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Impact of short-term flavanol supplementation on fasting plasma trimethylamine N-oxide concentrations in obese adults

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ABSTRACT

The gut microbiome metabolizes choline and carnitine to release trimethylamine (TMA), which subsequently undergoes hepatic conversion to trimethylamine N-oxide (TMAO). Elevated TMAO levels are associated with cardiovascular disease and all-cause mortality risk. Dietary flavanols modulate the composition and function of the gut microbiome. Therefore, the possibility exists that these compounds could reduce intestinal TMA production and lower circulating TMAO. However, this hypothesis has never been tested in humans. A secondary analysis was performed on blood samples from a clinical study in which obese subjects at risk for insulin resistance consumed tea or cocoa flavanols in a randomized crossover design while consuming a controlled diet. These subjects generally had elevated TMAO levels (~5 μM) compared to levels previously measured in healthy subjects (~1 μM). None of the interventions significantly altered TMAO levels. Individual variability for choline and carnitine was relatively low. However, TMAO exhibited somewhat greater inter-individual variability. No differences in mean TMAO concentrations observed across interventions were seen based on separating subjects by glycemic status, body mass index (BMI), race, age, or gender. However, subject minimum and maximum values observed across the interventions appeared to be more strongly associated with glycemic status and age than mean values across interventions, suggesting that average TMAO values over time may be less useful than maximum or minimum values as markers of disease risk. Traditional physiological characteristics do not appear to predict TMAO responsiveness to flavanol interventions. However, African-American subjects appeared less responsive compared to non-Hispanic white subjects for both green tea and high cocoa treatments, and female subjects appeared less responsive than males for the high cocoa treatment. The present results suggest that a short-term flavanol intervention does not generally reduce fasting TMAO levels in subjects with elevated circulating TMAO.

Keywords: green tea, cocoa, choline, carnitine, atherosclerosis, cardiovascular

INTRODUCTION

In the past decade, there has been increasing appreciation for an association between the gut microbiota and numerous cardiovascular and metabolic phenotypes. One of the most prominent has been the link between gut microbial metabolism of trimethylamine (TMA) moieties from dietary TMA-containing substrates (choline, phosphatidylcholine, L-carnitine, etc.) and CVD, particularly atherosclerosis[1,2]. These TMA-containing substrates are present in most animal products including eggs, meat, liver, fish, and dairy, and also a limited number of plant products. The western diet is rich in phosphotidylcholine (PC), the primary phospholipid in membranes and the major source of choline in the diet of omnivores[3]. Gut microbes metabolize these dietary substrates to release TMA via the action of specific bacterial enzymes (**Figure 1**); TMA is absorbed from the gut, and then oxidized by hepatic flavin monooxygenases (FMO3) to form trimethylamine N-oxide (TMAO)[1,2,4].

Accumulating evidence suggests that increased circulating TMAO is causally linked to glucose intolerance and atherosclerosis in animal models[1–3,5–7]. Elevated TMAO is also associated with increased carotid intimal thickness[8], a marker of early atherosclerosis, and the extent of atherosclerotic plaque burden[9] in humans. Furthermore, TMAO has been reported to be associated with type 2 diabetes and independently predictive of CVD and mortality risk [10–12]. TMAO has recently been identified as a potential therapeutic target for type-2 diabetes[13].

There are presently very few efficacious interventions for reducing TMAO in humans. The most obvious, and also most problematic in terms of feasibility, is to limit intake of dietary TMA precursors by limiting red meat, milk, eggs and other sources[14]. Traditional approaches to alter gut microbiome composition and function (prebiotics and probiotics) may be useful, but thus far have not been studied extensively for TMAO reduction. We previously demonstrated that probiotic supplementation did not significantly reduce TMAO production in humans[15]. Recently, a choline analog (3,3-dimtheylbutanol, DMB) has emerged as a potential inhibitor of TMAO production[16]. However, to date its use has been limited to animals. There is thus a need for translatable dietary interventions to reduce TMAO production. Inhibition of TMAO formation as a means to prevent or reduce the risk of atherosclerosis is a potentially attractive mechanism to target with dietary polyphenols. As the majority of the TMAO biosynthetic pathway is located in the lumen of the gut, there is no physical barrier limiting the concentrations of dietary polyphenols to which the target microbiota that carry out these reactions are exposed. Thus, processes that limit the bioavailability of dietary polyphenols (absorption, efflux, Phase-II metabolism, and elimination) are not factors that limit the ability of these compounds to inhibit the initial steps of TMAO formation. This is particularly critical for compounds with very poor systemic bioavailability following ingestion, such as quercetin, large procyanidins, theaflavins, curcuminoids and bound polyphenols[17–19]. Therefore, targeting gut luminal activities poses fewer challenges than targeting activities in peripheral tissues. Numerous studies have proposed that alterations to the composition and function of the gut microbiome could be a mechanism by which dietary polyphenols exert their beneficial activities. However, most studies have focused on community composition or host physiology (gut barrier function, etc.)[20,21], as opposed to direct inhibition of microbial metabolism and reduction of specific metabolites.

Very few existing studies have tested the hypothesis that dietary polyphenols could modulate TMAO production, with somewhat mixed results[22–27]. The compounds tested vary widely, and some of the studies reporting positive results used extremely high doses in animals[25] or humans[23]. To the best of our knowledge, no data exist regarding the impact of flavanol interventions on circulating TMAO levels in humans. Much research remains to be conducted to

determine whether dietary polyphenols, and particularly those with poor bioavailability, are able to modulate TMAO production and circulating levels.

Therefore, the objective of this study was to examine the potential for commonly consumed dietary polyphenols administered at nutritionally relevant doses, to reduce pro-atherogenic TMAO and a related pro-atherogenic microbial metabolite (γ-butyrobetaine) in a human population with generally elevated TMAO concentrations. In order to do this, a secondary analysis was performed on samples from a previous human clinical study employing controlled feeding (and therefore consistent levels of TMAO precursors choline, carnitine and betaine) on the impacts of green tea and cocoa supplementation in obese subjects[28]. Furthermore, secondary objectives were to examine inter- and intra-individual variability of TMAO concentrations and to add to the growing body of literature regarding the relationship between TMAO and subject characteristics (age, body weight, BMI, diabetes progression, etc.).

MATERIALS AND METHODS

Experimental design

The original human study[28], completed at the Food Components and Health Laboratory, Agricultural Research Service, United States Department of Agriculture (Beltsville, MD), sought to determine the impact of short-term flavanol supplementation on glucose homeostasis in obese adults at risk for insulin resistance. Twenty subjects (10 females, 10 males) age 25 to 55 years were recruited from the greater Washington DC, USA metropolitan area. Subjects were obese adults at risk for insulin resistance. A tree-based classification model was used to determine risk for insulin resistance on the basis of routine clinical measurements, including body mass index (BMI), waist circumference, fasting blood glucose concentration, blood insulin concentration, blood lipid and lipoproteins, blood pressure, and family history of diabetes mellitus following established criteria[29]. Exclusion criteria included a BMI <27 kg/m^2, antibiotic use within the previous 6 months, reported tobacco use, recent pregnancy, or lactation, history of cardiovascular diseases, diabetes, kidney diseases, liver diseases and certain cancers. Prebiotic or probiotic use prior to the study was not ascertained. However, we recently demonstrated that prebiotic or probiotic use are not likely to affect TMAO levels [15,30]. Study entry was approved by a physician on the basis of the subjects' medical history, blood, and urine test results at screening, and a physical exam. Subject baseline characteristics are shown in **Table 1**.

The study had a crossover design with five 5-day treatment periods. Subjects, investigators and staff were blinded to the flavanol content of the three cocoa treatments. However, subjects could potentially differentiate cocoa vs tea treatments due to differences in appearance. The subjects were randomly assigned to one of two balanced Latin squares (William's design for five treatments and five periods; ten subjects per square, two subjects per sequence within a square). Each treatment period was followed by a 10-day washout. Subjects consumed two servings of the treatment (cocoa or green tea beverage) per day in the context of a controlled diet (5-day menu rotation). The composition of the controlled diet is provided has been reported previously[28] and is provided in **Table 2**. The 5-day menu and associated nutrition information are presented in **Supplementary Information**. The TMAO substrate (choline, L-carnitine, etc.) levels are not available for these diets. However, the diets were uniform for all subjects, normalized by energy needs. Antibiotic use during the study was not permitted. Subjects were instructed to discontinue vitamin/mineral and herbal supplement use 2 weeks before the study, caffeine, except as provided through the study, for 4 days before the start and during the treatment periods.

One serving of cocoa powder and tea weighed 28 g and 1.2 g, respectively. The cocoa beverages provided flavanols at 30 mg (control, Ctrl), 180 mg (low. L), 400 mg (medium, M) and 900 mg (high, H) per day (Mars Inc., Hackettstown, NJ, USA). Tea was commercially available green tea (Lipton Green Tea To Go, Unilever, Englewood Cliffs, NJ, USA). The green tea (GT) treatment was chosen to reflect similar monomer content to that of the high-flavanol cocoa dose (high-flavanol cocoa: 236 mg, green tea: 297.9 mg). These doses were nutritionally relevant flavanol doses that can reasonably be consumed in typical human diets. For comparison, a single 30 g serving of dark chocolate (the official serving size in the United States) contains anywhere from ~28-600 mg flavanols [31–34]. Treatment beverages were prepared from standardized dry powders in individual packets and reconstituted at time of consumption with water. The cocoa treatments were formulated to be similar in total kilocalories, macronutrients, micronutrients, theobromine and caffeine. Daily intake of the green tea provided 36 g of caffeine and 42 kJ of energy (10 kcal). Caffeine was similar across all the treatments.

During the treatment periods, subjects consumed a controlled low-polyphenol diet on a 5-day menu cycle as described previously[28]. At the end of each treatment period, subjects underwent basic physiological measures, and provided fasting blood samples, and plasma was stored at −80°C. The original investigation was approved by the MedStar Research Institute Institutional Review Board (IRB, approval #2005-252). Secondary analysis of samples was approved by the Virginia Tech IRB (approval # 17-231) and performed at Virginia Tech. Written informed consent was obtained from all subjects, and all institutional and governmental (incl. United States Code of Federal Regulations, 45CFR46) regulations and laws, respectively, governing human subjects research were complied with. This study was registered with clinicaltrials.gov (NCT00668928).

UPLC-MS/MS analysis of plasma samples

TMAO, L-carnitine, choline, betaine and γ-butyrobetaine were measured as described previously [14,34] with minor modifications. Immediately prior to sample preparation, 1 mL of an internal standard (IS) stock solution (25 μM choline chloride-d_9, 25 μM betaine HCl-d_9, 25 μM TMAO-d_9, and 120 μM L-carnitine-d_9 in water; TMAO-d_9 and L-carnitine-d_9 from Cambridge Isotope Laboratories, Tewksbury, MA, all others from Sigma, St. Louis, MO) was diluted 100-fold with acetonitrile (ACN). Samples were thawed at room temperature, and 25 μL plasma was combined with 300 μL diluted IS solution. Samples were vortexed, centrifuged (17,000 x *g*, 3 min, room temperature), and the supernatant was filtered using a PTFE (4 mm, 0.2 μm) filter into a certified Waters HPLC vial (Milford, MA) with a 150 μL deactivated glass insert. Samples (5 μL) were immediately analyzed by UPLC-MS/MS on a Waters Acquity H-class UPLIC with triple quadrupole (TCD) detector. UPLC separations were performed with a Waters BEH HILIC column (2.1 x 100 mm; 1.7 μm particle size) with a BEH HILIC VanGuard pre-column (2.1 x 5 mm; 1.7 μm). Column and sample temperatures were 30 and 10°C, respectively. The mobile phases were 15 mM ammonium formate, pH 3.5 (phase A) and ACN (phase B). The flow rate was 0.65 mL/min, and isocratic elution was achieved (20% A/80% B) over 3 min. Following separation, analyte and IS compounds were quantified using electrospray ionization (ESI) in (+)-mode. Source and capillary temperatures were 150 and 400°C, respectively. Capillary voltage was +0.60 kV, and desolvation and cone gas (both N_2) flow rates were 800 and 20 L/h, respectively. Compounds were quantified using optimized multi-reaction monitoring (MRM) functions shown in **Table 3**. MRMs were optimized to achieve 12 points/10 s peak, and the detection span will be ±0.2 amu. Quantification was performed using ratio of the target analyte

and respective IS peak areas, based on authentic external standard curves prepared using a wide range of target analyte concentrations (~500 μM-0.1 nM; TMAO, betaine, L-carnitine, choline, and γ-butyrobetaine HCl from Sigma) and the same IS concentrations used to prepare the plasma samples.

Data Analysis and statistics

Statistical analyses were performed using Prism v6.0f (GraphPad, La Jolla, CA). Values are presented as mean ± SEM, except where individual values are specified. For overall intervention treatment effects, data for each compound were analyzed by 1-way repeated measures ANOVA. If a significant overall treatment effect was detected, Tukey's HSD post hoc test was performed to determine significance of all possible treatment comparisons. Values sharing a common letter superscript are not significantly different ($P<0.05$). For analysis of minimum and maximum TMAO values by various subject characteristics, significance between min and max values within grouping was determined by the Holm-Sidak method without assuming equal SD. Significance between min values across groupings, or between min values across groupings, was determined by unpaired t-tests. Power analyses were conducted using G*Power v3.1.9.3 (Düsseldorf, Germany).

RESULTS AND DISCUSSION

Analyte quantification

A representative chromatogram showing MRM traces of analytes and internal standards from a plasma sample from this study is shown in **Figure 2**.

Effect of intervention

Plasma levels of choline, carnitine, betaine, γ-butyrobetaine (γBB, a proatherogenic intermediate metabolite produced by the gut microbiome during conversion of carnitine to TMAO[35]) and TMAO are shown in **Figure 3**. Results shown are from fasting plasma samples collected after each intervention. Due to the design of the original study, baseline (pre-intervention) blood samples were not available for analysis, so we do not know the starting TMAO concentrations in these subjects. As shown in **Figures 3A-C**, there was essentially no variation in mean plasma levels of choline, carnitine and betaine across the five interventions. This finding was expected due to the controlled feeding and crossover designs of the original study. Furthermore, this observation verifies the effectiveness of dietary control and compliance in these subjects. As shown in **Figure 3D**, there was also essentially no variation in mean circulating levels of γBB across the interventions. Since γBB is a metabolite produced exclusively by the gut microbiota, this suggests that there were no alterations to the capacity of the gut microbiota to metabolize carnitine via the pathways that lead to γBB and TMAO. Furthermore, the general ranges of levels detected in these subjects (~1 μM) are similar to levels previously reported in healthy humans[36] as well as pre- and post-operative bariatric surgery patients[37]. Most importantly, no significant differences were detected in circulating TMAO concentrations (**Figure 3E**) across the interventions. This indicates that these interventions did not significantly alter the net production of TMAO (microbial metabolism of substrates to free trimethylamine (TMA) in the gut, gut uptake of TMA into circulation, and hepatic conversion of TMA into TMAO).

Statistical power

In order to determine whether this study provided sufficient power to detect statistically significant differences using this design, a *post hoc* power analysis was performed using circulating TMAO levels observed for all treatments (1-way repeated measures ANOVA, observed F=0.6596, α=0.05, 1 group, *n*=20, 5 measurements, observed correlation among measurements = 0.7977, Geisser-Greenhouse sphericity ε=0.4991). Based on these values, the statistical power was 100%, indicating that the sample size was sufficient to detect statistically significant differences if they were indeed present.

Inter-individual variability

The inter-individual variability of all analytes across the five interventions is shown in **Figure 4**. Individual variability for dietary precursors (choline, carnitine, and betaine) was relatively low (**Figures 4A-C**), further confirming the tight dietary control. However, the metabolites γBB and TMAO exhibited somewhat greater inter-individual variability (**Figures 4D-E**). The greater variation in TMAO over time, compared to dietary precursors, has previously been recorded[6,38]. However, the present study is unique in demonstrating such wide TMAO variation over such a short period of time.

The individual TMAO levels found in this investigation ranged from 1.6 to 19 μM. It is useful to note the extreme differences in terms of absolute concentrations observed, as well as the distinct patterns between individual subjects. For example, subject #12 had comparatively low TMAO levels compared to the others, and also has essentially no variation in TMAO concentrations across the 5 interventions. On the other end of the spectrum, subject #2 exhibited high TMAO levels, with extreme variation among interventions (~4.5-19 μM). In between these extremes, there were subjects with intermediate to high TMAO levels who exhibited comparatively little variation across intervention (#3-6, 9-11, 14-16, 20) and those that exhibited broad variation (#1, 7, 10, 18). There was no clear pattern regarding the effects of intervention. However, some subjects (#7, 10, 14, 18) appeared to respond to the interventions (the control cocoa had the highest TMAO concentrations, which appeared to be lowered by the flavanol interventions). It is important to note that variability in TMAO concentrations did not appear to be due to variability in dietary precursors or γBB.

Health status and other predictors

In view of the observed lack of effect of flavanol supplementation on TMAO concentrations, we wished to determine whether this was due to true lack of effect, or whether our analysis was not sensitive enough to determine physiologically relevant differences. In order to demonstrate the ability of the method to determine physiologically relevant differences in TMAO levels, we assumed that there was indeed no effect of treatment and treated the five intervention periods as replicate measurements of TMAO status in the subjects. Using these assumptions, we compared mean measured TMAO concentrations for subjects with different characteristics that may affect TMAO concentrations (**Figure 5**). No differences in mean TMAO concentrations observed across the 5 interventions were seen based on separating subjects by glycemic status (**Figure 5A**), BMI (**Figure 5B**), by race (**Figure 5C**), by age (**Figure 5D**), or by gender (**Figure 5E**). The grouping that most closely approached significance was between subjects above and below the median age (P=0.108).

In view of the variability observed in measured TMAO concentrations within subjects over time (**Figure 4**), it is possible that mean values may obscure physiologically important

spikes (maximum levels), or minimum levels that may correlate better with subject characteristics. We wished to test the hypothesis that the minimum or maximum observed TMAO concentrations might better correspond to subject characteristics than mean TMAO concentrations. Therefore, we compared the maximum and minimum observed TMAO levels observed for each individual subject grouped into the various categories (**Figure 6**). For all factors and within each subject category of that factor (for example, for glycemic status with subjects grouped as normoglycemic, insulin resistant or diabetic, we compared the minimum and maximum TMAO levels within each of the three groupings, **Figure 6A**) measured, the minimum and maximum observed TMAO levels for each individual subject were significantly different, suggesting that TMAO may not be a stable biomarker but rather susceptible to extreme variation over time even during controlled feeding. This is key, as these biomarkers are often measured in distinct groups at a single time (cross-sectional), whereas strict longitudinal studies over time are not as common. The controlled feeding for all interventions and short study duration (10 weeks maximum for any one subject) further strengthen the argument that TMAO may be highly variable within individuals. Therefore, our data support the conclusion that a more appropriate assessment of TMAO status may be to take several samples over the course of weeks or months and calculate average levels as well as maximum levels[38].

We also compared minimum observed TMAO levels between groups within each factor, as well as maximum observed TMAO levels between groups within each factor. It is important to note that there were two classifications with n=1, and therefore statistics were not possible for these groupings (1 diabetic subject and 1 Hispanic subject). For example, for glycemic status with subjects grouped as normoglycemic, insulin resistant or diabetic, we compared the minimum TMAO levels between groups and then compared the maximum levels between groups (**Figure 6A**). For glycemic status (**Figure 6A**), there was no difference in minimum observed TMAO levels between groups. However, the maximum observed TMAO levels were bordering on significantly higher in the insulin resistant group compared to the normoglycemic group (P=0.085). Conversely, for age (**Figure 6D**), the minimum TMAO levels were significantly higher in those above the median age (45.5 years) than below it, whereas there was no difference in the maximum TMAO levels between the two age groups. No such differences between minimum or maximum TMAO values were observed when subjects were split on the basis of BMI (median: 36.7 kg/m^2, **Figure 6B**), race (**Figure 6C**), or gender (**Figure 6E**). The observed association of TMAO with glycemic status agrees with previously published data[39,40]. These results suggest that the method can in fact detect physiologically relevant differences in TMAO, and that our observed lack of effect of short-term flavanol intervention on measured TMAO reflects a true lack of effect in terms of physiological relevance of TMAO.

Responders vs. non-responders

In order to further probe possible subject characteristics that predict effectiveness of the interventions, we identified "responders" and "non-responders" to the GT and H treatments (the highest flavanol doses) based on the ratio of the fasting TMAO concentration observed following those treatments and the control cocoa (Ctrl) treatment (the lowest flavanol doses). Responders were defined as the lowest quartile of the GT or H/Ctrl TMAO ratios, and non-responders were defined as the highest quartile. Ratios and characteristics of responders vs. non-responders are shown in **Figures 7-8**. As shown in **Figure 7A**, we were able to identify responders and non-responders to the GT treatment (relative to Ctrl), and the TMAO ratios (GT/Ctrl) were significantly different between responders and non-responders. However, no quantitative

characteristics (BMI, body mass, age, fat mass or lean mass) were significantly different between responders and non-responders (**Figure 7B-F**). In terms of quantitative characteristics: 3/5 responders were insulin resistant (compared to 4/5 non-responders), 0/5 responders were African-American (compared to 4/5 non-responders), and 2/5 responders were female (compared to 3/5 non-responders).

As shown in **Figure 8A**, we were able to identify responders and non-responders to the H treatment (relative to Ctrl), and the TMAO ratios (GT/Ctrl) were significantly different between responders and non-responders. Similar to GT, no quantitative characteristics (BMI, body mass, age, fat mass or lean mass) were significantly different between responders and non-responders (**Figure 8B-F**). However, body mass and lean mass were slightly higher, approaching significance, in responders (**Figure 8C, F**). In terms of quantitative characteristics: 4/5 responders were insulin resistant (compared to 3/5 non-responders), 0/5 responders were African-American (compared to 3/5 non-responders, and one non-responder was Hispanic), and 0/5 responders were female (compared to 4/5 non-responders).

Taken together, these results suggest that traditional physiological characteristics do not appear to predict TMAO responsiveness to flavanol interventions. However, interestingly, African-American subjects appeared less responsive compared to non-Hispanic white subjects for both GT and H treatments, and female subjects appeared less responsive than males for H.

Discussion

This study adds new findings to the very small body of literature examining the potential for dietary polyphenols to reduce TMAO production. Specifically, this is the first study to examine the impact of dietary polyphenols on blood TMAO levels in humans; previous human studies have measured only urinary levels of TMAO degradation products[23,26]. The present results suggest that a short-term flavanol intervention does not reduce fasting TMAO levels in subjects with elevated circulating TMAO. The average TMAO levels were ~4-5 μM across treatments. This highlights the elevation of TMAO levels in these subjects, reflecting elevated CVD and mortality risk due to their overall metabolic health status for which we specifically recruited (obesity and risk for insulin resistance as determined by BMI, waist circumference, fasting blood glucose and insulin, blood lipids, blood pressure, and family history of diabetes). These levels closely mirror those previous detected in obese subjects with and without type-2 diabetes. In previous investigations of healthy males (age 18-30 years) in our laboratory, we detected mean fasting TMAO concentrations of ~0.5-1.5 μM using the same stable isotope dilution method and instrument[30,35]. The finding that mean TMAO levels in these subjects were ~4-5 μM, compared to previous findings of ~1 μM in healthy subjects, is significant. A meta-analysis of clinical studies determined that every 10 μM increase in circulating TMAO is thought to increase relative risk for all-cause mortality by 7.6%[41]. Therefore, TMAO levels in this investigation were high overall compared to healthy subjects, indicating elevated risk and also that reductions back to healthy levels were theoretically possible. Thus, the lack of an effect in this study is likely due to inherent inefficacy of these treatments, as opposed to a flawed study design in which TMAO was not an alterable target (if TMAO levels had already been in the normal/healthy range at the start of the study, treatments other than antibiotics or reduction of TMA precursors would be unlikely to reduce TMAO levels). Therefore, this is not a limitation of the present study.

Another factor that influences the efficacy of any intervention is the duration of the intervention. It may be possible that flavanol exposure requires longer than 5 d to significantly

alter fasting TMAO levels. However, we have previously seen that TMAO production is a biomarker that can be rapidly modified by dietary interventions (specifically, 5 days of high-fat feeding, [34]). We have seen changes in dietary interventions as short as 5 d. However, the present study did not incorporate a choline/carnitine challenge or a meal, which is often needed to detect differences. For example, our previous 5-day high-fat feeding intervention did not show changes in fasting TMAO concentrations but did show significant differences in postprandial TMAO levels. Therefore, short-term flavanol supplementation may similarly alter only postprandial levels, which unfortunately we are unable to measure due to the design of the original study.

Extreme inter-variation in TMAO levels was observed in some subjects, while comparatively little was observed in others. Factors that may have dictated these data distributions, and variations among subjects, are differences in gut microbiota composition and function (presence of specific strains of bacteria, expression of specific enzymes that release TMA from dietary precursors) other biochemical factors (hepatic FMO3 expression and activity, etc.), dietary compliance, etc. However, dietary compliance does not appear to be the issue, as reflected in the relatively tight levels of dietary precursors (**Figures 4A-C**). While such variability often makes testing of specific dietary interventions difficult, understanding the source(s) of this variability and how it affects intervention efficacy can provide insights into the mechanisms governing TMAO production and suggest successful intervention strategies. Further research would be useful to identify the attributes of those subjects that exhibited extremely wide vs. tight TMAO distributions, as well as those who appeared to respond positively to the flavanol interventions. Furthermore, our results suggest that race and sex may influence TMAO response to flavanols more than traditional obesity and glycemia biomarkers; identifying the mechanism behind this finding will further illuminate the factors that predict responsiveness to flavanol interventions. If flavanols ultimately do show promise for altering TMAO levels, identification of attributes that facilitate flavanol-mediated reduction of TMAO could be used to personalize strategies to achieve the desired outcomes. This strategy has already been applied to identifying microbiome characteristics that predict the efficacy of diet-based weight loss programs[40]. Measurements that might prove useful would be baseline and post-intervention 1) levels (DNA abundance by 16S rDNA sequencing) and activity (metabolic activity by converting 16S rRNA to cDNA and sequencing) of the strains of bacteria identified as releasing TMA from dietary precursors[4], 2) targeted functional metagenomics and metatranscriptomics of microbial genes in the biosynthetic pathway[16], 3) *ex vivo* assessment of the capacity for TMA release in subjects fecal samples, and 4) hepatic FMO3 expression levels. The significant intra-individual variation observed for TMAO concentrations could be due differences in the TMA-releasing capacity of the gut microbiome and hepatic FMO3 expression. While these data are not available from this study, this will be an important consideration for future studies.

Previous studies have been somewhat inconclusive with respect to the effect of phenolic compounds on TMAO. Solanky *et al.*[22] dosed rats acutely with epicatechin at a dose equivalent to 10 cups of green tea in a human and observed a decrease in urinary TMAO levels. Van Dorsten *et al.*[23] administered 12 cups/d of green and black teas for 2 d to human subjects in a randomized crossover study, and observed that both teas increased dimethylamine (DMA, a urinary metabolite of TMAO) compared to caffeine placebo. An *et al* [24] reported that 40 mg/kg quercetin in rats increased urinary TMAO levels, potentially due to quercetin-induced upregulation of hepatic FMO3 expression. Chen *et al.*[25] reported that dietary resveratrol inhibited TMA and TMAO formation following both acute and chronic choline administration. However, this study used extremely high levels of resveratrol (0.4% in the diet), and therefore the

relevance of these results to attainable levels of resveratrol in humans remains to be seen. Ostertag *et al.*[26] reported that human subjects fed flavanol-rich dark chocolate had reduced urinary DMA levels. Finally, Liu *et al.*[27] demonstrated that a polyphenol-rich *Lonicera caerulea* berry extract reduced TMAO levels in rats.

The existing body of literature is therefore sparse and somewhat inconsistent. However, the promising findings that have been published do suggest that more thorough investigations of the potential for dietary polyphenols to modulate TMAO production are warranted. Factors that should be prioritized for investigation are 1) systematic comparison of various classes of polyphenols for their efficacy in a uniform model, 2) dose-dependence and use of doses equivalent to nutritionally relevant human doses, 3) the duration needed to detect an effect of polyphenols on TMAO levels, 4) whether polyphenols can modulate fasting TMAO levels, vs. postprandial TMAO production, or both, and 5) whether an acute choline (or other TMA precursor) challenge is needed in order to observe a preventative effect.

CONCLUSION

In this investigation, short-term intake of cocoa and green tea flavanols did not reduce plasma TMAO levels in individuals at risk for T2D. Future studies are needed to identify interventions that effectively target TMA-releasing bacteria and reduce TMAO. Furthermore, we report the novel finding that minimum and/or maximum TMAO values observed over time may in fact be better correlated with subject characteristics such as glycemic status and age than mean TMAO values.

Conflict of Interest. There are no conflicts to declare.

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FIGURE CAPTIONS

Figure 1. Biosynthetic pathway of trimethylamine N-oxide (TMAO) from 2 representative dietary precursors (phosphatidylcholine and choline).

Figure 2. Representative UPLC-MS/MS multireaction monitoring (MRM) chromatograms showing separation and detection of dietary precursors (choline, carnitine, betaine) and metabolites [γ-butyrobetaine (GBB), trimethylamine N-oxide (TMAO)] and their deuterated internal standards (d_9) in a plasma sample from this study.

Figure 3. Levels of choline (A), carnitine (B), betaine (C), γ-butyrobetaine (D) and trimethylamine N-oxide (TMAO, E) following each of the five interventions. Values are presented as mean ± SEM. Data were analyzed by 1-way repeated measures ANOVA. If a significant overall treatment effect was detected, Tukey's post hoc test was performed to

461 determine significance of all possible treatment comparisons. Values sharing a common letter
462 superscript are not significantly different (P<0.05).
463
464 **Figure 4.** Inter-individual variability of all analytes across the five interventions. Data points
465 represent individual fasting measurements following each intervention.
466
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470 B) by BMI [median = 36.7 kg/m^2 (n=10 > median, n=10 < median)], C) by race [AA: African-
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473 Values are presented as mean ± SEM. Lack of error bar indicates only 1 subject in the specified
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504
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647

TABLES

Table 1. Subject characteristics (n=20, values expressed as mean ± SD)

Measurement	Value
Age (years)	45 ± 10.1
Height (cm)	171.9 ± 8.5
Weight (kg)	109.2 ± 17.9
BMI (kg/m^2)	36.8 ± 4.5
Fat Mass (kg)	48.7 ± 12.0
Lean Mass (kg)	59.6 ±10.4

Table 2. Composition of the controlled diet[a]

Parameter	Value
Protein (% kcal)	14
Fat (% kcal)	32
Carbohydrate (% kcal)	54[b]
Sugar (g/d)	188
Dietary fiber (g/d)	24
Sodium (mg/d)	3156
Calcium (mg/d)	1046
Vitamin A (IU/d)	14703
Vitamin C (mg/d)	171
Cholesterol (mg/d)	297

[a]Based on average intake of subjects in this study (2700 kcal/d)
[b]Total dietary fat had a ratio of polyunsaturated: monounsaturated: saturated fatty acids of 0.7:0.8:1.1

Table 3. Multi-reaction monitoring (MRM) settings for UPLC-MS/MS detection of analytes in plasma

Compound	Retention time (min)	MW (g/mol)	Parent $[M+H]^+$ (m/z)	Daughter (m/z)	Cone voltage (V)	Collision energy (eV)
Carnitine	2.09	161.20	162.26	84.99	84.99	34
Carnitine-d_9	2.08	170.25	171.28	84.99	84.99	34
Betaine	1.25	117.15	118.24	59.42	59	44
γ-Butyrobetaine	0.98	145.20	146.27	87.00	26	16
Betaine-d_9	1.25	126.14	127.30	68.10	68	46
Choline	1.13	103.16	104.20	60.02	60	38
Choline-d_9	1.11	112.16	113.32	69.08	69	40
TMAO	2.01	75.11	76.16	58.91	59	40
TMAO-d_9	1.98	84.12	85.22	68.10	68	40

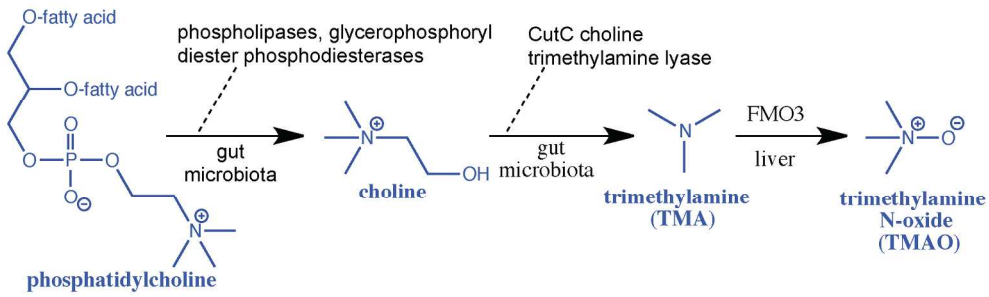

Figure 1. Biosynthetic pathway of trimethylamine N-oxide (TMAO) from 2 representative dietary precursors (phosphatidylcholine and choline).

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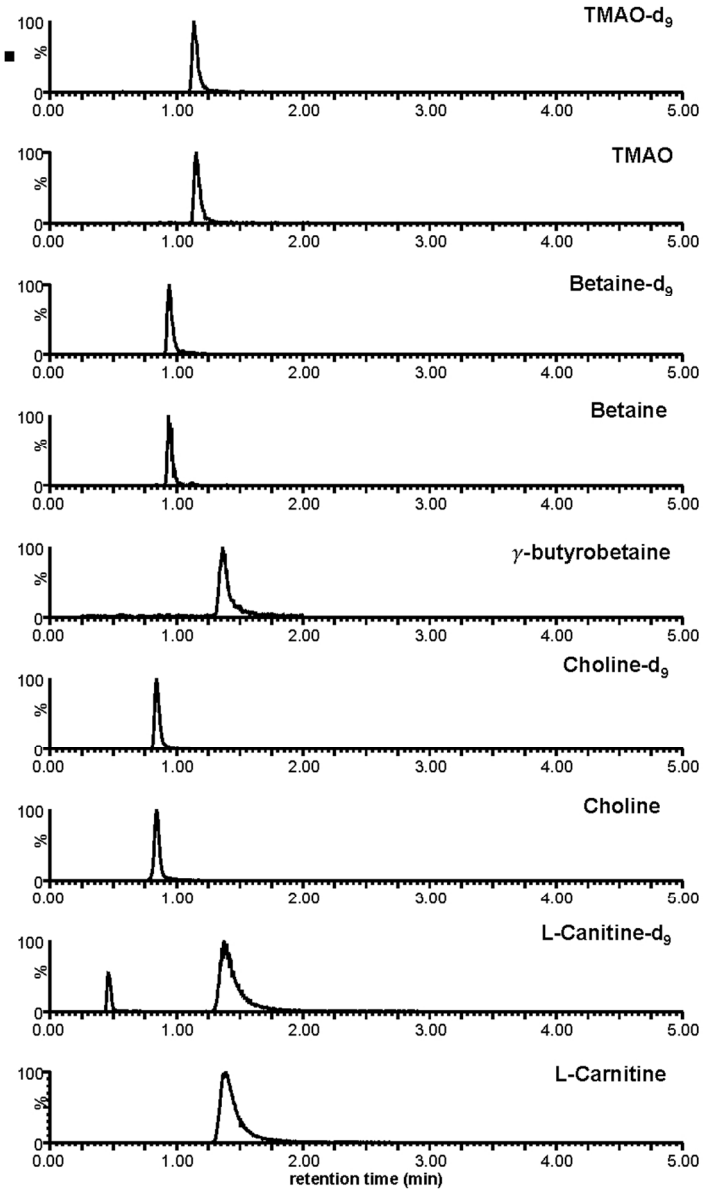

Figure 2. Representative UPLC-MS/MS multireaction monitoring (MRM) chromatograms showing separation and detection of dietary precursors (choline, carnitine, betaine) and metabolites [γ-butyrobetaine (GBB), trimethylamine N-oxide (TMAO)] and their deuterated internal standards (d9) in a plasma sample from this study.

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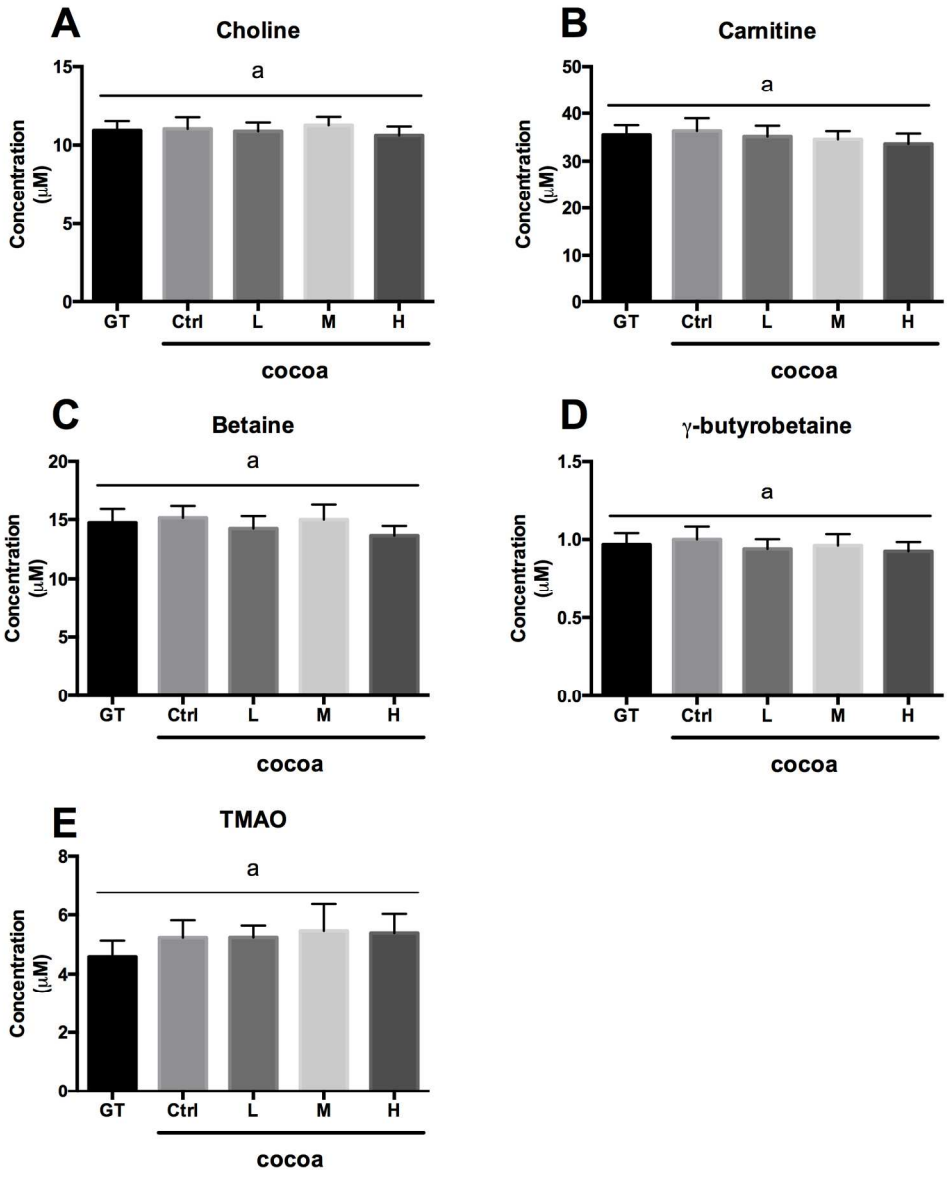

Figure 3. Levels of choline (A), carnitine (B), betaine (C), γ-butyrobetaine (D) and trimethylamine N-oxide (TMAO, E) following each of the five interventions. Values are presented as mean ± SEM. Data were analyzed by 1-way repeated measures ANOVA. If a significant overall treatment effect was detected, Tukey's post hoc test was performed to determine significance of all possible treatment comparisons. Values sharing a common letter superscript are not significantly different ($P<0.05$).

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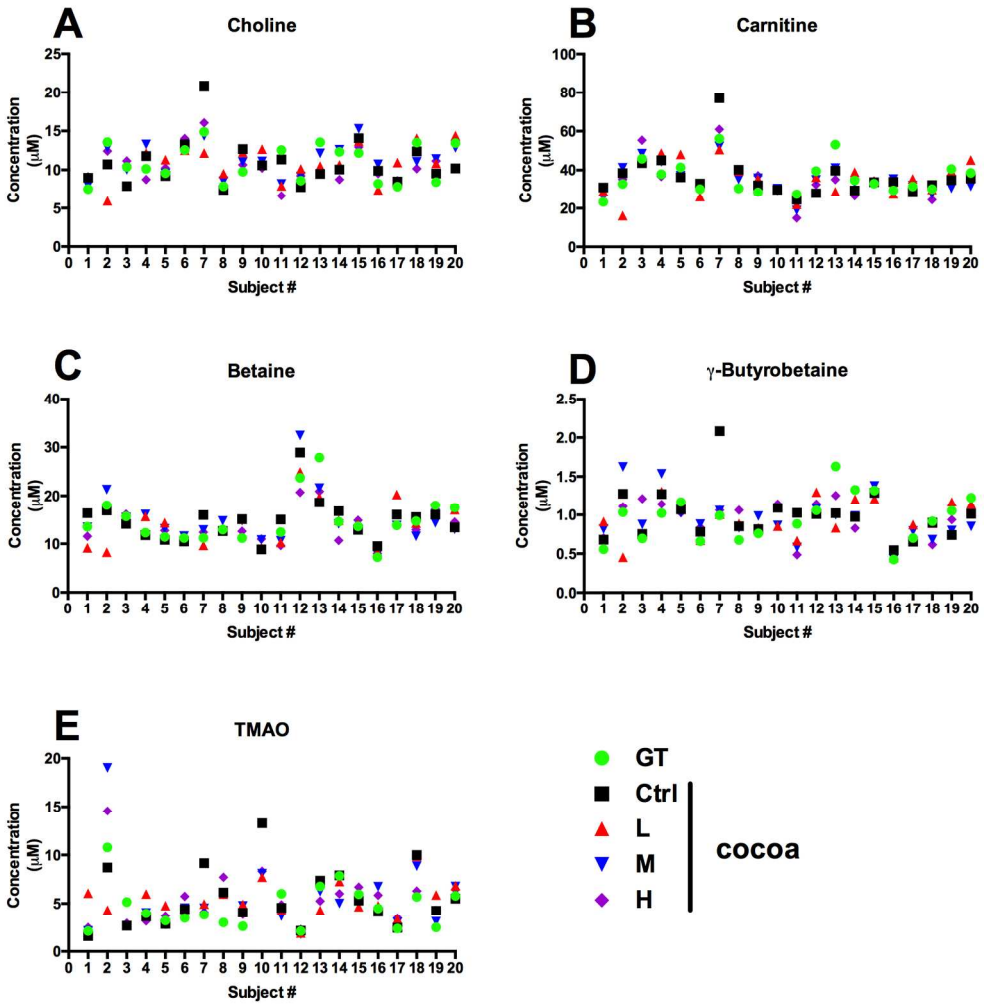

Figure 4. Inter-individual variability of all analytes across the five interventions. Data points represent individual fasting measurements following each intervention.

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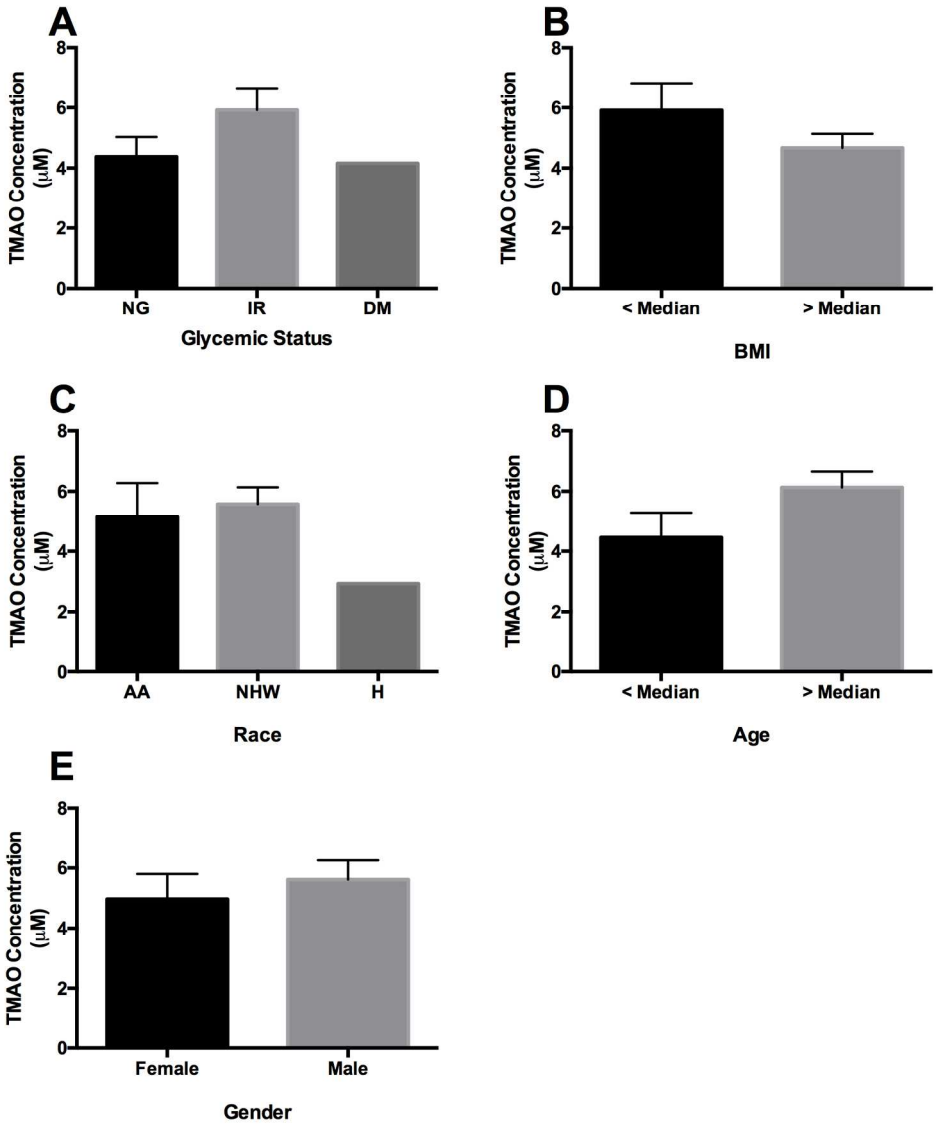

Figure 5. Mean TMAO concentrations observed for each subject across the five interventions groups, grouped by subject characteristics as follows: A) by glycemic status upon recruitment [NG: normoglycemic (n=7), IR: insulin resistance (n=12), DM: diabetes mellitus, type-2 (n=1)], B) by BMI [median = 36.7 kg/m2 (n=10 > median, n=10 < median)], C) by race [AA: African-American (n=7), NHW: non-Hispanic white (n=12), H: Hispanic (n=1)], D) by age [median = 45.5 years (n=10 > median, n=10 < median)], E) by gender [M: male (n=10), F: female (n=10)]. Values are presented as mean ± SEM. Lack of error bar indicates only 1 subject in the specified category. Significance between groupings within characteristics were determined by unpaired t-tests (statistical comparisons were not possible for those groupings with only one subject). * indicates $P<0.05$.

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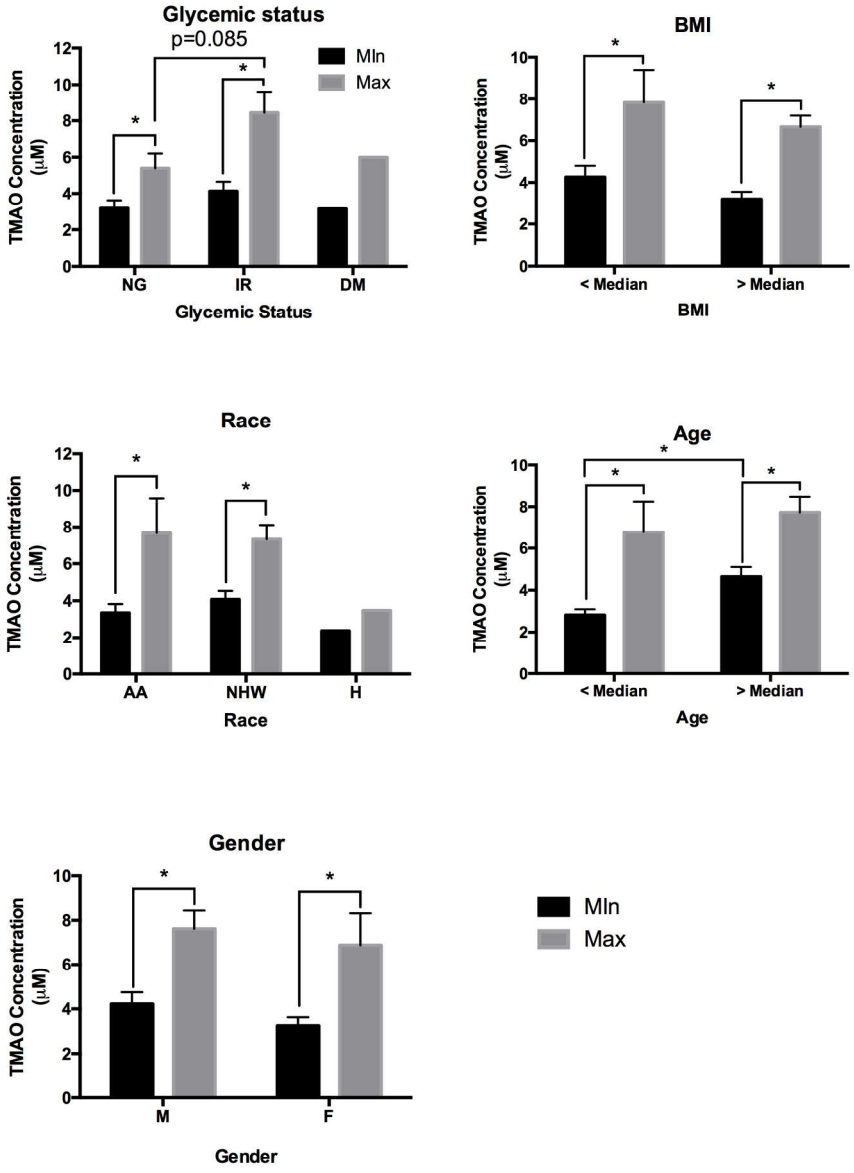

Figure 6. Mean minimum (min) and maximum (max) TMAO concentrations observed for subjects across the five interventions groups, grouped by subject characteristics as follows: A) by glycemic status upon recruitment [NG: normoglycemic (n=7), IR: insulin resistance (n=12), DM: diabetes mellitus, type-2 (n=1)], B) by BMI [median = 36.7 kg/m2 (n=10 > median, n=10 < median)], C) by race [AA: African-American (n=7), NHW: non-Hispanic white (n=12), H: Hispanic (n=1)], D) by age [median = 45.5 years (n=10 > median, n=10 < median)], E) by gender [M: male (n=10), F: female (n=10)]. Values are presented as mean ± SEM. Lack of error bar indicates only 1 subject in the specified category. Significance between min and max values within grouping was determined by the Holm-Sidak method without assuming equal SD. Significance between min values across groupings, or between min values across groupings, was determined by unpaired t-tests. * indicates P<0.05.

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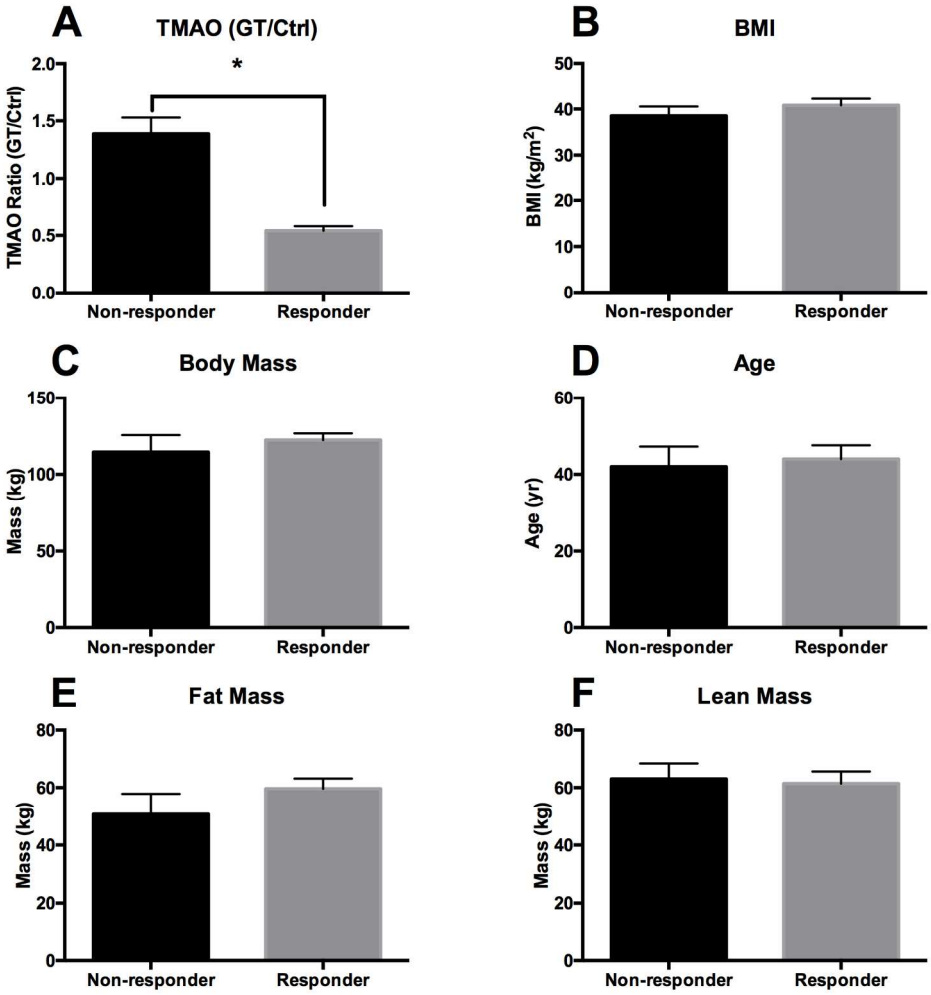

Figure 7. Characteristics of responders and non-responders to the green tea (GT) treatment, as defined by the ratio of the fasting TMAO concentration observed following the GT and control (Ctrl) treatments. Responders were defined as the lowest quartile of the GT/Ctrl TMAO ratio, and non-responders were defined as the highest quartile. A) GT/Ctrl TMAO ratio, B) BMI, C) body mass, D) age, E) fat mas, and F) lean mass. Values are presented as mean ± SEM. Significance between responders and non-responders was determined by t-tests. * indicates $P<0.05$.

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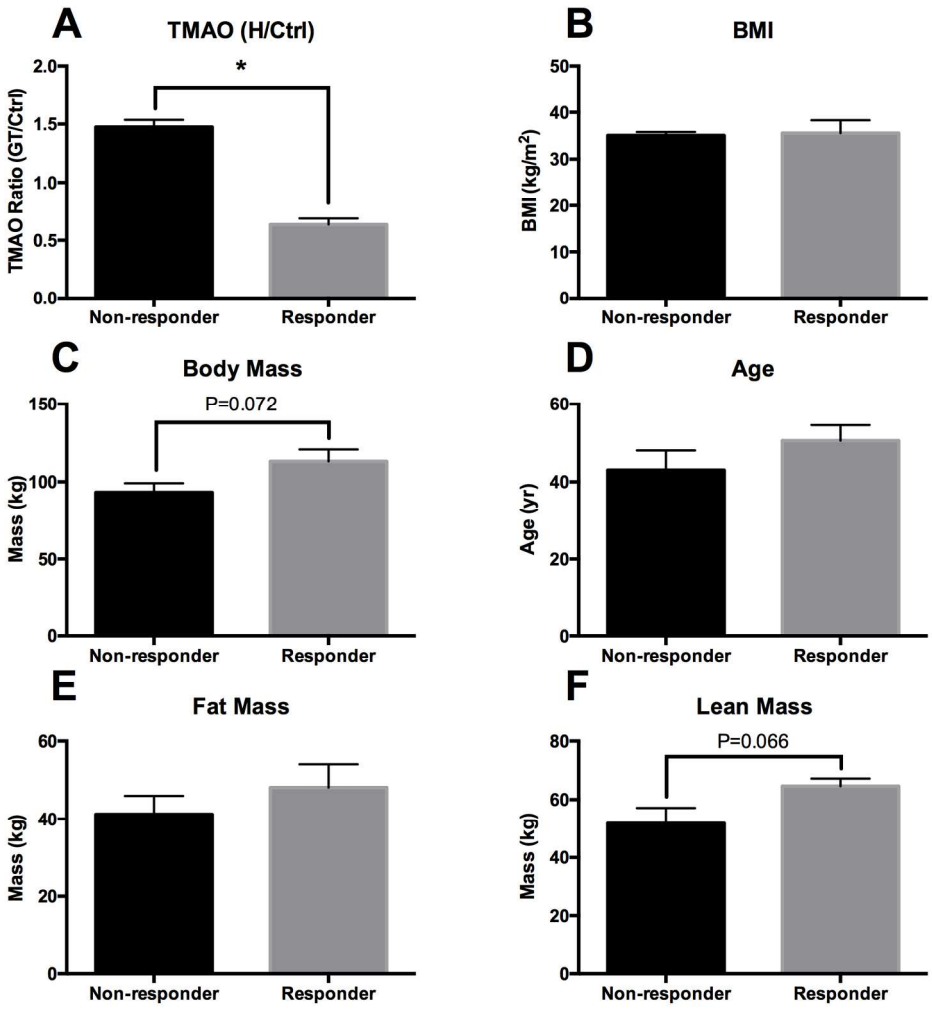

Figure 8. Characteristics of responders and non-responders to the High cocoa (H) treatment, as defined by the ratio of the fasting TMAO concentration observed following the H and control (Ctrl) treatments. Responders were defined as the lowest quartile of the H/Ctrl TMAO ratio, and non-responders were defined as the highest quartile. A) GT/Ctrl TMAO ratio, B) BMI, C) body mass, D) age, E) fat mas, and F) lean mass. Values are presented as mean ± SEM. Significance between responders and non-responders was determined by t-tests. * indicates $P<0.05$.

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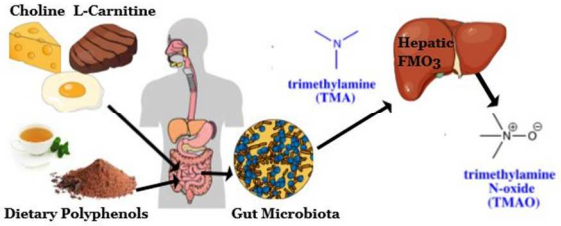

Short-term flavanol supplementation does not reduce levels of proatherogenic TMAO in adults at risk for insulin resistance