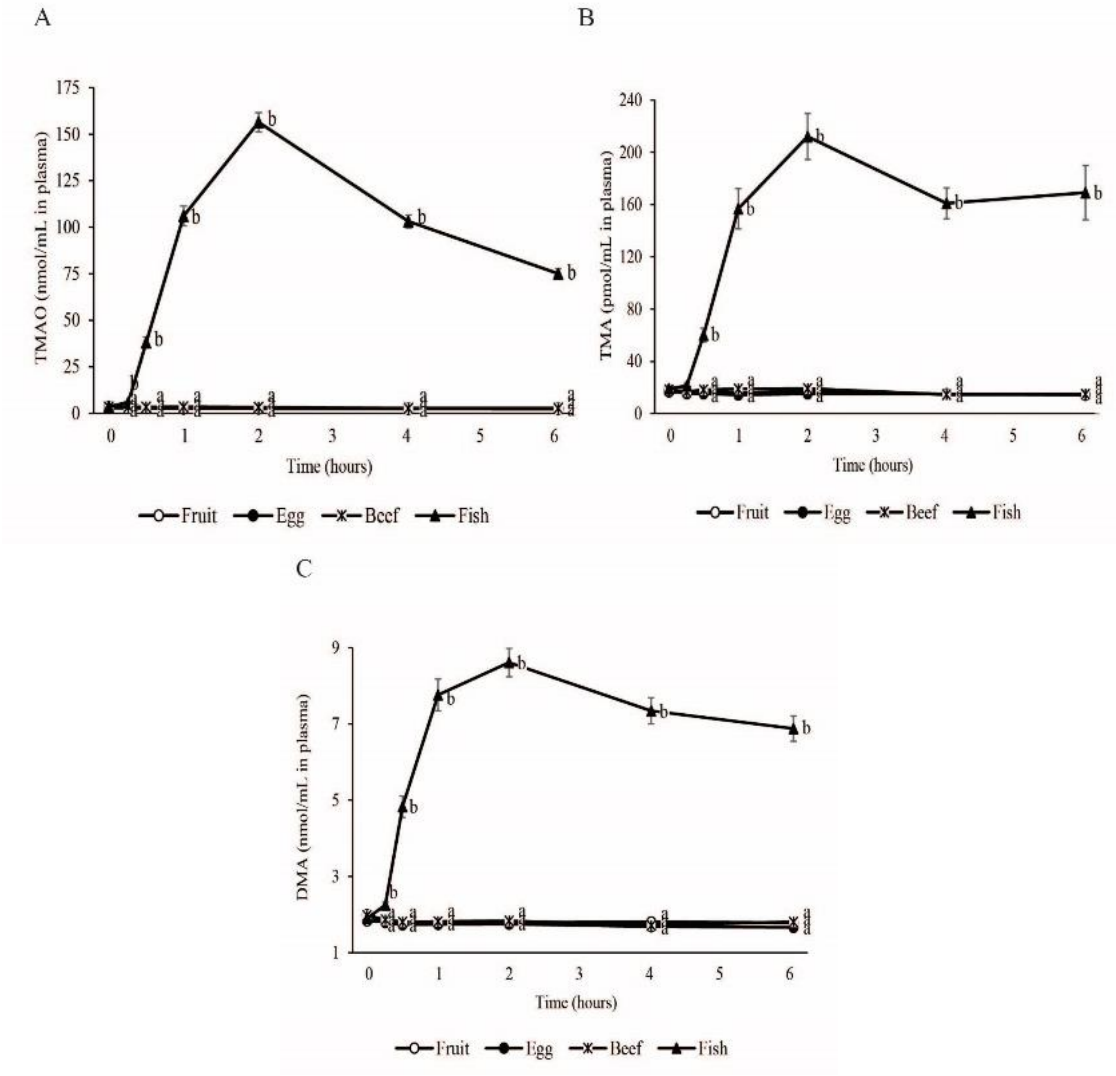

Figure 1.2 Effects of the study meals on plasma concentrations of trimethylamine-*N*-oxide (TMAO) (A), trimethylamine (TMA) (B) and dimethylamine (DMA) (C) across the 6-h study period. Different letter superscripts show a significant effect of study meal at each time point (one-way ANOVA, Tukey-Kramer post-hoc test). Values are mean ± SEM, n = 40 per study meal.

effect of FMO3 G472A on TMAO biomarker response was significant therefore this genotype was used as a covariate.

Similar to plasma levels, study meal interacted with time ($P < 0.005$) to influence urinary TMAO and methylamine derivative concentrations (**Table 1.3**). As compared to egg, beef and fruit control, fish consumption resulted in urinary excretions that were 46-51 times higher for TMAO ($P < 0.0001$), 9-12 times higher for TMA ($P < 0.0001$) and 6 times higher for DMA ($P < 0.0001$) as compared to egg, beef and fruit control during the 6-h study period ($P < 0.0001$).

Because of the substantial TMAO response following consumption of the fish study meal, we were unable to detect differences in TMAO response among the egg, beef and fruit study meals. Thus, we assessed TMAO response for eggs, beef and fruit separately from fish, and compared the response to study-baseline (**Figure 1.3**). A greater increase in plasma TMAO at each time point was detected for eggs and beef compared to the fruit control ($P < 0.05$). Likewise, the change in urinary TMAO excretion across the 6-h study period was greater following the consumption of eggs and beef compared to the fruit control ($P = 0.03$).

Notably, the individual variations in urinary TMAO response after the consumption of eggs and beef ranged from -30% to 270% **(Figure 1.4**). We therefore stratified our participants into high-TMAO producers (n = 11; those with ≥20% i ncrease in urinary TMAO in response to eggs and beef) versus low-TMAO producers (n = 15; those with <20% increase in urinary TMAO in response to eggs and beef) for the microbiome analyses (see next section). We also assessed for differences in the baseline characteristics of the TMAO response groups but none were detected (see Supplementary Table 1.1).

Table 1.3 Effects of the study meals on urinary concentrations of trimethylamine-N-oxide (TMAO) and the methylamine derivatives trimethylamine (TMA), dimethylamine (DMA) and methylamine (MA) adjusted for creatinine (Cr) at study-baseline (0 min) and across the 6-h study period. Two-way ANOVA showed a significant study meal-by-time interaction ($P < 0.005$). One-way ANOVA, Tukey-Kramer post-hoc test showed a significant effect of diet for pooled study urine across 6-h indicated by different letter superscripts. NS denotes not significant. Values are mean ± SEM, n = 40 per study meal.

(nmol/mmol Cr in urine)	**Fruit**	**Egg**	**Beef**	**Fish**	**P value**
TMAO					
0 min	28.4 ± 2.1	24.5 ± 1.9	26.0 ± 2.5	27.9 ± 1.8	NS
Study (0-6 h)	29.0 ± 1.8[a]	32.2 ± 2.7[a]	31.9 ± 3.0[a]	1486.9 ± 57.4[b]	P < 0.0001
TMA					
0 min	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	NS
Study (0-6 h)	0.2 ± 0.1[a]	0.1 ± 0.0[a]	0.1 ± 0.0[a]	1.7 ± 0.6[b]	P < 0.0001
DMA					
0 min	27 ± 1	27 ± 1	27 ± 1	27 ± 1	NS
Study (0-6 h)	30 ± 1[a]	31 ± 1[a]	28 ± 1[a]	180 ± 9[b]	P < 0.0001
MA					
0 min	3.8 ± 0.5	3.3 ± 0.4	5.5 ± 1.2	4.0 ± 0.4	NS
Study (0-6 h)	4.6 ± 0.4	6.2 ± 0.8	5.9 ± 0.7	6.2 ± 0.6	NS

1.3.4 Gut microbiota composition

To address whether microbiota composition was a determinant of TMAO response, we obtained a one-time baseline stool sample from each participant. Of the 40 stool samples, 26 samples were used in the analyses after amplification and quality control yielding 6,770,441 high-quality gene sequences with average sequences lengths (mean ± standard deviation) of 254 ± 10. High-TMAO producers had lower α-diversity

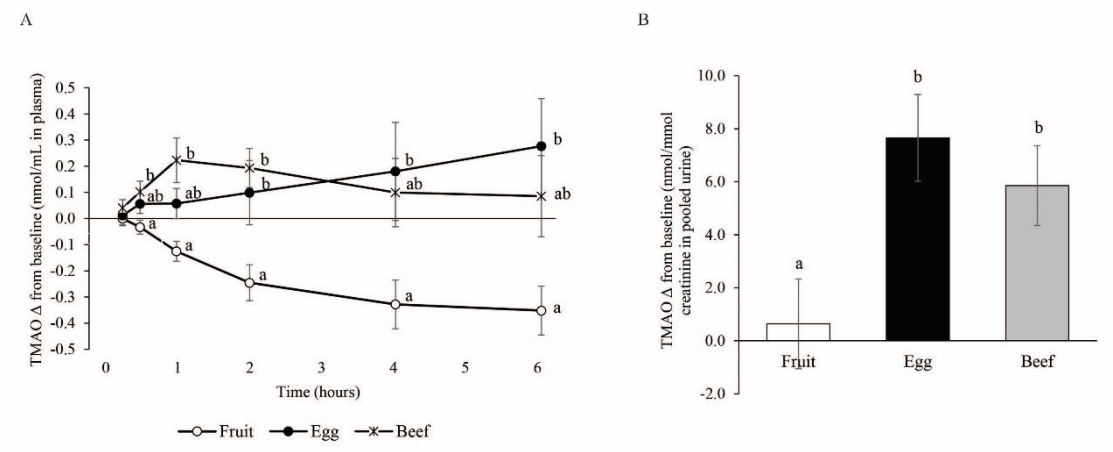

Figure 1.3 Effects of the study meals (eggs, beef and fruit only) on trimethylamine-*N*-oxide (TMAO) concentrations in serially-collected plasma across time (A) and pooled urine (B). Data are expressed as changes from study-baseline (0 min). Different letter superscripts show a significant effect of study meal (one-way ANOVA, Tukey-Kramer post-hoc test). Values are mean ± SEM, n = 40 per study meal.

(within-individual) measure than low-TMAO producers (P = 0.03 with 999 permutations by Monte carlo method; **Figure 1.5**). PCoA analysis of the unweighted UniFrac distances and relative abundance of the bacteria showed distinct bacterial profiles between low-TMAO producers and high-TMAO producers (P = 0.04, R^2 = 0.11 with 999 permutations by anosim method; **Figure 1.6**).

We then visualized gut microbiota differences between high-TMAO producers and low-TMAO producers using a heatmap (**Figure 1.7**). A table of representative OTUs is included as Supplementary Table 1.2 (https://onlinelibrary.wiley.com/doi/10.1002/mnfr.201600324). High-TMAO producers had 58.1% Firmicutes to 32.6% Bacteroidetes (~2:1 Firmicutes:Bacteroidetes) whereas low-TMAO producers had 47.7% Firmicutes to

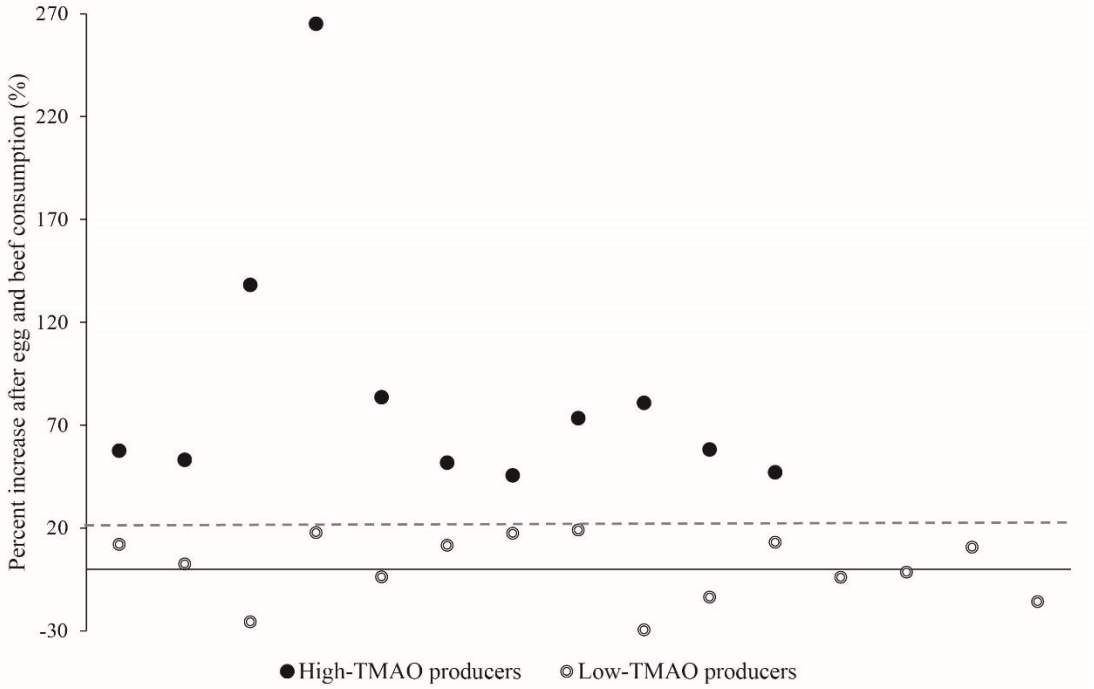

Figure 1.4 Percent variation of urinary trimethylamine-*N*-oxide (TMAO) concentration after the consumption of egg and beef study meals. The urinary TMAO response was averaged between egg and beef consumption. The variation ranged from -30% to 270% among individuals. The participants were stratified into high-TMAO producers (n = 11) shown as black circles and defined as having ≥20% increase in urinary TMAO concentrations after egg and beef consumption, versus low-TMAO producers (n = 15) in white circles and defined as having <20% increase in urinary TMAO concentrations.

47.2% Bacteroidetes (1:1 Firmicutes:Bacteroidetes). High-TMAO producers were represented by Clostridiales within the Firmicutes phylum of which were Bacteroidaceae and Prevotellaceae. The Archaea phylum was represented in the low-TMAO producers but absent in the high-TMAO producers.

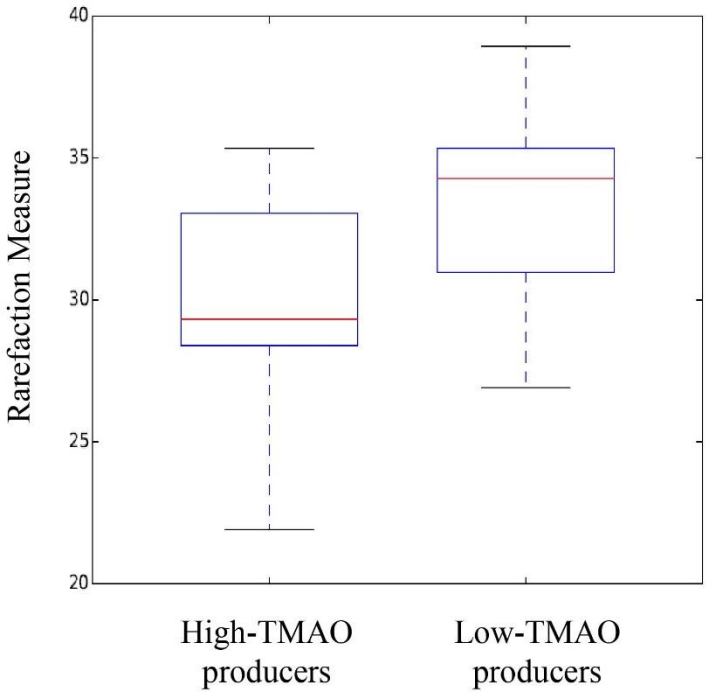

Figure 1.5 Within-individual difference shown as box-plots (mean ± standard deviation) for the 16S rRNA sequence data from baseline stool samples of healthy male participants (n = 26). Alpha diversity metrics were computed for rarefied operational taxonomic unit (OTU) at maximum sampling depth of 60,000 sequences per sample. High-trimethylamine-*N*-oxide (TMAO) producers (n = 11) were defined as having ≥20% increase in urinary TMAO concentrations after egg and beef consumption, whereas low-TMAO producers (n = 15) were defined as having <20% increase in urinary TMAO concentrations. P = 0.03 with 999 permutations by Monte carlo method.

1.4 Discussion

This study sought to advance understanding of the effects of animal source foods on TMAO production, and to determine whether this response was influenced by an individual's gut microbiome. Three main findings emerged: (i) fish consumption yielded several-fold higher quantities of TMAO metabolites than either eggs or beef;

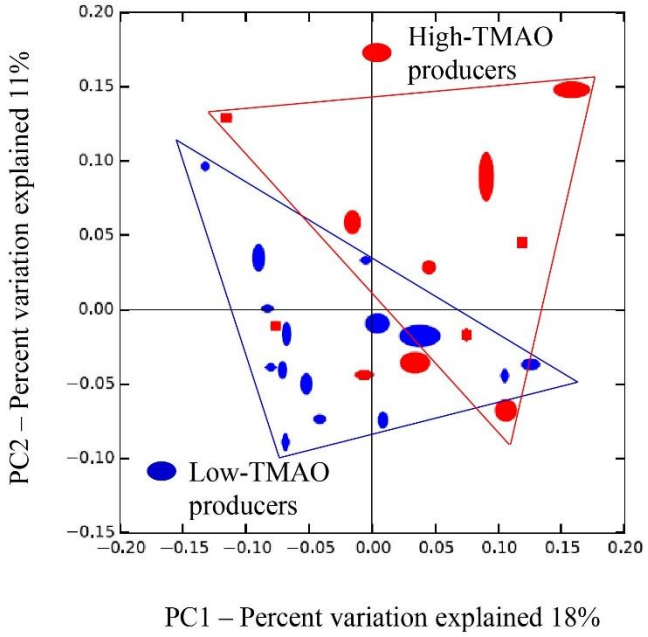

Figure 1.6 Principal coordinates analysis (PCoA) of the unweighted UniFrac distances for the 16S rRNA sequence data from baseline stool samples of healthy male participants (n = 26). Beta diversity metrics (between-individual differences) were jackknifed by repeatedly re-sampling at 60,000 sequences per sample. Axes show the percent variation explained by the principal components (PCs). High-trimethylamine-*N*-oxide (TMAO) producers (n = 11) were defined as having ≥20% increase in urinary TMAO concentrations after egg and beef consumption, whereas low-TMAO producers (n = 15) were defined as having <20% increase in urinary TMAO concentrations. The size of the symbols indicates operational taxonomic unit (OTU) clustering in the microbiomes with red points representing those from high-TMAO producers and the blue points from low-TMAO producers. A distinct grouping of the gut microbiota profiles is indicated by the red triangle outline for high-TMAO producers versus the blue triangle outline for low-TMAO producers. $P = 0.04$, $R^2 = 0.11$ with 999 permutations by anosim method.

(ii) dietary TMAO can be absorbed intact without processing by gut microbes; and (iii) high-TMAO producers (≥20% increase in urinary TMAO in response to eggs and beef) had more Firmicutes than Bacteroidetes and a less diverse gut microbiome.

Figure 7

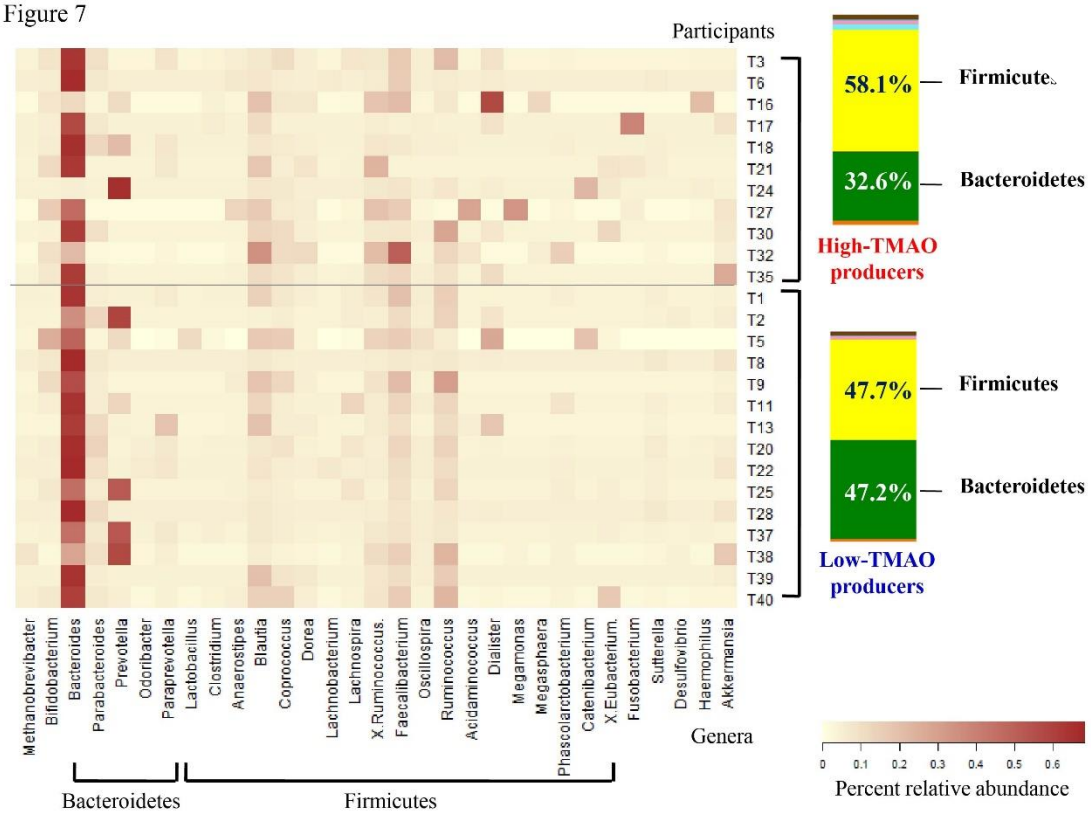

Figure 1.7 Heatmap of relative operational taxonomic unit (OTU) abundances for the 16S rRNA sequence data from baseline stool samples of male participants (n = 26) to differentiate between high-trimethylamine-*N*-oxide (TMAO) producers (n = 11; those having ≥20% increase in urinary TMAO concentrations after egg and beef consumption) versus low-TMAO producers (n = 15; those having <20% increase in urinary TMAO concentrations). The taxonomic assignment of each OTU represents the gut microbiota at the genus level which is grouped into the phylum level as indicated with black square brackets. The color key corresponds to percent relative abundance of the gut microbiota of OTU at each expression level.

1.4.1 Fish consumption yields the highest concentrations of plasma and urinary TMAO concentrations compared to all other study meals

Fish consumption yielded ~50 times higher circulating concentrations of TMAO than either eggs or beef which is consistent with previous reports of substantially higher urinary TMAO concentrations [21, 22] following the consumption of fish compared to meat, dairy, fruits, vegetables or grain. Notably, plasma TMAO was elevated within 15 minutes of fish consumption indicating that the absorption of dietary TMAO may occur without processing to TMA by the gut microbes as previously proposed [23]. The sustained elevation in circulating TMAO in response to fish consumption, despite substantial urinary excretion, suggests that a portion of the TMAO is retained by the body possibly due to its functions as an osmolyte [4] and protein stabilizer [24]. Other methylamine derivatives influenced by diet, albeit to a lesser extent than TMAO, included TMA and DMA, both of which showed higher levels in response to fish versus eggs and beef.

In light of the recent proposed role of TMAO as a causative agent for cardiovascular disease [7, 23], and a recent report of higher serum TMAO concentrations and accelerated aortic plaque formation in apoE null mice with increased fish intake [25], some researchers have advocated for the restriction of animal source foods that raise circulating TMAO concentrations [7]. However, these animal source foods are enriched in nutrients that are required for optimal health [26], and fish consumption is well-known for its cardio-protective attributes in humans [27]. Moreover, circulating TMAO and carnitine concentrations were inversely associated or showed no association with the development of chronic diseases [28, 29]. Thus,

caution is warranted when proposing dietary recommendations that restrict the intakes of animal source foods because of their TMAO-raising characteristics.

1.4.2 TMAO response to dietary precursors may be a biomarker of the gut microbiota composition

Gut microbes are known to participate in modulating TMAO response to its dietary precursors and potentially chronic disease susceptibility [6, 23, 30]. However, TMAO response is highly variable and it is unclear whether this variation arises from individual differences in gut microbiota composition. To address this question, we used urinary TMAO excretion to stratify our participants into "high" (≥20% increase) or "low" (<20% increase) TMAO producers, and examined differences in the gut microbiota composition between the two groups.

Although differences in the gut microbiota were subtle at the individual OTU level, high-TMAO producers were characterized by enriched ratios of Firmicutes to Bacteroidetes compared to low-TMAO producers (Figure 1.4), which is consistent with previous reports that TMAO production potential is detected in Firmicutes but absent in Bacteroidetes [31]. Furthermore, low-TMAO producers had more *archaea Methanobrevibacter*, which has been proposed to deplete host TMA levels [32]. Lower α- diversity (within-individual difference) was also observed among high-TMAO producers indicating that TMAO production may be driven by a select set of bacteria (e.g., Firmicutes).

Distinct gut microbiota profiles between high-TMAO compared to low-TMAO producers in response to its dietary precursors indicate that circulating TMAO may be

a biomarker of the gut microbiota. As such, higher circulating concentrations of TMAO in a disease versus non-disease state may reflect differences in gut microbe composition, rather than indicating a causative role of TMAO in the disease process. A greater ratio of Firmicutes to Bacteroidetes has previously been associated with increased risk of obesity and metabolic syndrome [33], and this gut microbiota characterization may also be linked to atherosclerosis-associated dysbiosis. The significance of these findings is that circulating TMAO may be used to identify individuals with microbiota profiles that increase disease susceptibility, and thereby inform the development of dietary and pharmaceutical strategies aimed at increasing gut microbiota diversity and restoring the symbiotic relationship between the gut microbes and their host.

1.5 Study limitations and future directions

This was a short-term feeding study that focused on advancing current understanding of TMAO metabolism in healthy male adults. As such, the findings may not reflect long-term effects of the diet and may not be generalizable to other segments of the population including women and those with health conditions. In addition, pre-disease clinical endpoints, such as markers of inflammation, were not evaluated. More studies are needed to address these important issues and evaluate the clinical utility of lowering circulating concentrations of TMAO as a means to improve human health.

1.6 Conclusion

Consumption of fish, which is high in TMAO, yielded substantially greater increases in circulating TMAO than eggs or beef, which contain high amounts of dietary TMAO precursors. The rapid rise in circulating TMAO in response to fish consumption demonstrated that the absorption of intact dietary TMAO occurs in a manner that is independent of the gut microbes. The higher Firmicutes to Bacteroidetes microbial enrichment among men exhibiting greater response to dietary TMAO precursor intake indicates that circulating TMAO can reflect a person's microbiota which may in turn modulate their risk of disease.

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AUTHOR CONTRIBUTIONS

CEC contributed to the study design, collected the data, conducted the statistical analyses, interpreted the data and prepared the manuscript. ST contributed to the study design, collected the data, and assisted in the statistical analyses and data interpretation. OVM, EB and JY provided technical assistance and contributed to the data collection. NFT assisted in the data collection and study meal metabolite measurements. JLS provided technical assistance for the 16S rRNA gene sequencing. MAC conceived the study, contributed to the data interpretation and critically reviewed the manuscript. All authors read and approved the final manuscript.

REFERENCES

[1] Barrett, E. L., Kwan, H. S., Bacterial reduction of trimethylamine oxide. *Annu Rev Microbiol* 1985, *39*, 131-149.

[2] Bain, M. A., Fornasini, G., Evans, A. M., Trimethylamine: metabolic, pharmacokinetic and safety aspects. *Curr Drug Metab* 2005, *6*, 227-240.

[3] Al-Waiz, M., Mitchell, S. C., Idle, J. R., Smith, R. L., The relative importance of N-oxidation and N-demethylation in the metabolism of trimethylamine in man. *Toxicology* 1987, *43*, 117-121.

[4] Lidbury, I., Murrell, J. C., Chen, Y., Trimethylamine N-oxide metabolism by abundant marine heterotrophic bacteria. *Proc Natl Acad Sci U S A* 2014, *111*, 2710-2715.

[5] Overland, M., Rorvik, K. A., Skrede, A., Effect of trimethylamine oxide and betaine in swine diets on growth performance, carcass characteristics, nutrient digestibility, and sensory quality of pork. *J Anim Sci* 1999, *77*, 2143-2153.

[6] Wang, Z., Klipfell, E., Bennett, B. J., Koeth, R., *et al.*, Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature* 2011, *472*, 57-63.

[7] Tang, W. H., Wang, Z., Levison, B. S., Koeth, R. A., *et al.*, Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. *N Engl J Med* 2013, *368*, 1575-1584.

[8] Bae, S., Ulrich, C. M., Neuhouser, M. L., Malysheva, O., *et al.*, Plasma choline metabolites and colorectal cancer risk in the Women's Health Initiative Observational Study. *Cancer Res* 2014, *74*, 7442-7452.

[9] Turkanoglu Ozcelik, A., Can Demirdogen, B., Demirkaya, S., Adali, O., Flavin containing monooxygenase 3 genetic polymorphisms Glu158Lys and Glu308Gly and their relation to ischemic stroke. *Gene* 2013, *521*, 116-121.

[10] Yan, J., Jiang, X., West, A. A., Perry, C. A., *et al.*, Maternal choline intake modulates maternal and fetal biomarkers of choline metabolism in humans. *Am J Clin Nutr* 2012, *95*, 1060-1071.

[11] Guinotte, C. L., Burns, M. G., Axume, J. A., Hata, H., *et al.*, Methylenetetrahydrofolate reductase 677C-->T variant modulates folate status response to controlled folate intakes in young women. *J Nutr* 2003, *133*, 1272-1280.

[12] Solis, C., Veenema, K., Ivanov, A. A., Tran, S., *et al.*, Folate intake at RDA levels is inadequate for Mexican American men with the methylenetetrahydrofolate reductase 677TT genotype. *J Nutr* 2008, *138*, 67-72.

[13] Johnson, D. W., A flow injection electrospray ionization tandem mass spectrometric method for the simultaneous measurement of trimethylamine and trimethylamine N-oxide in urine. *J Mass Spectrom* 2008, *43*, 495-499.

[14] Koc, H., Mar, M. H., Ranasinghe, A., Swenberg, J. A., Zeisel, S. H., Quantitation of choline and its metabolites in tissues and foods by liquid chromatography/electrospray ionization-isotope dilution mass spectrometry. *Anal Chem* 2002, *74*, 4734-4740.

[15] Holm, P. I., Ueland, P. M., Kvalheim, G., Lien, E. A., Determination of choline, betaine, and dimethylglycine in plasma by a high-throughput method based on

normal-phase chromatography-tandem mass spectrometry. *Clin Chem* 2003, *49*, 286-294.

[16] Yan, J., Wang, W., Gregory, J. F., 3rd, Malysheva, O., *et al.*, MTHFR C677T genotype influences the isotopic enrichment of one-carbon metabolites in folate-compromised men consuming d9-choline. *Am J Clin Nutr* 2011, *93*, 348-355.

[17] Park, C. S., Chung, W. G., Kang, J. H., Roh, H. K., *et al.*, Phenotyping of flavin-containing monooxygenase using caffeine metabolism and genotyping of FMO3 gene in a Korean population. *Pharmacogenetics* 1999, *9*, 155-164.

[18] Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., *et al.*, Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci U S A* 2011, *108 Suppl 1*, 4516-4522.

[19] Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., *et al.*, QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010, *7*, 335-336.

[20] Cashman, J. R., Zhang, J., Leushner, J., Braun, A., Population distribution of human flavin-containing monooxygenase form 3: gene polymorphisms. *Drug Metab Dispos* 2001, *29*, 1629-1637.

[21] Mitchell, S. C., Zhang, A. Q., Smith, R. L., Dimethylamine and diet. *Food Chem Toxicol* 2008, *46*, 1734-1738.

[22] Zhang, A. Q., Mitchell, S. C., Smith, R. L., Dietary precursors of trimethylamine in man: a pilot study. *Food Chem Toxicol* 1999, *37*, 515-520.

[23] Koeth, R. A., Wang, Z., Levison, B. S., Buffa, J. A., *et al.*, Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med* 2013, *19*, 576-585.

[24] Ma, J., Pazos, I. M., Gai, F., Microscopic insights into the protein-stabilizing effect of trimethylamine N-oxide (TMAO). *Proc Natl Acad Sci U S A* 2014, *111*, 8476-8481.

[25] Yazdekhasti, N., Brandsch, C., Schmidt, N., Schloesser, A., *et al.*, Fish protein increases circulating levels of trimethylamine-N-oxide and accelerates aortic lesion formation in apoE null mice. *Mol Nutr Food Res* 2016, *60*, 358-368.

[26] Caudill, M. A., Gregory, J.F. III, Miller, J., Shane, B., *Folate, choline, vitamin B-12 and vitamin B-6. In: Stipanuk, M.H., Caudill, M.A. (eds.), Biochemical, Physiological, & Molecular Aspects of Human Nutrition, 3rd edition*, Elsevier Saunders, St Louis, MO 2012.

[27] Galli, C., Rise, P., Fish consumption, omega 3 fatty acids and cardiovascular disease. The science and the clinical trials. *Nutr Health* 2009, *20*, 11-20.

[28] Collins, H. L., Drazul-Schrader, D., Sulpizio, A. C., Koster, P. D., *et al.*, L-Carnitine intake and high trimethylamine N-oxide plasma levels correlate with low aortic lesions in ApoE transgenic mice expressing CETP. *Atherosclerosis* 2015, *244*, 29-37.

[29] Skagen, K., Troseid, M., Ueland, T., Holm, S., *et al.*, The Carnitine-butyrobetaine-trimethylamine-N-oxide pathway and its association with cardiovascular mortality in patients with carotid atherosclerosis. *Atherosclerosis* 2016, *247*, 64-69.

[30] Gregory, J. C., Buffa, J. A., Org, E., Wang, Z., *et al.*, Transmission of atherosclerosis susceptibility with gut microbial transplantation. *J Biol Chem* 2015, *290*, 5647-5660.

[31] Falony, G., Vieira-Silva, S., Raes, J., Microbiology Meets Big Data: The Case of Gut Microbiota-Derived Trimethylamine. *Annu Rev Microbiol* 2015, *69*, 305-321.

[32] Brugere, J. F., Borrel, G., Gaci, N., Tottey, W., *et al.*, Archaebiotics: proposed therapeutic use of archaea to prevent trimethylaminuria and cardiovascular disease. *Gut Microbes* 2014, *5*, 5-10.

[33] Ley, R. E., Backhed, F., Turnbaugh, P., Lozupone, C. A., *et al.*, Obesity alters gut microbial ecology. *Proc Natl Acad Sci U S A* 2005, *102*, 11070-11075.

CHAPTER 2

THE METABOLIC FATE OF ISOTOPICALLY LABELED TRIMETHYLAMINE-*N*-OXIDE (TMAO) IN HUMANS

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ABSTRACT

Trimethylamine-N-oxide (TMAO) is associated with chronic disease risk. However, little is known about the metabolic fate of dietary TMAO. This study sought to quantitatively elucidate the metabolic fate of orally consumed TMAO in humans. As part of a crossover feeding study, healthy young men (n = 40) consumed 50 mg deuterium-labeled methyl d9-TMAO (d9-TMAO), and enrichments of TMAO and its derivatives were measured in blood for 6 h, urine and stool, as well as skeletal muscle in a subset of men (n = 6). Plasma d9-TMAO was detected as early as 15 min, increased until 1 h, and remained elevated through the 6-h period. TMAO exhibited an estimated turnover time of 5.3 h, and ~96% of the dose was eliminated in urine by 24-h, mainly as d9-TMAO. No d9-TMAO was detected in feces. Notably, d9-TMAO and d9-trimethylamine were detected in skeletal muscle (n = 6) at 6-h, and the enrichment ratio of d9-TMAO to d9-trimethylamine was associated with a genetic variant in flavin-containing monooxygenase isoform 3 (*FMO3* G472A). These results suggest that the absorption of orally consumed TMAO is near complete and may not require

processing by gut microbes. TMAO exhibits fast turnover in the circulation with the majority being eliminated in urine within 24-h. A small portion of the dose, however, is taken up by extrahepatic tissue in a manner that is associated with *FMO3* G472A polymorphism. This trial was registered at clinicaltrials.gov as NCT02558673.

2.1 Introduction

Elevated circulating concentrations of trimethylamine-N-oxide (TMAO) have been linked to increased risk of chronic kidney disease [1,2] greater development and progression of cardiovascular disease [1,3,4] and higher risk of colorectal cancer among post-menopausal women [5]. TMAO is derived from consumption of choline (abundant in eggs) and L-carnitine (abundant in red meat) [4,6]. Dietary choline and L-carnitine are metabolized by gut microbiota to trimethylamine (TMA) [4,6] and possibly to small amounts of dimethylamine (DMA) and methylamine (MA) [7,8]. These methylamines are absorbed via enterohepatic circulation, and TMA is primarily oxidized to TMAO by hepatic flavin-containing monooxygenases (FMOs) [9]. TMAO and its derivatives are then released into systemic circulation and suggested to be excreted in urine [10,11].

Interestingly, fish and seafood, which are recognized for their cardio-protective benefits [12], are the richest food sources of TMAO [10]. Results from our recent crossover feeding trial showed that fish consumption increased post-prandial plasma and urinary TMAO concentrations over 50 times more than eggs or beef [13]. This increase underscores the substantial impact of dietary TMAO on circulating TMAO concentrations. In spite of this, the metabolism of dietary TMAO in humans is poorly

understood, although it has been purported to enter the same metabolic pathway as its precursors [14].

To quantitatively elucidate the metabolic fate of orally consumed TMAO, the current study employed stable isotope methodology using deuterium-labeled methyl d9-TMAO (d9-TMAO) in 40 healthy men. Enrichments of d9-TMAO, along with its methylamine derivatives, were measured in blood, skeletal muscle, urine and stool. The association of a polymorphism (G472A) in *FMO* isoform 3 coding gene (*FMO3*) with the metabolic fluxes of labeled TMAO metabolites was also examined.

2.2 Materials and methods

2.2.1 Participants

The participants were healthy men (n = 40) aged 21-50 y with a body mass index (BMI) of 20-29.9 kg/m2, whom were recruited from Cornell University and the Ithaca area from May-July 2014 as previously described [13]. Women, vegetarians, and smokers were excluded from the study, as were men with a BMI ≥ 30 kg/m2, age over 50 y, or had values outside of normal range for indices of organ function (based on their blood chemistry profile). Men who indicated in a health history questionnaire having gastrointestinal diseases or complaints, chronic illnesses or other metabolic diseases, or taking nutritional supplements, antibiotics or probiotics within 2 months of recruitment were also excluded. The study was approved by the Institutional Review Board for Human Study Participants at Cornell University (Protocol ID#:

1403004534), and all participants signed a written informed consent prior to study enrollment.

2.2.2 Study design and protocol

The study is a single-arm feeding trial embedded within a randomized trial. The original study was a four-arm crossover feeding study with one-week washout periods in which study participants consumed one of the following meals in random order: eggs, beef, fish and fruit-control [13]. At the fruit-control study meal, a TMAO tracer was administered to enable investigation of its metabolic fate, which is the focus of the current study.

A day prior to the fruit-control study session, participants were advised to avoid consumption of foods that can decrease FMO enzyme activity and alter TMAO metabolism (i.e., grapefruit juice and indole-containing vegetables including broccoli, Brussels sprout, cabbage, cauliflower, kale, and bok choy) [15]. The participants were also asked to maintain their normal dietary and lifestyle routine, including their exercise routine the day prior to the study session.

The deuterium-labeled methyl-d9-TMAO (d9-TMAO) stock solution was prepared by dissolving 1 g d9-TMAO powder (Cambridge Isotopes, Tewksbury, MA) in 10 mL drinking water, dispensing 500 μL (50 mg) of it into 50-mL conical tubes, and storing at -80°C. Immediately before consumption, the 50-mg d9-TMAO concentrate was thawed and mixed with drinking water to yield the final volume of 1 cup (237 mL). A dose of 50 mg d9-TMAO was provided because i) it achieves enrichment levels in blood and urine that are readily measured by liquid chromatography-tandem mass

spectrometry (LC-MS/MS), ii) it is a physiologically relevant amount, and iii) it is well within the range of deuterium that can be administered safely [16].

On the session day, participants arrived at the Human Research Metabolic Unit (HMRU) at Cornell University between 07.00 and 10.00 in the fasted state (10 h). Participants completed a 24-h dietary recall to ensure compliance to the food restriction and were queried about deviations from their usual dietary and lifestyle routine. Participants who did not comply with the food restriction or who deviated from their usual dietary and lifestyle routine were rescheduled. Participants then consumed 1 cup (237 mL) of water containing 50 mg d9-TMAO along with 2 single-serve packages of applesauce (Mott's natural applesauce; Tops), the non-TMAO containing control food of the parent study [13]. Following the consumption of the d9-TMAO tracer, serial blood samples were collected at 15, 30 min, 1, 2, 4 and 6 h, and urine voids produced during this 6-h study period were pooled. At 4.5 h, participants were provided a fruit snack (2 single-serve packages of applesauce) to ease hunger without interfering with the study protocol. The participants remained at the testing site throughout the study period, and they were not allowed food or beverages (except water) outside of those provided by study personnel. Following the testing session, the participants turned in self-collected 6-24-h post study urine and a stool sample (collected in whole from the first bowel movement).

2.2.3 Sample collection and processing

At screening, fasting serum and buffy coat were collected for blood chemistry profiling [17] and *FMO3* G472A (rs 2266782) genotyping. During the study period,

whole blood was collected in EDTA-coated tubes (Becton, Dickinson and Company) and processed on-site for plasma metabolite measurements as previously described [18]. Urine was collected in wide-mouth polyethylene bottles (1 L; Nalgene) during the 6-h study period and the 6-24 h post-study, and the volumes were recorded separately. Additionally, participants collected a complete bowel movement in a provided bag, kept the stool frozen, and delivered it in insulated thermal bags. Thawed stool weight was recorded. All samples were de-identified, distributed and stored at -80 °C. Personnel involved in sample collection and processing were blinded to participant identities.

2.2.4 Muscle biopsy

A subset of participants (n = 6) volunteered to undergo skeletal muscle biopsy 6 h after the fruit + d9-TMAO tracer consumption so that tissue uptake could be examined. The inclusion criteria for muscle biopsy were participants with no history of a negative or allergic reaction to local anesthetics, with no tendency toward easy bleeding or bruising, not currently on any medications that may increase the chance of bleeding or bruising (e.g., Aspirin, Coumadin, Anti-inflammatories, Plavix), not currently on any immunosuppressive medications (e.g., glucocorticoid steroids, chemotherapy), with no current disease processes that would impair the healing process (e.g., diabetes, cancer, keloids, hereditary healing disorders, jaundice, alcoholism, HIV/AIDS), and with no history of fainting when undergoing or watching medical procedures. All participants signed a written informed consent prior to the procedure.

The skeletal muscle biopsy was performed at Gannett Health Services at Cornell University by a trained nurse practitioner. Using aseptic techniques, local anesthetic (Lidocaine hydrogen chloride 1%) was applied to numb the area of the muscle above the knee. A small 1 cm incision was made using a scalpel through the skin, and the 5 mm Bergstrom muscle biopsy needle was inserted into the muscle belly. Suction was applied using a syringe to obtain a small sample of muscle tissue (~200 mg) which was then distributed into cryogenic tubes (Fisher Scientific), rapidly frozen in liquid nitrogen, and stored at -80 °C. Immediately following the biopsy, pressure was applied, and thin adhesive strips and pressure dressing were used to close the wound.

2.2.5 Metabolite measurements

TMAO and its derivatives TMA, DMA and MA were extracted from plasma, muscle, urine, and stool samples as previously described [13]. Unlabeled metabolite concentrations in plasma and muscle were measured by isotope-dilution LC-MS/MS protocol [13] using Surveyor HPLC system TSQ Quantum Ultra mass spectrometer (ThermoElectron Corp), while those in urine and stool were measured using an Accela HPLC system TSQ Quantum Access mass spectrometer (ThermoElectron Corp). At that time, a separate run without internal standards was used to determine the relative intensities of unlabeled and labeled metabolites. The following precursor → product ion pairs were used for detection of these metabolites: *m/z* 76.3 → 58.4 for TMAO, *m/z* 79.3 → 61.4 for d3-TMAO, *m/z* 82.3 → 64.4 for d6-TMAO, and *m/z* 85.3 → 66.4 for d9-TMAO. TMA, DMA and MA derivatized with EBA had transitions as follow: *m/z* 146.1 → 118.1 for TMA-EBA, *m/z* 149.1 → 121.1 for d3-TMA-EBA, *m/z* 152.1

→ 124.1 for d6-TMA-EBA, *m/z* 155.1 → 127.1 for d9-TMA-EBA, *m/z* 132.1 → 58.1 for DMA-EBA, *m/z* 135.1→ 107.1 for d3-DMA-EBA, *m/z* 138.1 → 64.2 for d6-DMA-EBA, *m/z* 118.1 → 44.3 for MA-EBA, and *m/z* 121.1 → 47.3 for d3-MA-EBA. Peak area under the chromatography curve of the labeled and unlabeled metabolites was acquired and analyzed using the XCalibur program (ThermoElectron Corp).

2.2.6 Calculation of metabolite enrichments, concentrations, and pharmacokinetics

Isotopic enrichment percentages were calculated by dividing the peak area under the chromatography curve of the labeled metabolite by the total area of the labeled plus unlabeled metabolites. These enrichments were used to monitor the flow of orally consumed TMAO (d9-TMAO) through the TMAO pathway. Because isotopic enrichments account for inter-person variation in metabolite pool size, they provide a less biased indication of the flow than absolute concentrations. Metabolite enrichment ratios were also examined as indicators of the flow of the tracer through specific steps of the TMAO pathway.

In addition, the partitioning of the tracer among body compartments shown in Table 2.1 was determined by calculating the concentrations of labeled metabolites, which were derived from the concentrations of the unlabeled metabolites measured in the parent trial [13] using similar protocols and the same instrumentation. This derivation was comprised of two steps. The first step was to determine the concentrations of the labeled metabolite by using the following equation: peak area of labeled metabolite/peak area of unlabeled metabolite × unlabeled metabolite concentration.

The second step involved estimating the total amount of labeled metabolites in each body compartment (i.e., plasma, muscle, urine and stool), which equaled the average labeled metabolite concentration × the average size of a body compartment. The latter was specified as follows: plasma, 41.25 mL/kg body weight (assuming plasma is 55% v/v of blood and average blood volume is 75 mL/kg body weight); muscle, 33.7 kg based on our participants' average body weight and age [20]; urine, recorded volumes; stool, recorded weights.

As an exploratory analysis, turnover rate (h-1), the rate at which the whole pool of a metabolite is renewed, was calculated by dividing the pool size of total TMAO enrichment (d9 + unlabeled TMAO = 100%) by the formation rate of plasma d9-TMAO (% per h). The latter was estimated from the slope of d9-TMAO enrichments from 30 min to 2 h (Figure 2.1A), with the assumption that equilibrium of TMAO was established at 1 h based on the d9-TMAO response curve shown in Figure 2.1A. Turnover time was the reciprocal of the turnover rate.

2.2.7 Genotyping

DNA was extracted from monocytes collected at screening according to the manufacturer's protocol (Dneasy Tissue kit, Qiagen Science). The most common variant flavin-containing monooxygenase isoform 3 (*FMO3*) G472A (rs2266782; E158K) reported to influence *N*-oxygenation of TMA [19] was determined for each participant by endpoint genotyping using a fluorescent Taqman SNP genotyping assay kit (Thermo Scientific, catalog no. 4362691) on the LightCycler® 480 real-time PCR instrument (Roche). The genotyping was performed according to the manufacturer's

protocol with cycling conditions of 10 min hold at 95°C, followed by 34 cycles of 92°C for 15 s and 62°C for 90 s. Samples were run in duplicate with in-run standards and a negative water control.

2.2.8 Quantitative real-time RT-PCR

RNA was extracted from frozen muscle biopsy samples (~30mg) using TRIzol reagent (Invitrogen) and the RNeasy Mini Kit (Qiagen). RNA concentration and quality were assessed with a NanoDrop ND-1000 spectrophotometer (Thermo). All samples had A260/A280 ratios above 1.8. One sample showed contamination and thus was excluded from subsequent procedures. Reverse transcription was performed on all five samples using the ImProm-II Reverse Transcription System (Promega). Quantitative PCR was performed using LightCycler 480 SYBR Green I Master (Roche) in a LightCycler 480 instrument (Roche). Primers were designed using NCBI Primer-BLAST. Sequences were as follows: *FMO3*, F/5' GAAGAAAGTGGCCATCATTGGAG 3', R/ 5' AATTTCCACAGGCCCCCAA 3'; *GAPDH*, F/ 5' TGTTGCCATCAATGACCCCTT 3', R/ 5' CTCCACGACGTACTCAGCG 3'. Reaction conditions were as follows: 95°C for 5 minutes, followed by 40 cycles with 15 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C. All samples were run in triplicate. A melting curve analysis was included at the end of the amplification cycles to verify successful amplification of the PCR product and specificity was confirmed by running products on agarose gels. Expression levels of *FMO3* were calculated by the ΔCt method normalized to the expression of reference gene *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase).

2.2.9 Statistical analyses

Metabolite enrichments were analyzed descriptively by calculating averages and standard deviations at each time point for plasma, muscle, urine and stool. Furthermore, a repeated measures mixed linear model was employed to investigate enrichment percentage as a function of time and/or genotype for individual compartments. Tukey-Kramer post-hoc test was used to correct for multiple comparison among the time points and the genotypes. The residuals were inspected for violation of model assumptions, and the data was logarithmically transformed if needed. Significant differences are reported at $P < 0.05$. All model-based results are expressed as means ± SEM. All statistical analyses were conducted using SAS (version 9.4, SAS Institute, NC) with the PROC MIXED model procedure with random slopes and intercepts, and unstructured covariance structure.

2.3 Results

2.3.1 Labeled TMAO metabolites in the circulation

After oral consumption of the tracer, d9-TMAO was detected in plasma as early as 15 min, then increased rapidly during the first hour and remained elevated at 66–75% enrichment throughout the 6-h study period (Figure 2.1A). Based on this observation, further exploratory analyses were conducted to derive an estimated turnover rate and turnover time of 0.19 h-1 and 5.3 h, respectively for TMAO (see methods section for calculations). Compared to d9-TMAO, d9-TMA and d6-DMA appeared in plasma more gradually. Plasma d9-TMA continuously increased at most time points, achieving 52% enrichment at 6-h, while plasma d6-DMA was not

detected until after 2-h, when it increased significantly and rather exponentially, achieving 3% enrichment at 6-h (Figure 2.1). Notably, enrichments all of the labeled TMAO metabolites in the circulation varied greatly among participants, ranging from 26 to 92% for TMAO, 12 to 82% for TMA, and 0 to 10% for DMA at 6 h.

To examine TMAO pool size in relation to the inflow of the tracer, total TMAO concentration (labeled + unlabeled) was calculated and compared with d9-TMAO enrichment (Figure 2.2). Although the former exhibited a slight logarithmic increase, the latter, which reflects the flux of labeled TMAO, increased more rapidly as indicated by the steeper slope. Unlabeled (i.e., endogenous) TMAO concentrations remained constant throughout this time period (Supplementary Figure 2.1).

2.3.2 Skeletal muscle uptake of labeled TMAO metabolites and modulation by *FMO3* G472A

At 6 h after tracer consumption, d9-TMAO comprised 36% enrichment in skeletal muscle, while d9-TMA comprised 3.3% enrichment. Moreover, d9-TMAO and d9-TMA in muscle correlated rather strongly with those in plasma, but statistical significance was not achieved (d9-TMAO Pearson's r = 0.65, P = 0.16; d9-TMA Pearson's r = 0.70, P = 0.12). Neither d9-TMAO nor d9-TMA was associated with *FMO3* G472A polymorphism in our sample of three heterozygous GA and three homozygous wild-type GG genotypes. However, the GA genotype had a lower muscle d9-TMAO:d9-TMA (product: precursor) ratio than the GG genotype (P = 0.047) (Supplementary Figure 2.2) despite a very low mRNA expression level (0.000464 fold over GAPDH expression) of FMO3 in muscle. Paradoxically, no effect of the *FMO3*

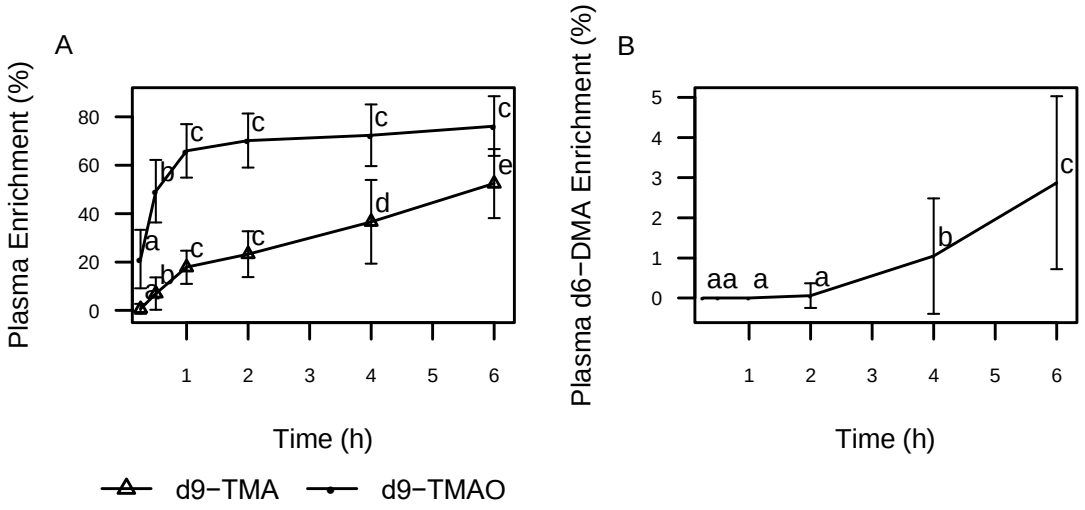

Figure 2.1 A) Plasma d9-TMAO (circle), d9-TMA (triangle) and B) d6-DMA enrichments increased within 6 h after consumption of the 50 mg d9-TMAO tracer. Isotopic enrichment percentage was calculated by dividing the peak area under the chromatography curve of the labeled metabolite by the sum of the peak area of the labeled plus unlabeled metabolites. For each metabolite, significant differences ($P < 0.05$) among the timepoints were determined using repeated measure mixed linear model and the Tukey-Kramer post-hoc test for multiple comparison correction, and are denoted by different letters. Values are mean ± SD; n = 40 per time point per metabolite. Abbreviation: d6-DMA = Deuterium-labeled methyl d6-dimethylamine; d9-TMA = d9-trimethylamine; d9-TMAO = d9-trimethylamine-N-oxide

G472A polymorphism was detected in other compartments when all participants were included in the analysis, as opposed to just those who had undergone a muscle biopsy. Other labeled metabolites (i.e., d6-DMA and d3-MA) were not detected in muscle.

2.3.3 Excretion of labeled TMAO metabolites

d9-TMAO enrichment in urine was higher during 0-6 h (67%) than 6-24 h (52%) after consumption of the tracer (Figure 2.3). d9-TMA enrichment did not differ during

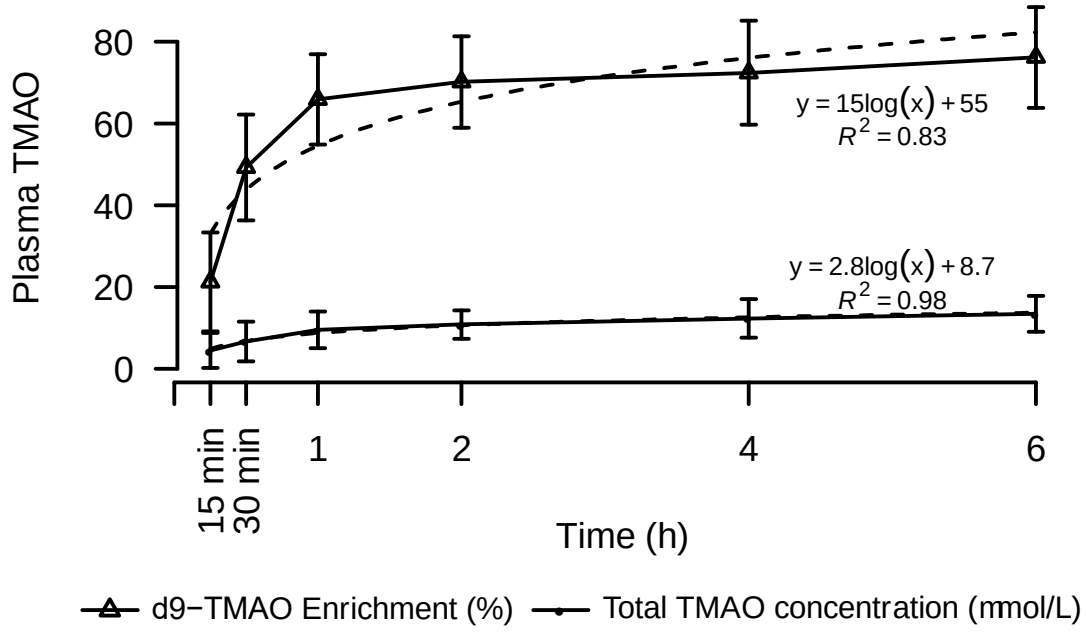

Figure 2.2 Following oral administration of the 50 mg deuterium-labeled methyl d9-trimethylamine-*N*-oxide (d9-TMAO) tracer, plasma d9-TMAO enrichment (triangle) increased more rapidly than total TMAO concentration (circle) [d9 + unlabeled TMAO]. d9-TMAO enrichment percentage was calculated by dividing the peak area under the chromatography curve of the labeled metabolite by the sum of the peak area of the labeled plus unlabeled metabolites. d9-TMAO concentration was derived from data published separately [13] by using the peak area of d9-TMAO / peak area of unlabeled TMAO × unlabeled TMAO concentration. The units of the y-axis are shown below the plotting area. Dashed lines are best-fitted logarithmic trend lines with indicated equations and R^2-values. Values are mean ± SD, n = 40 per time point.

these periods (~48%). Urinary d6-DMA enrichment was lower during the 0-6 h (1%) than the 6-24 h (4%) periods. In addition, compared to urine, d9-TMA enrichment in stool was lower (4% vs. 46%), while d6-DMA was higher (15% vs. 4%). d9-TMAO and other labeled metabolites were not detected in stool.

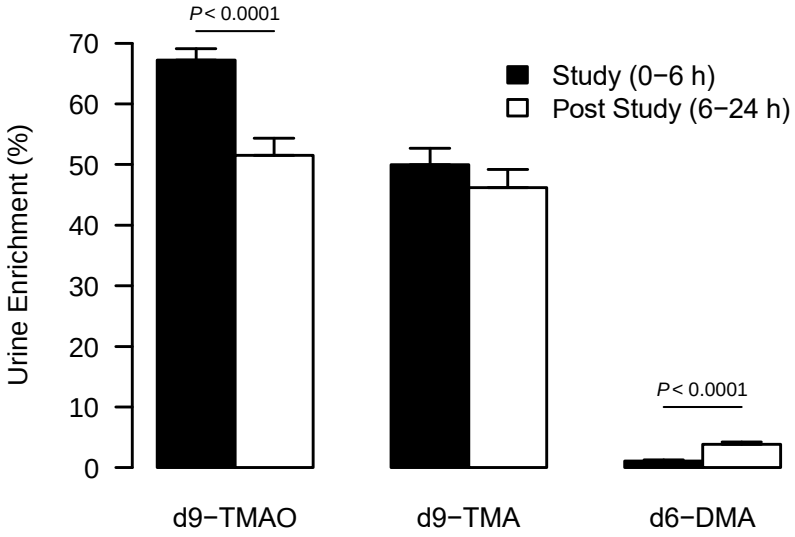

Figure 2.3: The 50 mg d9-TMAO tracer was excreted as d9-TMAO, d9-TMA and d6-DMA in urine 0-24 h after oral administration. d9-TMAO enrichments were higher during 0-6 h (black) than 6-24 h (white), while d9-TMA enrichments did not differ between the two periods. d6-DMA enrichments were higher during 6-24 h. Isotopic enrichment percentage was calculated by dividing the peak area under the chromatography curve of the labeled metabolite by the sum of the peak area of the labeled plus unlabeled metabolites. Significant differences ($P < 0.05$) between the two periods are determined using repeated measure mixed linear model for each metabolite. Values are mean ± SEM; $n = 40$ per time period per metabolite. Abbreviation: d6-DMA = Deuterium-labeled methyl d6-dimethylamine; d9-TMA = d9-trimethylamine; d9-TMAO = d9-trimethylamine-*N*-oxide

2.3.4 Estimated distribution of labeled TMAO metabolites

Table 2.1 shows estimates of d9-TMAO and methylamine derivatives as percentages of the tracer dose for each body compartment. At 6-h, an estimated 5.6% of the dose was present in plasma, 19% in skeletal muscle, and 42% in urine, mostly as d9-TMAO. An estimated 0.15% of the dose was detected in stool mainly as d9-

Table 2.1: The distribution of the 50 mg d9-TMAO oral dose within body compartments

	% of d9-TMAO oral dose[1] ± SEM			
	d9-TMAO	d9-TMA	d6-DMA	Total[2]
Plasma at 6 h[3]	5.5 ± 0.3	0.01 ± 0.001	0.03 ± 0.004	5.6
Muscle at 6 h[3]	19 ± 5.6	0.14 ± 0.03	-	19
Urine				
0-6 h[3]	42 ± 2.7	0.08 ± 0.02	0.18 ± 0.04	42
6-24 h[3]	52 ± 3.9	0.16 ± 0.03	1.5 ± 0.16	53
Total (0-24 h)	94 ± 5.1	0.24 ± 0.05	1.7 ± 0.18	96
Stool after 6 h[4]	-	0.13 ± 0.05	0.02 ± 0.01	0.15

[1] Percentage of dose was estimated from the concentrations of labeled metabolites, which were derived from the concentrations of the unlabeled metabolites measured in the parent trial [13] using the following equation: peak area of labeled metabolite/peak area of unlabeled metabolite × unlabeled metabolite concentration. Next, the total amount of labeled metabolites in each body compartment were estimated using the average labeled metabolite concentration × the average size of a body compartment. The latter was specified as follows: plasma, 41.25 mL/kg body weight (assuming plasma is 55% v/v of blood and average blood volume is 75 mL/kg body weight); muscle, 33.7 kg based on our participants' average body weight and age [20]; urine, recorded volumes; stool, recorded weights. Finally, the total amount of labeled metabolites was converted to percentage of the 50-mg d9-TMAO dose. Total methylamines equal the sum of d9-TMAO, d9-TMA and d6-DMA. Abbreviation: d6-DMA = Deuterium-labeled methyl d6-dimethylamine; d9-TMA = d9-trimethylamine; d9-TMAO = d9-trimethylamine-*N*-oxide

[2] n = 40 per compartment per metabolite

TMA. After 24-h, an estimated 96% of the administered dose was excreted in urine mainly as d9-TMAO, leaving 4% (2 mg) retained in the body.

2.4 Discussion

This study traced the metabolic fate of orally consumed TMAO in humans using a stable isotope approach. Our findings demonstrate that orally consumed TMAO i) is largely absorbed and does not require processing; ii) has a high turnover and rapid clearance and iii) is taken up by extrahepatic tissue. The proposed fate of labeled TMAO based on the study findings is illustrated in Figure 2.4.

2.4.1 Absorption of orally consumed TMAO is near complete and does not require processing by the gut microbes and hepatic FMOs

The orally consumed d9-TMAO tracer was rapidly absorbed, achieving ~20% enrichment in the systemic circulation within 15-min of consumption (Figure 2.1A). This timing of events suggests that a portion of orally consumed TMAO can be absorbed from the intestine independent of its conversion to TMA by gut microbiota and subsequent reversion to TMAO by hepatic FMOs. Furthermore, d9-TMAO occupied more than half of the endogenous pool slightly after 30 min, at which time d9-TMA was barely detected in plasma, thereby providing additional support that the majority of orally consumed TMAO is absorbed intact.

Although the majority of TMAO appeared to be absorbed intact, detection of d9-TMA and d6-DMA in plasma, urine and stool suggests that microbial and/or hepatic processing also occurs to a limited extent. These metabolites may be formed when d9-TMAO passes through the gastrointestinal tract or when absorbed d9-TMAO is secreted to the intestine. Nonetheless, d6-DMA was not detected in plasma until two

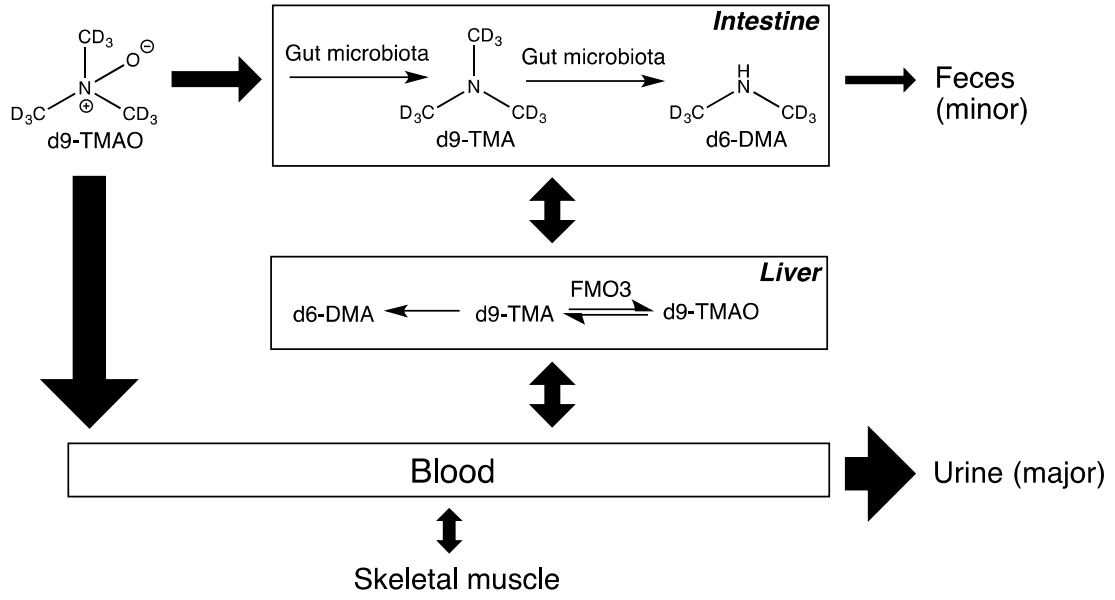

Figure 2.4: A simplified schema of the metabolic fate of orally consumed TMAO and its methylamine derivatives. The majority of d9-TMAO is absorbed intact into the systemic circulation. A portion of the tracer undergoes microbial conversion to form d9-TMA and d6-DMA. Minor portions of these metabolites are lost in feces, but the majority is absorbed into the liver, where hepatic enzyme FMO3 converts d9-TMA mainly to d9-TMAO. These metabolites subsequently enter the systemic circulation. Circulating TMAO exhibits fast turnover with the majority being eliminated in urine. However, a small portion of the dose, potentially the portion generated by hepatic FMO3, is taken up by extrahepatic tissue. The direction of the arrows indicates the flow of the labeled metabolites through the body. The size of the arrows indicates relative magnitude of the flow. Abbreviation: d6-DMA = Deuterium-labeled methyl d6-dimethylamine; d9-TMA = d9-trimethylamine; d9-TMAO = d9-trimethylamine-*N*-oxide; FMO3 = Flavin-containing monooxygenase isoform 3

hours post consumption (Figure 2.1B) suggesting that conversion of TMA to DMA (and/or absorption of DMA) is slow within the gut, or that this conversion mostly transpires in the liver. Importantly, the absorption of d9-TMAO, d9-TMA and d6-

DMA was near complete (~96% of the dose) with no d9-TMAO detected in feces (Table 2.1). It is also worth noting that although TMAO molecules bear structural similarity to choline, we found no evidence for the use of TMAO's methyl groups in one-carbon metabolism (e.g., d3-methionine was not detected in any compartments investigated).

2.4.2 TMAO exhibits a high turnover rate and rapid clearance

Incorporation of the label into the TMAO pool occurred rapidly such that d9-TMAO comprised 66% enrichment within the first hour and reached 76% 6 h after tracer consumption (Figure 2.1A). These enrichments are indicative of a high turnover rate such that new TMAO molecules are largely replacing the endogenous TMAO pool. Although the total pool size of TMAO (d9 + d0 TMAO) also expanded (Figure 2.2), plasma d9-TMAO enrichments, which indicate the influx of d9-TMAO molecules, increased at a much greater rate than the pool size. Indeed the increase in pool size was due to the rapid increase in d9-TMAO enrichment, given the lack of change in unlabeled TMAO concentrations during this period (Supplementary Figure 2.1). Given that the initial increase (15 min-1 h) of d9-TMAO enrichment was approximately linear, and the new equilibrium was established at 1 h, we were able to calculate the formation rate of plasma TMAO and the steady-state pool size. From these data, we identified a plasma TMAO turnover rate of 0.19 h^{-1} and a turnover time of 5.3 h. This is slower compared to TMAO turnover rates in rat models [21] possibly because of the larger pool size in humans. Nonetheless, compared to those of many common medicinal agents, TMAO turnover rate is relatively fast [22,23].

In addition to a high turnover rate in plasma, orally consumed TMAO is also rapidly and efficiently cleared in urine within 24 h of consumption. d9-TMAO comprised 52-67% enrichment within 24-h of tracer consumption (Figure 2.3), indicating rapid elimination of TMAO. In addition, urinary d9-TMAO enrichment was highest during 0-6 h after consumption, suggesting that elimination is greatest during this period. On the other hand, TMA and DMA excretion appear to be slower than that of TMAO as indicated by their lower urinary enrichments. Slower excretion of TMA and DMA is consistent with the order of their metabolic production (Figure 2.4). This sequential production and excretion were also observed in changes in urinary enrichments over time (Figure 2.3). Specifically, d9-TMA was excreted constantly, while d6-DMA had prolonged excretion with greater elimination rates in the 6-24 h period as compared to the 0-6 h period. Notably, approximately 96% of the dose was detected mainly as d9-TMAO within 24 h (Table 2.1), indicating that orally consumed TMAO is efficiently cleared in urine.

2.4.3 TMAO is taken up by extrahepatic tissue

d9-TMAO and d9-TMA were detected in skeletal muscle within 6 h of administering the tracer, indicating for the first time that these metabolites can be taken up by extrahepatic tissue in humans. Interestingly, in our cohort, the *FMO3* G472A genotype was associated with the skeletal muscle ratio of TMAO:TMA (and TMA:TMAO) (Supplementary Figure 2.2). Specifically, a lower d9-TMAO:d9-TMA enrichment ratio was detected among men with the heterozygous GA genotype as compared to those with the wild-type GG genotype. Given the very low level of

FMO3 transcript abundance in the skeletal muscle of our participants, which is consistent with an absence of skeletal muscle FMO3 protein as reported by others [24], our findings suggest that the effect modification of the d9-TMAO:d9-TMA ratio by genotype in muscle reflects the hepatic activities of FMO3 in the GG and GA genotypes. As such, these data imply that the plasma TMAO pool is comprised of at least two kinetic pools that differ in their metabolic fates: one that bypasses the liver and is largely excreted and one that is generated from hepatic FMO3 and imported into tissues.

2.4.4 Study limitations

Our study illustrates the metabolic fate of TMAO and uncovers potentially novel aspects of TMAO metabolism. However, the current study design did not permit calculation of absolute bioavailability, which is the fraction of the oral dose that reaches the systemic circulation relative to an intravenous dose. In addition, the turnover parameters, which are often calculated using an elimination curve, were estimated based on the formation curve, and the results presented in Table 2.1 are crude estimates calculated using linear curves for unlabeled metabolites. We also had limited power to detect the genetic effect of variants in FMO genes on TMAO metabolism. Finally, the reported kinetics of TMAO solutions may differ from that of TMAO in food matrices.

2.4.5 Conclusion and clinical implications

Absorption of orally consumed TMAO is near complete, and may occur largely by a process that is independent of gut microbes and hepatic FMOs. In healthy men,

TMAO exhibits fast turnover in the circulation with the majority being eliminated in urine. A small portion of the dose, however, is taken up by extrahepatic tissue in a manner that appears to be under the influence of *FMO3* G472A polymorphism. From a clinical perspective, our findings show that circulating TMAO concentrations in a healthy population are temporarily elevated as a result of intake of the intact TMAO molecules, which are abundant in fish and seafood. This elevation does not necessarily depend on gut microbiota as observed upon consumption of dietary precursors of TMAO like choline. Moreover, TMAO is rapidly eliminated in healthy adults which is in contrast to the sustained elevation of TMAO in atherosclerotic patients [3,4,6] and elevated TMAO concentrations due to gut microbial processing of TMAO precursors in disease animal models [4,6]. Finally, the discovery of TMAO uptake by extrahepatic tissue may signify a physiological role of this metabolite in humans.

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AUTHOR CONTRIBUTIONS

ST, CEC, JY, AETM, and MAC designed research. ST, CEC, OVM, EB and JHK conducted research. ST analyzed data. ST and MAC wrote paper. CEC and JHK assisted in manuscript preparation. All authors read and approved the final manuscript.

REFERENCES

[1] Stubbs JR, House JA, Ocque AJ, Zhang S, Johnson C, Kimber C, et al. Serum Trimethylamine-N-Oxide is Elevated in CKD and Correlates with Coronary Atherosclerosis Burden. J Am Soc Nephrol 2016;27:305–13. doi:10.1681/ASN.2014111063.

[2] Tang WHW, Wang Z, Kennedy DJ, Wu Y, Buffa JA, Agatisa-Boyle B, et al. Gut microbiota-dependent trimethylamine N-oxide (TMAO) pathway contributes to both development of renal insufficiency and mortality risk in chronic kidney disease. Circ Res 2015;116:448–55. doi:10.1161/CIRCRESAHA.116.305360.

[3] Tang WHW, Wang Z, Levison BS, Koeth R a, Britt EB, Fu X, et al. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med 2013;368:1575–84. doi:10.1056/NEJMoa1109400.

[4] Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 2011;472:57–63. doi:10.1038/nature09922.

[5] Bae S, Ulrich CM, Neuhouser ML, Malysheva O, Bailey LB, Xiao L, et al. Plasma Choline Metabolites and Colorectal Cancer Risk in the Women's Health Initiative Observational Study. Cancer Res 2014;74:7442–52. doi:10.1158/0008-5472.CAN-14-1835.

[6] Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat Med 2013;19:576–85. doi:10.1038/nm.3145.

[7] Asatoor AM, Simenhoff ML. The origin of urinary dimethylamine. Biochim Biophys Acta 1965;111:384–92.

[8] Al-Waiz M, Mitchell SC, Idle JR, Smith RL. The relative importance of N-oxidation and N-demethylation in the metabolism of trimethylamine in man. Toxicology 1987;43:117–21. doi:10.1016/0300-483X(87)90001-1.

[9] Krueger SK, Williams DE. Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism. Pharmacol Ther 2005;106:357–87. doi:10.1016/j.pharmthera.2005.01.001.

[10] Zhang AQ, Mitchell SC, Smith RL. Dietary Precursors of Trimethylamine in Man: A Pilot Study. Food Chem Toxicol 1999;37:515–20. doi:10.1016/S0278-6915(99)00028-9.

[11] Smith JL, Wishnok JS, Deen WM. Metabolism and excretion of methylamines in rats. Toxicol Appl Pharmacol 1994;125:296–308. doi:10.1006/taap.1994.1076.

[12] Kris-Etherton PM. Fish Consumption, Fish Oil, Omega-3 Fatty Acids, and Cardiovascular Disease. Circulation 2002;106:2747–57. doi:10.1161/01.CIR.0000038493.65177.94.

[13] Cho CE, Taesuwan S, Malysheva OV, Bender E, Tulchinsky NF, Yan J, et al. Trimethylamine-N-oxide (TMAO) response to animal source foods varies among healthy young men and is influenced by their gut microbiota composition: a randomized controlled trial. Mol Nutr Food Res 2016. doi:10.1002/mnfr.201600324.

[14] Al-Waiz M, Ayesh R, Mitchell SC, Idle JR, Smith RL. Disclosure of the metabolic retroversion of trimethylamine N-oxide in humans: A pharmacogenetic approach. Clin Pharmacol Ther 1987;42:608–12. doi:10.1038/clpt.1987.207.

[15] Bain M, Fornasini G, Evans A. Trimethylamine: Metabolic, Pharmacokinetic and Safety Aspects. Curr Drug Metab 2005;6:227–40. doi:10.2174/1389200054021807.

[16] Koletzko B, Sauerwald T, Demmelmair H. Safety of stable isotope use. Eur J Pediatr 1997;156 Suppl:S12-7.

[17] Yan J, Jiang X, West AA, Perry CA, Malysheva OV, Devapatla S, et al. Maternal choline intake modulates maternal and fetal biomarkers of choline metabolism in humans. Am J Clin Nutr 2012;95:1060–71. doi:10.3945/ajcn.111.022772.

[18] Guinotte CL, Burns MG, Axume JA, Hata H, Urrutia TF, Alamilla A, et al. Methylenetetrahydrofolate reductase 677C-->T variant modulates folate status response to controlled folate intakes in young women. J Nutr 2003;133:1272–80.

[19] Yamazaki H, Shimizu M. Survey of variants of human flavin-containing monooxygenase 3 (FMO3) and their drug oxidation activities. Biochem Pharmacol 2013;85:1588–93. doi:10.1016/j.bcp.2013.03.020.

[20] Janssen I, Heymsfield SB, Wang Z, Ross R. Skeletal muscle mass and distribution in 468 men and women aged 18-88 yr. J Appl Physiol 2000;89:81–8.

[21] Nnane IP, Damani LA. Pharmacokinetics of trimethylamine in rats, including the effects of a synthetic diet. Xenobiotica 2001;31:749–55. doi:10.1080/00498250110065586.

[22] Baselt RC. Disposition of Toxic Drugs and Chemicals in Man. 8th ed. Foster City: Biomedical Publications; 2008.

[23] Hammett-Stabler CA, Dasgupta A. Therapeutic Drug Monitoring Data: A Concise Guide. 3rd ed. Washington: AACC Press; 2007.

[24] Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Tissue-based map of the human proteome. Science 2015;347:1260419–1260419. doi:10.1126/science.1260419.

CHAPTER 3

RELATIONSHIP OF CHOLINE INTAKE WITH BLOOD PRESSURE IN THE NATIONAL HEALTH AND NUTRITION EXAMINATION SURVEY 2007-10

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ABSTRACT

Dietary choline is a precursor of trimethylamine *N*-oxide (TMAO), a metabolite that has been associated with an increased risk of cardiovascular disease. The mechanism underlying this association is unknown, but may include TMAO effects on blood pressure. This study assessed the association of choline intake with hypertension and blood pressure (BP) using cross-sectional data from the 2007 to 2010 National Health and Nutrition Examination Survey (NHANES). Modifying factors (sex, race/ethnicity) and dietary versus supplemental sources of choline intake were also investigated. The associations of total (dietary + supplemental) and dietary choline intake with the prevalence odds of hypertension differed by sex (n=9,227; $P_{interaction}$=0.04). In women, both total and dietary choline intake had a borderline inverse association with hypertension (n=4,748; prevalence odds ratio [OR] per 100 mg of total choline and 95% confidence interval [95% CI] was 0.89 [0.77 – 1.02], P < 0.10), but no association was observed in men (n=4,479; P=0.54). Use of choline supplements was inversely associated with hypertension in both sexes (prevalence OR

[95% CI], 0.68 [0.49 – 0.92]; P=0.01). There was little to no association of total, dietary, or supplemental choline intake with systolic or diastolic BP (n=6,554; mmHg change in BP associated with 100 mg difference in total choline ± SEM was -0.26±0.22 mmHg and -0.29±0.19 mmHg, respectively). In summary, the cross-sectional NHANES data do not support the hypothesis of a positive association between choline intake and BP.

3.1 Introduction

Hypertension affects 85.7 million Americans or 34% of the population [1] and is a major risk factor for atherosclerosis and cardiovascular disease (CVD). Genetic predisposition combined with exposure to environmental factors such as unhealthy diets and lifestyles can lead to abnormal regulation of blood pressure and development of hypertension. Elevated blood pressure stimulates collagen production, which contributes to intima-media thickening, vascular stiffening, and endothelial dysfunction with increasing age [2]. This vascular wall remodeling promotes expression of pro-inflammatory cytokines, growth factors, adhesion molecules, as well as infiltration of macrophages and vascular smooth muscle cells [2]; all of which are predominant pathophysiologic features in the development of atherosclerosis.

Choline, a nutrient found mostly in animal source foods, was recently shown to predict CVD risk in humans and to promote atherosclerosis in mice through its gut-dependent metabolism to trimethylamine-*N*-oxide (TMAO) [3]. Upon consumption, unabsorbed choline is metabolized by gut microbiota to trimethylamine and subsequently, by hepatic enzyme flavin-containing monooxygenases (FMOs), to

TMAO [3,4]. Epidemiologic evidence links elevated TMAO concentrations to adverse cardiovascular events [5], but whether CVD is associated with dietary intake of choline is unclear [6]. Mechanistic studies in apolipoprotein E knockout mice supplemented with choline and/or TMAO reported enhanced macrophage foam cell formation [3] and reduced reverse cholesterol transport [7], although in mice expressing human cholesteryl ester transfer protein, TMAO was inversely correlated with aortic lesion size [8], TMAO was also shown to prolong the hypertensive effects of angiotensin II in rats [9] suggesting a possible effect of the choline-derived metabolite on blood pressure.

In this study, we investigated the association of choline intake with both blood pressure and the prevalence odds of hypertension, using data from the 2007 to 2010 cross-sectional National Health and Nutrition Examination Survey (NHANES) [10,11].

3.2 Methods

3.2.1 Study population

The study population was drawn from the cross-sectional, publicly-accessible NHANES survey data from 2007 to 2010 (n = 20,686) [10,11]. The programming code that produced the findings described herein is available by request. NHANES used a complex, multistage probability sampling design to obtain a representative sample of the civilian, noninstitutionalized U.S. population [12]. National Center for Health Statistics Research Ethics Review Board approved all NHANES protocols, and

all participants gave informed consent [12]. The current study included non-pregnant individuals aged ≥ 20 y who completed two 24-h recalls with non-extreme caloric intakes (n = 9,559; Supplementary Figure 3.1). The sample was further refined using *a priori* criteria to yield the final analytic samples for the evaluation of the hypertension and blood pressure outcomes. For the hypertension outcome, individuals were considered to be hypertensive (n = 3,933) or non-hypertensive (n = 5,626) based on the definitions of hypertension described below. Non-hypertensive individuals with incomplete data on the variables used to define the presence/absence of hypertension were omitted from further consideration (n = 332), yielding 9,227 participants in the analysis of choline—hypertension. For the analysis of the choline—blood pressure association, participants with missing or non-positive blood pressure (n = 314) or who reported using anti-hypertensive medications (n = 2,691) were omitted from further consideration (n = 6,554 for analysis).

3.2.2 Choline intake measurement

Two 24-h dietary recalls were collected using the validated Automated Multiple Pass Method by trained interviewers [13,14]. Immediately after the dietary recalls, 24-h dietary supplement usage was collected using a similar protocol. The first dietary recall was conducted in person at the mobile examination centers, while the second occurred via follow-up phone calls. Total amounts of dietary choline were determined using nutrient values from the Food and Nutrient Database for Dietary Studies [13], and amounts of supplemental choline were calculated from a database of label information. The sum of dietary and supplemental choline yielded total choline intake

for each recall day. The average total and dietary choline intake across the two recall days were used as continuous variables in all analyses.

Use of dietary supplements during the past 30 days was collected at the participants' homes using a questionnaire administered by trained interviewers. Amounts of supplemental choline were calculated from supplement labels. Supplemental choline variables in both the 24-h recalls and the 30-day questionnaire were modeled as binary variables (user vs. non-user).

3.2.3 Covariates

The covariates considered as possible confounding variables were sex, age, race/ethnicity (non-Hispanic White; non-Hispanic Black; Hispanic, Mexican American, and others), ratio of family income to poverty level, education (< 9th grade, 9-11th grade, high school graduate, some college, college graduate), marital status (yes, no), physical activity (h/wk of moderate-vigorous activities), smoking (never, former, current), menopausal status and hormone replacement therapy usage; all variables were self-reported (Supplementary Table 3.1). Weight and height were measured using standardized methods [15] and used to calculate body mass index (BMI, kg/m^2). Total calories, protein and fat, cholesterol, sodium, folate, vitamin B6 and vitamin B12 intakes from foods and supplements were obtained from the average of two 24-h recalls, with calculations as described above for choline.

Covariates that were associated with choline intake, causally associated with blood pressure or hypertension, and that had a demonstrable influence on the effect estimates

were retained in the models. Final model covariates were: age, sex, race/ethnicity, BMI, total calories, education and poverty ratio.

3.2.4 Outcomes

The primary outcomes were hypertension prevalence and systolic and diastolic blood pressure (SBP and DBP, respectively). Hypertension status was assessed using data collected during the interview and examination. The protocol for blood pressure measurement is reported elsewhere [15]. Hypertensive individuals were defined by the following criteria: 1) reported having been told by doctors 2+ times of hypertension diagnosis, or 2) reported using antihypertensive medication(s), or 3) had mean SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg (calculated from 1-3 readings at one sitting). Non-hypertensive individuals were defined as participants without any evidence of hypertension, i.e., not meeting the above criteria. In addition to models estimating the association of choline with prevalent hypertension, separate statistical models estimated the association of choline with SBP and DBP.

3.2.5 Statistical analysis

Sample weights were used according to the NHANES analytic guidelines to account for complex survey design in all analyses [16]. Population means, proportions, and standard deviations of baseline characteristics in Supplementary Table 3.1 were estimated [17]. Population means of dietary variables were adjusted for total calories using the residual method [18] and standardized to 2,000 kcal. Associations between choline intake and the prevalence odds of hypertension and blood pressure were assessed using logistic regression and multiple linear regression,

respectively. All analyses were adjusted for the above-mentioned covariates unless otherwise noted; about 10% of participants were missing on covariates in each analysis (n = 878 missing in hypertension models; n = 615 missing in blood pressure models). Estimated regression coefficients for choline were based on participants with complete data on all covariates, while the variances of the estimates were adjusted for missing values in the covariates (NOMCAR option). In addition, sex, race/ethnicity, BMI, post-menopausal status and use of hormone replacement therapy were tested as effect modifiers of the choline—hypertension/blood pressure associations. Further analyses considered the associations of dietary intake or supplemental intake of choline in separate models. Sensitivity analyses considered similarity of associations when supplemental choline intake data were obtained from 24-h recalls versus the 30-day questionnaire.

The primary analyses and statistically significant results were followed by three planned sensitivity analyses. First, participants with <3 blood pressure readings were excluded. Second, participants with CVD endpoints defined as self-reported congestive heart failure, coronary heart disease, angina, heart attack or stroke were excluded. Finally, hypertension status was categorized into “definite” (2 or all 3 classification features), “probable” (1 or 2 classification features) or “possible” (1 classification feature) based on each participant’s available data (Supplemental Methods). All analyses were conducted using SAS (version 9.4, SAS Institute, NC) with a significant threshold of two-sided $P < 0.05$ and a trended association at $P < 0.1$.

3.3 Results

3.3.1 Associations between choline intake and hypertension

The prevalence of hypertension during 2007-10 in the analytic sample was 43%. Hypertensive participants had 12 mg higher energy-adjusted choline intake, a lower proportion of supplemental choline users, and higher SBP and DBP, compared to non-hypertensive participants (Supplementary Table 3.1). Additionally, compared to the non-hypertensive group, the hypertensive group was more likely to be non-Hispanic White or non-Hispanic Black, older, married, post-menopausal, former smoker, high school or lower education level, higher BMI, and lower on physical activity. Compared to non-hypertensives, the hypertensive participants reported consuming fewer calories, but more nutrients overall.

In a multivariate-adjusted model the association of total choline intake with the prevalence odds of hypertension differed by sex ($P_{interaction}$ = 0.04, Figure 3.1). Specifically, women consuming higher amounts of choline tended ($P < 0.1$) to have lower odds of prevalent hypertension compared to women consuming less choline (OR per 100 mg of choline [95% CI], 0.89 [0.77 – 1.02]). In contrast, total choline intake had little to no association with the odds of hypertension in men ($P = 0.54$; graphed in Figure 3.2). There was no evidence for effect modification of the total choline intake—hypertension association by race/ethnicity, BMI or, among female participants, by post-menopausal status or use of hormone replacement therapy (Figure 3.1).

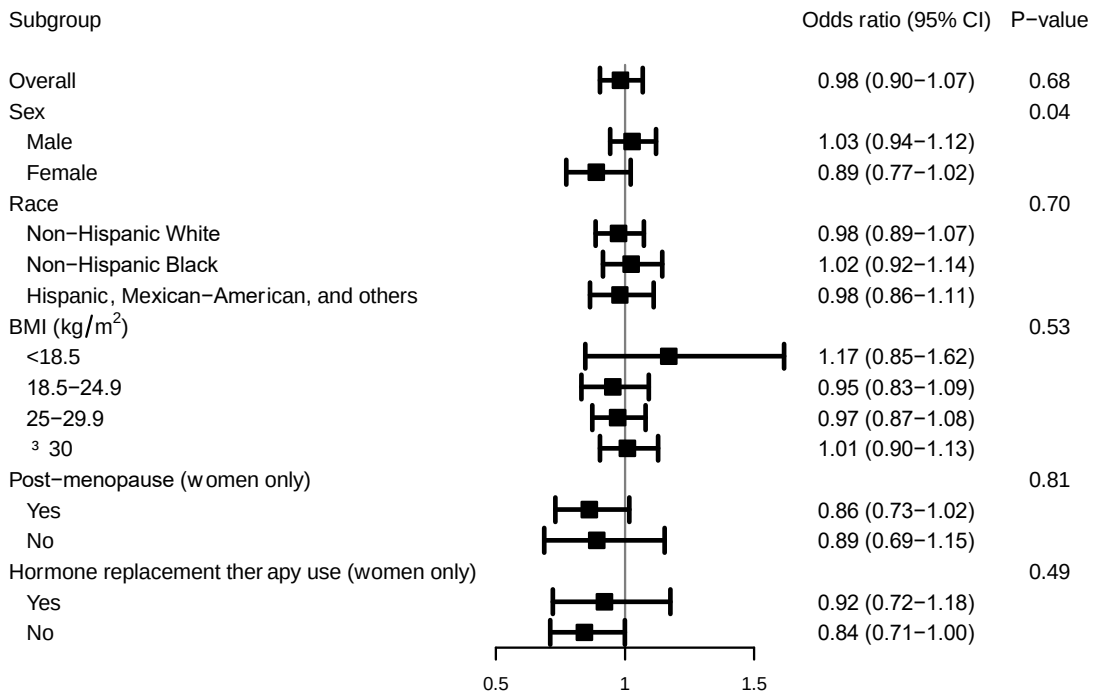

Figure 3.1 Associations between total choline intake and odds of hypertension in 2007-10 National Health and Nutrition Examination Survey by subgroups. All models were adjusted for age, sex, race, education, poverty ratio, BMI and total calories unless the covariate was tested as an effect modifier, in which case it was entered in an interaction term; odds ratios for sex, race and BMI were based on n= 8,349 due to missing data in the covariates; odds ratios for menopausal status and hormone replacement therapy use were based on n = 4,023 and n = 4,011 respectively; odds ratios are expressed per 100 mg of total choline

Because molecular forms of choline in diet could differ from supplemental forms, the separate associations of dietary and supplemental choline with hypertension were investigated. The dietary intake of choline—hypertension association was similar to the findings for total choline intake. Dietary choline was differentially associated with the odds of hypertension by sex ($P_{interaction}$ = 0.03), such that a protective association was observed only in women (0.89, [0.77 – 1.02]; Supplementary Figure 3.2 and Supplementary Figure 3.3).

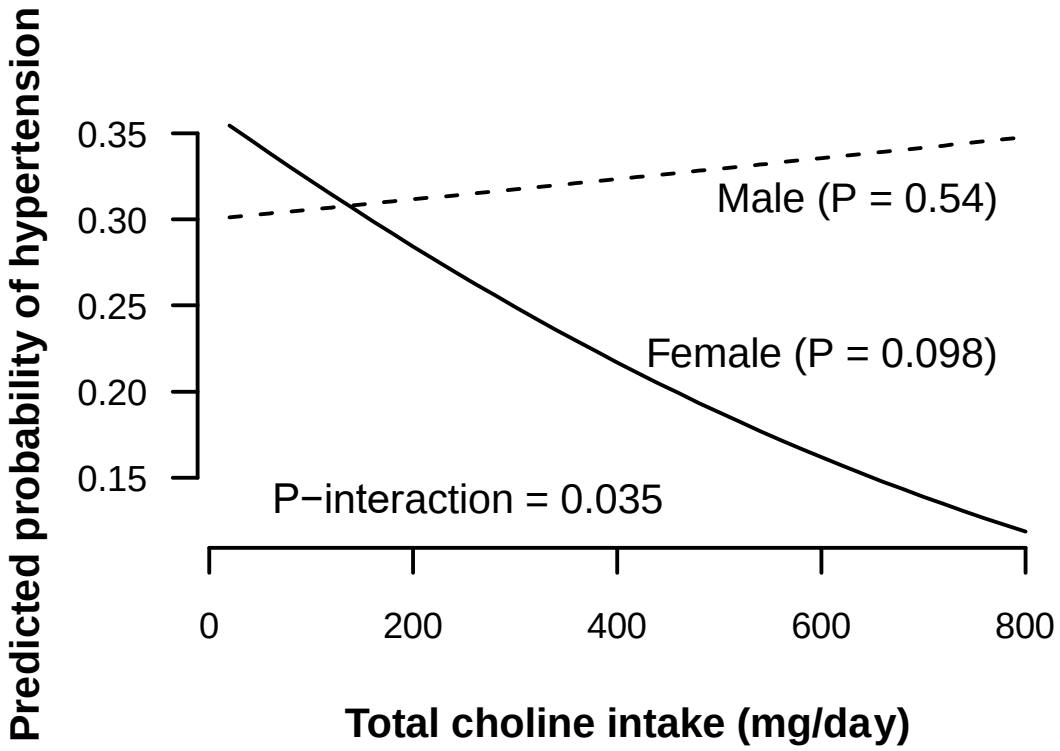

Figure 3.2 Example of predicted probabilities of having hypertension over a range of total choline intake for a male or female who were White, had a college degree, and had mean age (48 y), poverty ratio (3), BMI (28.7 kg/m^2) and total caloric intake (2086.6 kcal/d)

In 2007-10 NHANES the use of supplemental choline was rare, and doses were limited (Supplementary Table 3.2), hindering investigation of the dose-response relationships of supplemental choline with hypertension. Thus, supplemental choline intake was modeled as a binary variable (user vs. non-user). In contrast to the sex-dependent association of dietary choline and the odds of hypertension, supplemental choline use during the past 24 h was associated with lower odds of hypertension in both sexes (0.68 [0.49 – 0.92], Table 3.1). However, in the sensitivity analysis defining supplemental choline use during the past 30 d, the association was attenuated (0.83 [0.61 – 1.13]). In a further analysis, which limited supplement users and non-users to

Table 3.1 Association between total, dietary and supplemental choline intake and prevalent odds of hypertension in 2007-10 National Health and Nutrition Examination Survey (n = 9,227)

		Age-adjusted analysis			Multivariate analysis*		
Choline	Unit of comparison	Odds ratio	95% CI	P	Odds ratio†	95% CI	P
Total intake	1 mg/day	1.00	(1.0, 1.001)	0.50	1.00	(0.999, 1.001)	0.68
	100 mg/day‡	1.01	(0.97, 1.06)		0.98	(0.90, 1.07)	
Dietary intake	1 mg/day	1.00	(1.0, 1.001)	0.45	1.00	(0.999, 1.001)	0.72
	100 mg/day‡	1.02	(0.97, 1.06)		0.98	(0.90, 1.07)	
Supplemental	User vs. non-user	0.57	(0.44, 0.74)	<0.0001	0.68	(0.49, 0.92)	0.01

* Adjusted for age, sex, race, education, poverty ratio, BMI and total calories

† Estimates were based on n= 8,349 due to missing data in the covariates

‡ Estimates were calculated from 1 mg/day models

those participants with a consistent report of use in both the 24-h recalls and the 30-day questionnaire, a statistically significant association between supplemental choline use and the odds of hypertension persisted (0.67 [0.48 – 0.94]).

All results from the main analyses were confirmed in planned sensitivity analyses that first excluded participants with comorbidities, and second, limited the outcome definition to participants with 'definite' hypertension status.

3.3.2 Associations between choline intake and blood pressure

The majority of the participants included in the analysis of the blood pressure outcome (6,416 out of 6,554 total) were also included in the analysis of the hypertension outcome. The subset of participants who were included in the

hypertension outcome models, but who were excluded from the blood pressure outcome models (3,005 out of 9,227 total) were omitted because of the use of antihypertensive medications (n = 2,691) and zero or missing blood pressure (n = 314). Thus, as expected, participants in the blood pressure outcome models had a lower average age and corresponding differences in age-related indices (e.g., lower proportion married, lower proportion post-menopausal, lower SBP and BMI, greater physical activity and caloric intake) compared to participants in the hypertension models.

There was little to no association between total choline intake and blood pressure (change in SBP per 100 mg of choline ± SEM, -0.26 ± 0.22 mmHg, P = 0.23; change in DBP per 100 mg choline, -0.29 ± 0.19 mmHg, P = 0.12, Table 3.2). The findings for dietary choline and supplemental choline use were similarly null (Table 3.2), and there was no evidence for modification of the total choline intake—BP associations by sex, race/ethnicity, BMI or, among female participants, by post-menopausal status or use of hormone replacement therapy.

Because the participants included in the blood pressure outcome models were younger than the participants included in the hypertension models, we investigated age as an effect modifier of choline—blood pressure association to understand if the lack of an association might be age-related. Modeling age as a dichotomy (≥ 65 vs. < 65 y), the choline—SBP association differed by age ($P_{interaction}$ < 0.0001) such that total choline intake was inversely associated with SBP among participants age ≥ 65 y but not among younger participants (-3.29 ± 0.67 mmHg, P < 0.0001 and 0.40 ± 0.22

Table 3.2 Linear associations between total, dietary and supplemental choline intake and blood pressure in 2007-10 National Health and Nutrition Examination Survey (n = 6,554)

		Systolic blood pressure						Diastolic blood pressure					
		Age-adjusted analysis			Multivariate analysis*			Age-adjusted analysis			Multivariate analysis*		
Choline	Unit of comparison	β	SEM	P	β†	SEM	P	β	SEM	P	β†	SEM	P
Total intake	1 mg/day	0.007	0.002	<0.001	-0.003	0.002	0.23	0.005	0.001	0.0002	-0.003	0.002	0.12
	100 mg/day‡	0.653	0.158		-0.264	0.220		0.514	0.135		-0.291	0.185	
Dietary intake	1 mg/day	0.007	0.002	0.01	-0.002	0.002	0.26	0.005	0.001	0.0001	-0.003	0.002	0.13
	100 mg/day‡	0.666	0.159		-0.247	0.221		0.519	0.135		-0.284	0.185	
Supplement	User vs. non-user	-2.207	1.080	0.04	-0.635	1.060	0.55	-0.31	1.058	0.77	-0.645	1.135	0.95

* Adjusted for age, sex, race, education, poverty ratio, BMI and total calories

† Estimates were based on n= 5,939 due to missing data in the covariates

‡ Estimates were calculated from 1 mg/day models

mmHg, P = 0.07, respectively, Supplementary Table 3.3). Age did not modify the association of choline with DBP ($P_{interaction}$ = 0.86, Supplementary Table 3.3).

3.4 Discussion

This study investigated the association of choline intake with hypertension and blood pressure. Overall, the findings do not support an adverse effect of dietary choline on blood pressure, and do not fit the hypothesized direction of association. A higher total choline intake, comprised primarily of dietary sources of choline, tended to be associated with lower odds of hypertension limited to women only, and

supplemental choline use was statistically significantly associated with a lower odds of hypertension in both sexes. In analyses of choline and blood pressure, higher choline intake was associated with lower SBP, but only in adults aged ≥ 65 y.

The hypothesis that higher dietary choline intake associates with an increased risk of hypertension is based on a plethora of studies (both human and animal) that reported direct and positive associations between the choline-derivative TMAO and cardiovascular outcomes. However, our findings, which show an inverse association of total or dietary choline intake with odds of hypertension and blood pressure, are consistent with other human studies that have reported associations between dietary choline intake and risk of atherosclerosis [19,20] and the development of CVD [21–23]. A cross-sectional study in a Greek population showed that dietary choline intake was inversely associated with levels of inflammatory markers [19], while a cohort study observed no association between dietary choline and markers of inflammatory and atherosclerotic states [20]. Additionally, two other prospective cohort studies found no associations between dietary choline and incident CVD [21,22]. A cohort study in African Americans reported an inverse association of dietary choline with stroke incidence in the overall population, and with subclinical markers of CVD risk in women only [23]. To our knowledge, only one study reported that an increased dietary choline intake was associated with an increased CVD mortality, and the same study found no association with incident CVD [24]. Since a positive association was observed only with CVD mortality and not incidence, the dietary choline-CVD relationship may depend on the stage of the disease. For example, in patients with advanced CVD, dietary choline could exacerbate the risk of cardiovascular

complications, possibly through its role in hepatic lipid export [25,26] or via the production of TMAO. We attempted to test this hypothesis by excluding participants with existing CVD, but the effect estimates were unchanged. Furthermore, we did not observe differential choline-blood pressure associations across ranges of blood pressure (≥140/90 mmHg and <140/90 mmHg).

It is noteworthy that among women, both total and dietary choline intake tended ($P < 0.10$) to be inversely associated with odds of hypertension, while supplemental choline use by both sexes showed a significant inverse association with odds of hypertension. The results of models for the hypertension outcome were not consistent with the results of models for the blood pressure outcome. A possible explanation for this inconsistency in findings is the difference in the age distribution between the participants in the hypertension model and the participants in the blood pressure model. Compared to the participants in the hypertension model, participants in the blood pressure model were younger and had different distributions on age-related traits as well. The differences in findings between the hypertension and blood pressure outcomes may arise due to these differences. If the choline—BP association differed by age, then different age distributions would contribute to the inconsistent findings for choline—hypertension and choline—BP. Indeed, the finding that choline was inversely associated with SBP only in participants age ≥ 65 y supports this explanation for the pattern of findings.

Given the inverse associations, we speculated that a possible mechanism for the protective effect of dietary choline on blood pressure may involve increased endogenous production of a phosphatidylcholine (PC) molecule that is enriched in

docosahexaenoic acid (DHA). DHA has been shown to reduce blood pressure and heart rate [27] and improve vascular reactivity in generally healthy adults [28]. Endogenous synthesis of PC-DHA is catalyzed by phosphatidylethanolamine *N*-methyltransferase (PEMT), an enzyme that is regulated by estrogen [29] A higher dietary choline intake has been shown to enhance the activity of the PEMT pathway and increase hepatic PC-DHA export among women [30]. In addition, animal data have demonstrated that female mice use more dietary choline to generate PC via the PEMT pathway than male mice [31]. These findings may collectively explain our observation of a borderline significant inverse association between dietary choline intake among women, but not men. Other mechanisms by which choline may benefit cardiovascular health include the reduction of plasma homocysteine [21] and vasodilation of vascular smooth muscle by the neurotransmitter acetylcholine [32]. Notably, reduced synthesis of acetylcholine is particularly prevalent among older adults [33,34], and this may contribute to the inverse association of total choline intake with SBP in this age group.

Sources of choline might also affect its relationship with health outcomes. Based on 24-h supplement usage information, we observed 32% lower odds of hypertension among supplemental choline users as compared to non-users. This effect estimate was diluted when 30-day supplement use information was used, indicating that measurement error likely existed in the categorization of choline supplement use. To address measurement error, we restricted the analysis to individuals who reported supplement use consistently on both the 24-hr recall and the 30-day questionnaire; there was a 33% reduction in the odds of hypertension associated with the use of a

choline supplement. In addition to reducing measurement error, we tested for additional confounding by smoking status and by any dietary supplement use and found no change to the conclusion.

The beneficial association of supplemental choline with the risk of hypertension may be mediated by the release of free choline molecules from choline bitartrate, the prevalent form of supplemental choline reported by users. Free choline is absorbed more rapidly than PC [35], the main form of choline in foods. A large proportion of absorbed free choline can be irreversibly oxidized to betaine in the intestinal cells and the liver [36]. Betaine is a methyl donor and osmolyte whose plasma concentration was inversely associated with several CVD risk factors, including blood pressure [37]. A potential increase in this metabolite following choline supplement use may mediate the observed beneficial association with hypertension.

We acknowledge several limitations of the present study. First, the use of 24-hour recalls represents a snapshot in time of an individual's diet. Intra-individual variation may dilute the effect estimates and could account for the reported non-significant findings. Second, misclassification of hypertension status could also bias the effect estimates toward the null. However, this issue may have limited influence because we found similar effect estimates when excluding participants with a less definitive diagnosis. Third, the limited number of supplement users and severe skewness in supplement dose data prevented an investigation of a dose-response relationship between supplemental choline and the outcomes. Related to this issue, we acknowledge that the inverse association observed by comparing users vs. non-users of choline supplements may have been confounded by lifestyle factor differences

between these groups leading to reverse causality. Adjustment for lifestyle-related confounders did not change the conclusion, although residual confounding may still remain. Last, reverse causality may be a more general issue given that hypertensive individuals may have modified their dietary patterns or taken dietary supplements in response to their diagnosis. Excluding individuals with CVD-related comorbidities did not substantially change the effect estimates, but it is not possible to overcome this limitation in this study design.

Strengths of this study include use of both hypertension and blood pressure as outcomes to derive the conclusions, and testing for and demonstration of a sex-specific association. This study also highlights the possible importance of free choline found in supplements in relation to hypertension. Finally, by using samples drawn from a nationally representative dataset, results of this study are generalizable to the U.S. population.

In conclusion, data from this cross-sectional study do not support a positive association between choline intake and blood pressure. The tendency for an inverse association between total or dietary choline intake and odds of hypertension in women suggests that choline intake may have a beneficial association limited to this subgroup. Use of supplemental choline was associated with a lower odds of hypertension, suggesting that sources of choline may be important in determining the associations with disease risk.

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AUTHOR CONTRIBUTIONS

ST and PAC designed research. ST conducted research, analyzed data and wrote paper. FV and PAC assisted in data analysis. MAC assisted in manuscript preparation. All authors read and approved the final manuscript.

REFERENCES

[1] Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R, et al. Heart Disease and Stroke Statistics—2017 Update: A Report From the American Heart Association. 2017. doi:10.1161/CIR.0000000000000485.

[2] Zieman SJ, Melenovsky V, Kass DA. Mechanisms, pathophysiology, and therapy of arterial stiffness. Arterioscler Thromb Vasc Biol 2005;25:932–43. doi:10.1161/01.ATV.0000160548.78317.29.

[3] Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 2011;472:57–63. doi:10.1038/nature09922.

[4] Bennett BJ, de Aguiar Vallim TQ, Wang Z, Shih DM, Meng Y, Gregory J, et al. Trimethylamine-N-oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. Cell Metab 2013;17:49–60. doi:10.1016/j.cmet.2012.12.011.

[5] Heianza Y, Ma W, Manson JE, Rexrode KM, Qi L. Gut Microbiota Metabolites and Risk of Major Adverse Cardiovascular Disease Events and Death: A Systematic Review and Meta-Analysis of Prospective Studies. J Am Heart Assoc 2017;6:e004947. doi:10.1161/JAHA.116.004947.

[6] Meyer K, Shea J. Dietary Choline and Betaine and Risk of CVD: A Systematic Review and Meta-Analysis of Prospective Studies. Nutrients 2017;9:711. doi:10.3390/nu9070711.

[7] Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat Med 2013;19:576–85. doi:10.1038/nm.3145.

[8] Collins HL, Drazul-Schrader D, Sulpizio AC, Koster PD, Williamson Y, Adelman SJ, et al. L-Carnitine intake and high trimethylamine N-oxide plasma levels correlate with low aortic lesions in ApoE-/- transgenic mice expressing CETP. Atherosclerosis 2016;244:29–37. doi:10.1016/j.atherosclerosis.2015.10.108.

[9] Ufnal M, Jazwiec R, Dadlez M, Drapala A, Sikora M, Skrzypecki J. Trimethylamine-N-oxide: a carnitine-derived metabolite that prolongs the hypertensive effect of angiotensin II in rats. Can J Cardiol 2014;30:1700–5. doi:10.1016/j.cjca.2014.09.010.

[10] Centers for Disease Control and Prevention (CDC). National Center for Health Statistics (NCHS). National Health and Nutrition Examination Survey Data 2009.

[11] Centers for Disease Control and Prevention (CDC). National Center for Health Statistics (NCHS). National Health and Nutrition Examination Survey Data 2011.

[12] Zipf G, Chiappa M, Porter KS, Ostchega Y, Lewis BG, Dostal J. National health and nutrition examination survey: plan and operations, 1999-2010. Vital Health Stat 1 2013:1–37. doi:312'.0723s 80-607914.

[13] Raper N, Perloff B, Ingwersen L, Steinfeldt L, Anand J. An overview of USDA's Dietary Intake Data System. J Food Compos Anal 2004;17:545–55. doi:10.1016/j.jfca.2004.02.013.

[14] Moshfegh AJ, Rhodes DG, Baer DJ, Murayi T, Clemens JC, Rumpler W V, et al. The US Department of Agriculture Automated Multiple-Pass Method reduces bias in the collection of energy intakes. Am J Clin Nutr 2008;88:324–32.

[15] Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). National Health and Nutrition Examination Survey Examination Protocol 2007.

[16] John C, Paulose-Ram R, Ogden C. National Health and Nutrition Examination Survey: Analytic Guidelines, 1999–2010. Vital Heal Stat 2013;2.

[17] SAS Institute. SAS/STAT Estimating the Standard Deviation of a Variable in a Finite Population n.d. https://support.sas.com/rnd/app/stat/examples/SurveyStdDev/new_example/index.html (accessed March 14, 2018).

[18] Willett W, Stampfer MJ. Total energy intake: implications for epidemiologic analyses. Am J Epidemiol 1986;124:17–27.

[19] Detopoulou P, Panagiotakos DB, Antonopoulou S, Pitsavos C, Stefanadis C. Dietary choline and betaine intakes in relation to concentrations of inflammatory markers in healthy adults: the ATTICA study. Am J Clin Nutr 2008;87:424–30.

[20] Fargnoli JL, Fung TT, Olenczuk DM, Chamberland JP, Hu FB, Mantzoros CS. Adherence to healthy eating patterns is associated with higher circulating total and high-molecular-weight adiponectin and lower resistin concentrations in women from the Nurses' Health Study. Am J Clin Nutr 2008;88:1213–24. doi:10.3945/AJCN.2008.26480.

[21] Dalmeijer GW, Olthof MR, Verhoef P, Bots ML, van der Schouw YT. Prospective study on dietary intakes of folate, betaine, and choline and cardiovascular disease risk in women. Eur J Clin Nutr 2008;62:386–94. doi:10.1038/sj.ejcn.1602725.

[22] Bidulescu A, Chambless LE, Siega-Riz AM, Zeisel SH, Heiss G. Usual choline and betaine dietary intake and incident coronary heart disease: the Atherosclerosis Risk in Communities (ARIC) study. BMC Cardiovasc Disord 2007;7:20. doi:10.1186/1471-2261-7-20.

[23] Millard HR, Musani SK, Dibaba DT, Talegawkar SA, Taylor HA, Tucker KL, et al. Dietary choline and betaine; associations with subclinical markers of cardiovascular disease risk and incidence of CVD, coronary heart disease and stroke: the Jackson Heart Study. Eur J Nutr 2016:1–10. doi:10.1007/s00394-016-1296-8.

[24] Zheng Y, Li Y, Rimm EB, Hu FB, Albert CM, Rexrode KM, et al. Dietary phosphatidylcholine and risk of all-cause and cardiovascular-specific mortality

among US women and men. Am J Clin Nutr 2016:ajcn131771. doi:10.3945/ajcn.116.131771.

[25] Jacobs RL, Devlin C, Tabas I, Vance DE. Targeted deletion of hepatic CTP:phosphocholine cytidylyltransferase alpha in mice decreases plasma high density and very low density lipoproteins. J Biol Chem 2004;279:47402–10. doi:10.1074/jbc.M404027200.

[26] Noga AA, Vance DE. A gender-specific role for phosphatidylethanolamine N-methyltransferase-derived phosphatidylcholine in the regulation of plasma high density and very low density lipoproteins in mice. J Biol Chem 2003;278:21851–9. doi:10.1074/jbc.M301982200.

[27] Mori TA, Bao DQ, Burke V, Puddey IB, Beilin LJ. Docosahexaenoic Acid but Not Eicosapentaenoic Acid Lowers Ambulatory Blood Pressure and Heart Rate in Humans. Hypertension 1999;34:253–60. doi:10.1161/01.HYP.34.2.253.

[28] Mori TA, Watts GF, Burke V, Hilme E, Puddey IB, Beilin LJ. Differential Effects of Eicosapentaenoic Acid and Docosahexaenoic Acid on Vascular Reactivity of the Forearm Microcirculation in. Circulation 2000;102:1264–9.

[29] Resseguie M, Song J, Niculescu MD, da Costa K-A, Randall TA, Zeisel SH. Phosphatidylethanolamine N-methyltransferase (PEMT) gene expression is induced by estrogen in human and mouse primary hepatocytes. FASEB J 2007;21:2622–32. doi:10.1096/fj.07-8227com.

[30] West AA, Yan J, Jiang X, Perry CA, Innis SM, Caudill MA. Choline intake influences phosphatidylcholine DHA enrichment in nonpregnant women but not in pregnant women in the third trimester. *Am J Clin Nutr*. 2013;97(4):718-727. doi:10.3945/ajcn.112.050211.

[31] Chew TW, Jiang X, Yan J, et al. Folate intake, MTHFR genotype, and sex modulate choline metabolism in mice. *J Nutr*. 2011;141(8):1475-1481. doi:10.3945/jn.111.138859.

[32] Furchgott RF, Zawadzki J V. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*. 1980;288(5789):373-376. doi:10.1038/288373A0.

[33] Cohen BM, Renshaw PF, Stoll AL, Wurtman RJ, Yurgelun-Todd D, Babb SM. Decreased Brain Choline Uptake in Older Adults. *JAMA*. 1995;274(11):902. doi:10.1001/jama.1995.03530110064037.

[34] Gibson GE, Peterson C, Jenden DJ. Brain acetylcholine synthesis declines with senescence. *Science*. 1981;213(4508):674-676. doi:10.1126/SCIENCE.7256270.

[35] Cheng WL, Holmes-McNary MQ, Mar MH, Lien EL, Zeisel SH. Bioavailability of choline and choline esters from milk in rat pups. *J Nutr Biochem*. 1996.

[36] Flower RJ, Pollitt RJ, Sanford PA, Smyth DH. Metabolism and transfer of choline in hamster small intestine. *J Physiol*. 1972;226(2):473-489. doi:10.1113/jphysiol.1972.sp009994.

[37] Konstantinova S V., Tell GS, Vollset SE, Nygard O, Bleie O, Ueland PM. Divergent Associations of Plasma Choline and Betaine with Components of Metabolic Syndrome in Middle Age and Elderly Men and Women. *J Nutr*. 2008;138(5):914-920.

CONCLUSION

SUMMARY

In this dissertation, the roles of diet in the metabolism of trimethylamine-*N*-oxide (TMAO), an emerging factor in cardiovascular disease (CVD), are examined. In Chapter 1, we show that consumption of fish, which is high in TMAO, yields substantially greater increases in circulating TMAO than eggs or beef, which contain high amounts of dietary TMAO precursors. TMAO response to food sources varies as a function of gut microbiome. Specifically, men with higher Firmicutes to Bacteroidetes microbial ratios exhibit greater TMAO responses to dietary precursor intakes.

In Chapter 2, we show that absorption of orally consumed TMAO is near complete, and may occur largely by a process that is independent of gut microbes and hepatic flavin-containing monooxygenases (FMOs). In healthy men, TMAO exhibits fast turnover in the circulation with the majority being eliminated in urine. A small portion of the dose, however, is taken up by extrahepatic tissue in a manner that correlates with *FMO3* G472A polymorphism.

In Chapter 3, we further investigate the cross-sectional relationship between dietary choline and blood pressure in the 2007-10 National Health and Nutritional Examination Survey (NHANES). Despite being a precursor of TMAO, our data do not support a positive association between choline intake and blood pressure. Specifically, total or dietary choline intake tends to be inversely associated with odds of

hypertension in women, and use of supplemental choline is associated with lower odds of hypertension.

STUDY IMPLICATIONS

Evidence in this dissertation indicates that circulating concentrations of TMAO in healthy adults are temporarily elevated upon consumption of choline and TMAO food sources (eggs, beef and fish). Previous research has positively associated dietary choline with CVD risk due to its TMAO-raising characteristics. However, we demonstrate here that a source of TMAO in the body also comes from heart-healthy food such as fish. Food sources of choline and TMAO also contain nutrients that are critical for health, and thus caution is warranted when proposing dietary recommendations that restrict the intakes of certain foods because of their TMAO-raising characteristics.

Additionally, we show that FMOs and gut microbiota may influence TMAO metabolism. *FMO3* G472A polymorphism correlates with the uptake of TMAO relative to trimethylamine into skeletal muscle, while gut microbiome composition is associated with individual TMAO production from choline-rich foods. The higher Firmicutes to Bacteroidetes ratio among men with greater TMAO production has also been associated with increased risk of obesity and metabolic syndrome; supporting the notion that gut microbiota and FMOs influence plasma TMAO and potentially CVD risk.

The dietary choline/TMAO—CVD relationship may depend on the health status of individuals. To this end, we traced the metabolic fates of orally consumed TMAO and show that it is rapidly and efficiently eliminated in healthy men. These results suggest that dietary TMAO may not chronically elevate circulating TMAO in healthy individuals, unlike the elevated baseline TMAO status that is common among cardiac or renal patients. To further examine whether dietary sources of TMAO could be linked to disease in a non-patient population, we investigated the relationship of dietary choline with hypertension, a major public health concern and risk factor of CVD. Findings from the 2007-10 NHANES data do not support a positive association between choline intake and blood pressure or prevalence odds of hypertension.

SUPPLEMENTARY TABLES AND FIGURES

Chapter 1. Trimethylamine-N-oxide (TMAO) response to animal source foods varies among healthy young men and is influenced by their gut microbiota composition: a randomized controlled trial

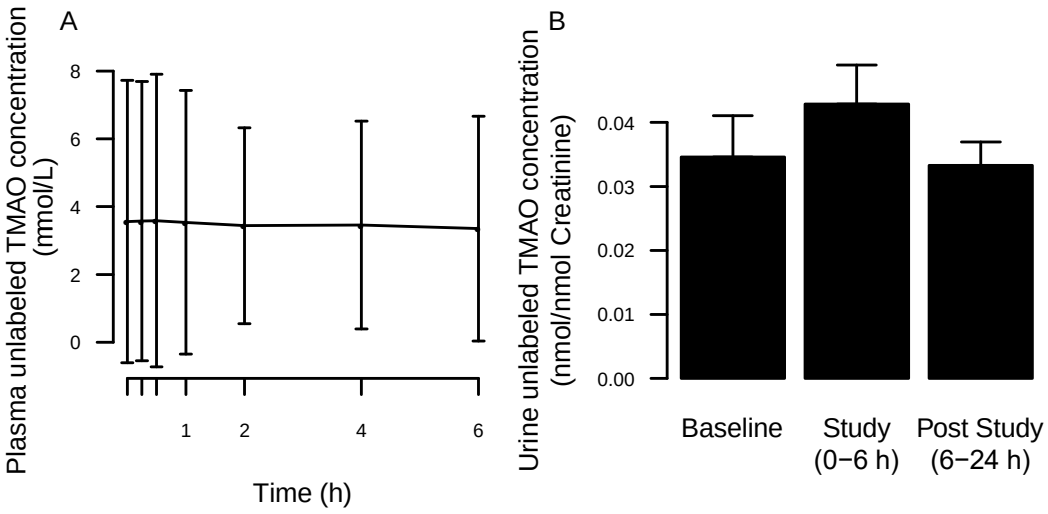
Supplementary Table 1.1 Participant characteristics and baseline measures for high- and low-TMAO producers

	High-TMAO Producers (n = 11)	Low-TMAO Producers (n = 15)[1]
Participant Characteristics		
Age (y)	29.2 ± 1.8	27.1 ± 1.6
BMI (kg/m^2)	24.8 ± 0.7	23.7 ± 0.6
Genotype		
GG %	36	40
GA %	55	47
AA %	9	13
Blood Chemistry Concentrations (all serum)		
HDL (mg/dL)	56 ± 5	56 ± 3
LDL (mg/dL)	117 ± 10	124 ± 6
Cholesterol (mg/dL)	176 ± 8	174 ± 8
Triglycerides (mg/dL)	74 ± 11	74 ± 9
Total bilirubin (mg/dL)	0.8 ± 0.1	0.8 ± 0.1
Direct bilirubin (mg/dL)	0.2 ± 0.0	0.2 ± 0.0
ALP (U/L)	70 ± 5	71 ± 3
Cr (mg/dL)	1.1 ± 0.0	1.1 ± 0.0
CK (U/L)	133 ± 16	147 ± 16

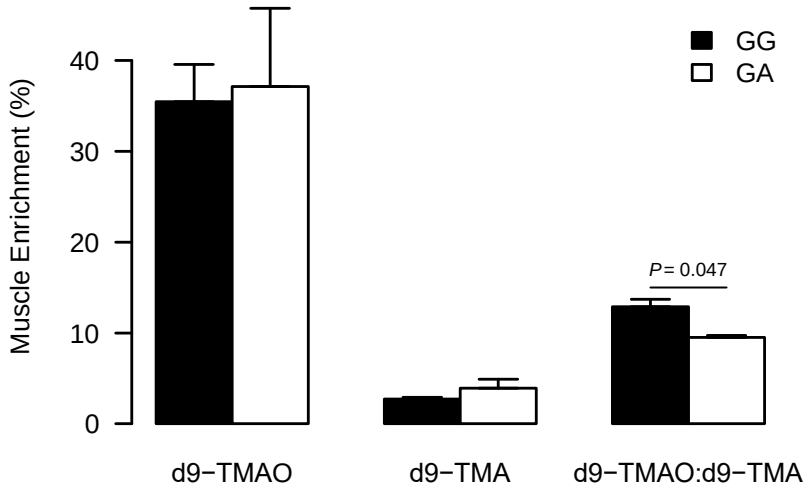
LDH (U/L)	161 ± 6	153 ± 4
Amylase (U/L)	56 ± 4	52 ± 5
Lipase (U/L)	135 ± 10	151 ± 14
AST (U/L)	22 ± 1	21 ± 1
ALT (U/L)	36 ± 2	32 ± 4
GGT (U/L)	35 ± 2	30 ± 2
Total protein (g/dL)	7.7 ± 0.1	7.6 ± 0.1
Albumin (g/dL)	4.2 ± 0.0	4.4 ± 0.0
BUN (mg/dL)	14 ± 1	14 ± 1
Cell Counts		
WBC ($\times 10^3/\mu L$)	5.6 ± 0.4	5.5 ± 0.2
Lymphocytes ($\times 10^3/\mu L$)	1.9 ± 0.2	1.9 ± 0.1
Monocytes ($\times 10^3/\mu L$)	0.2 ± 0.0	0.2 ± 0.0
Granulocytes ($\times 10^3/\mu L$)	3.5 ± 0.4	3.4 ± 0.2
RBC ($\times 10^3/\mu L$)	5.2 ± 0.1	5.2 ± 0.1
TMAO (nmol/mL)	3.4 ± 0.5	3.4 ± 0.2
TMA (pmol/mL)	19.0 ± 1.9	20.4 ± 1.4
DMA (nmol/mL)	1.9 ± 0.1	2.0 ± 0.0
MA (nmol/mL)	ND	ND
Urinary Methylamine Excretion		
TMAO (nmol/mmol Cr)	27.4 ± 2.1	28.7 ± 1.7
TMA (nmol/mmol Cr)	0.2 ± 0.0	0.1 ± 0.0
DMA (nmol/mmol Cr)	25.8 ± 0.5	27.7 ± 0.5
MA (nmol/mmol Cr)	5.1 ± 1.1	3.9 ± 0.4

[1] No differences were detected between high- and low-TMAO producers in the participant characteristics and baseline measures.

Chapter 2. The metabolic fate of isotopically labeled trimethylamine-N-oxide (TMAO) in humans



Supplementary Figure 2.1 A) Plasma unlabeled trimethylamine-N-oxide (d0-TMAO) concentration remained unchanged throughout the 6-h study period. B) Urinary unlabeled TMAO concentration did not change statistically from baseline to 24-h post study. Data was analyzed using repeated measure mixed linear model with P = 0.05 threshold and the Tukey-Kramer post-hoc test for multiple comparison correction. Values are mean ± SD for A) and SEM for B); n = 40 per time point.



Supplementary Figure 2.2 *FMO3* 472GA genotype exhibited a lower d9-TMAO:d9-TMA enrichment ratio than the wild-type GG genotype. Data was analyzed using t-test with P = 0.05 threshold and the Bonferroni adjustment for multiple comparison. Values are mean ± SEM; n = 3 per genotype per metabolite. Abbreviation: d9-TMA = Deuterium-labeled methyl d9-trimethylamine; d9-TMAO = d9-trimethylamine-N-oxide

Chapter 3. Relationship of choline intake with blood pressure in the National Health And Nutrition Examination Survey 2007-10

Supplementary Methods

The primary analyses and statistically significant results were followed by three planned sensitivity analyses. First, participants with <3 blood pressure readings were excluded. Second, participants with CVD endpoints defined as self-reporting of congestive heart failure, coronary heart disease, angina, heart attack or stroke were excluded. Finally, hypertension status was categorized into “definite”, “probable” or “possible” based on each participant’s available data. Definite was defined as the presence of 2 or all 3 classification features, including doctor’s diagnosis and medication use, or doctor’s diagnosis and high blood pressure, or doctor’s diagnosis, medication use and high blood pressure. Probable status was defined as presence of 1 or 2 classification features, including doctor’s diagnosis only, or medication use and high blood pressure. Possible status was defined as the remaining categories, including medication use only or high blood pressure only.

Supplementary Tables

Supplementary Table 3.1 Participant characteristics in 2007-10 National Health and Nutrition Examination Survey

	Hypertensive (n=3933)*		Non-hypertensive (n=5294)†		Total for hypertension analysis (n=9227)‡		Total for blood pressure analysis (n=6554)§	
	Means	SD	Means	SD	Means	SD	Means	SD
Exposure								
Energy-adjusted choline intake (mg/day)‖¶	326	107	314	112	319	110	316	112
Outcomes								
Systolic blood pressure (mmHg)‖	134	20	115	11	121	17	118	15
Diastolic blood pressure (mmHg)‖	73	14	69	10	70	12	71	11
Demographics								
Male (%)	47.5		48.0		47.8		49.4	
Age (y)‖	58.7	14.5	42.0	14.7	48.0	16.7	43.5	15.3
Race/ethnicity (%)‖								
Non-Hispanic White‖	73.7		69.7		71.2		70.0	
Non-Hispanic Black‖	13.9		9.3		11.0		9.8	
Mexican American, Hispanic, Others‖	12.4		21.0		17.9		20.2	
Ratio of family income to poverty	3.0	1.6	3.1	1.6	3.0	1.6	3.1	1.7
Education level (%)‖								
< 9th grade‖	7.6		5.1		6.0		5.4	

9-11th grade‖	14.2		11.5		12.5		11.8	
High school‖	26.6		22.7		24.1		23.0	
College	29.6		29.8		29.7		30.3	
Graduate‖	22.0		31.0		27.7		29.6	
Body mass index (kg/m^2)‖	30.9	7.1	27.5	6.0	28.7	6.6	27.8	6.2
Married (%)‖	61.2		55.8		57.8		55.8	
Moderate-vigorous activities (h/wk)‖	8.8	15.3	12.8	18.1	11.3	17.2	12.5	17.9
Smoking status (%)‖								
Non-smoker‖	50.5		56.3		54.2		55.4	
Former smoker‖	32.8		21.5		25.6		22.1	
Current smoker‖	16.6		22.2		20.1		22.4	
Post-menopausal (%)‖	78.3		31.2		48.6		35.7	
Hormone replacement therapy usage (%)‖	35.9		15.8		23.2		17.8	
Intakes per day¶								
Total energy (kcal)‖	1982	795	2143	844	2087	831	2146	853
Energy-adjusted fat (g)‖	77	17	74	18	75	18	74	18
Energy-adjusted cholesterol (g)‖	281	144	267	154	278	151	269	154
Energy-adjusted protein (g)	80	19	79	21	79	20	79	21
Energy-adjusted sodium (mg)	3355	871	3344	883	3348	879	3332	887
Energy-adjusted folate (dietary folate equivalents)‖	832	551	736	486	770	512	750	516
Energy-adjusted vitamin B6 (mg)	6.1	15.7	5.2	12.3	5.5	13.6	5.4	12.8

Energy-adjusted vitamin B12 (μg)‖	55.4	331.337.1	220.8 43.5	265.2	40.4	245.6

* Due to missing values, final sample sizes varied: systolic blood pressure (n = 3,841), diastolic blood pressure (n = 3,813), ratio of family income to poverty (n = 3,612), education (n = 3,926), body mass index (n = 3,868), % married (n = 3,931), moderate-vigorous activities (n = 3,921), % post-menopausal (women only; n = 1,920), hormone replacement therapy usage (women only; n = 1,911)

† Due to missing values, final sample sizes varied: ratio of family income to poverty (n = 4,834), education (n = 5,289), body mass index (n = 5,263), % married (n = 5,291), moderate-vigorous activities (n = 5,283), smoking status (n = 5,293), % post-menopausal (women only; n = 2,514), hormone replacement therapy usage (women only; n = 2,510)

‡ Due to missing values, final sample sizes varied: systolic blood pressure (n = 9,135), diastolic blood pressure (n = 9,107), ratio of family income to poverty (n = 8,446), education (n = 9,215), body mass index (n = 9,131), % married (n = 9,222), moderate-vigorous activities (n = 9,204), smoking status (n = 9,226), % post-menopausal (women only; n = 4,434), hormone replacement therapy usage (women only; n = 4,421)

§ Due to missing values, final sample sizes varied: ratio of family income to poverty (n = 5,985), education (n = 6,545), body mass index (n = 6,511), % married (n = 6,551), moderate-vigorous activities (n = 6,541), smoking status (n = 6,553), % post-menopausal (women only; n = 3,058), hormone replacement therapy usage (women only; n = 3,051)

|| Hypertensive group differed significantly ($p < 0.05$) from the non-hypertensive based on a Rao-Scott likelihood ratio chi-square test (categorical variables) or a T-test (continuous variables); all tests were adjusted for complex survey design

¶ Total intake (diet and supplement) was used and standardized to 2,000 kcal/d for all nutrients

Supplementary Table 3.2 Choline intake from diet and supplements in 2007-10 National Health and Nutrition Examination Survey

	Hypertensive (n=3933)*		Non-hypertensive (n=5294)†		Total for hypertension analysis (n=9227)‡		Total for blood pressure analysis (n=6554)§	
	Means (n)	SD	Means (n)	SD	Means (n)	SD	Means (n)	SD
Energy-adjusted dietary choline intake (mg/day)*	326 (3,933)	106	313 (5,294)	111	318 (9,227)	110	315 (6,554)	112
Supplemental choline use over 24 h (%)*	2.5 (3,933)		4.2 (5,294)		3.6 (9,227)		4.0 (6,554)	
Supplemental choline intake over 24 h (mg/day)	38 (100)	71	24 (176)	33	27 (276)	46	24 (219)	34
Supplemental choline use over 30 days (%)*	3.1 (3,932)		4.7 (5,292)		4.2 (9,224)		4.5 (6,552)	
Supplemental choline intake over 30 days (mg/day)	51 (106)	88	24 (213)	45	31 (319)	61	27 (263)	49

* Hypertensive group differed significantly ($p < 0.05$) from the non-hypertensive based on a Rao-Scott likelihood ratio chi-square test (categorical variables) or a T-test (continuous variables); all tests were adjusted for complex survey design

Supplementary Table 3.3 Age-dependent associations between total choline intake and blood pressure in 2007-10 National Health and Nutrition Examination Survey*

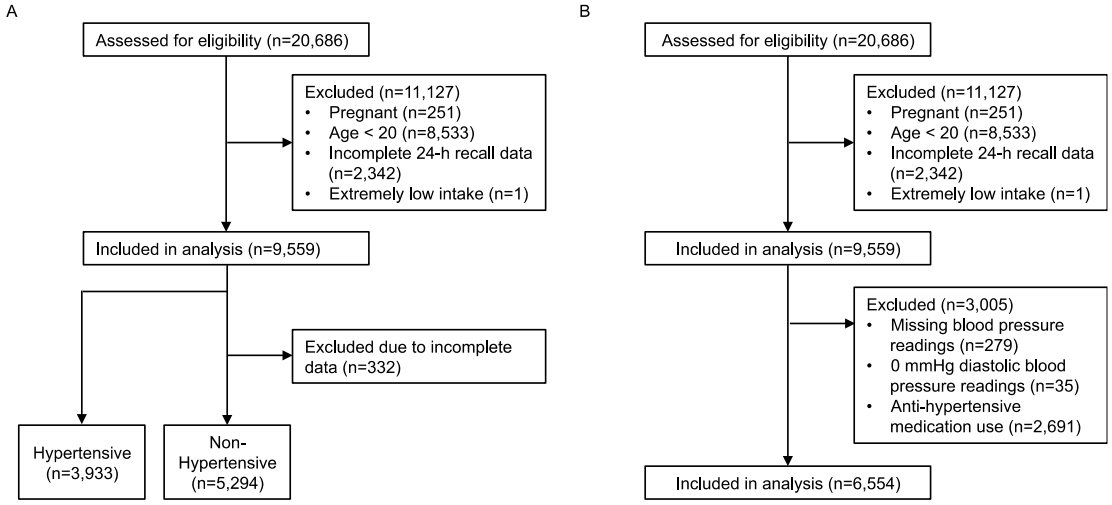
		Systolic blood pressure			Diastolic blood pressure		
	Unit of comparison	β†	SEM	P-interaction	β†	SEM	P-interaction
Age ≥ 65 y	1 mg/day	-0.033	0.007	< 0.0001	-0.001	0.002	0.86
	100 mg/day‡	-3.293	0.671		-0.147	0.194	
Age < 65 y	1 mg/day	0.004	0.002		-0.006	0.005	
	100 mg/day‡	0.405	0.222		-0.063	0.462	

* Adjusted for sex, race, education, poverty ratio, BMI and total calories

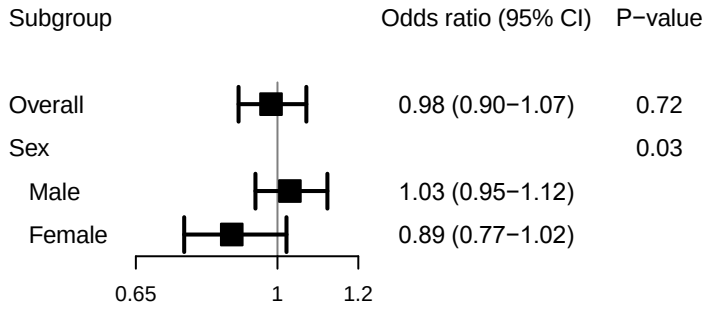
† Estimates were based on n= 5,939 due to missing data in the covariates

‡ Estimates were calculated from 1 mg/day models

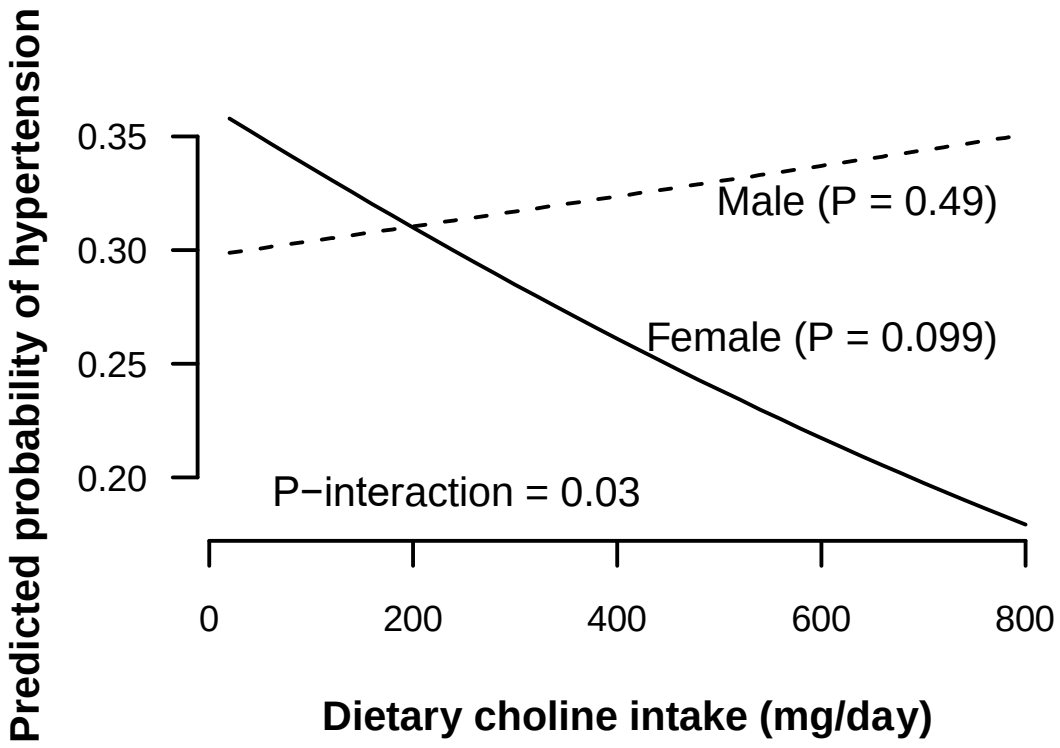
Supplementary Figures



Supplementary Figure 3.1 Participant flow charts for A) the analysis of hypertension and B) blood pressure



Supplementary Figure 3.2 Sex-dependent associations between dietary choline intake and odds of hypertension in 2007-10 National Health and Nutrition Examination Survey. Models were adjusted for age, sex, race, education, poverty ratio, BMI and total calories; odds ratios were based on n= 8,349 due to missing data in the covariates and are expressed per 100 mg of dietary choline



Supplementary Figure 3.3 Example of predicted probabilities of having hypertension over a range of dietary choline intake for a male or female who were White, had a college degree, and had mean age (48 y), poverty ratio (3), BMI (28.7 kg/m^2) and total caloric intake (2086.6 kcal/d)