Impact of acute choline loading on circulating trimethylamine N-oxide (TMAO) levels

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Despite recent efforts to reduce cardiovascular disease risk by dietary intervention (1), few markers are useful to assess the efficiency and progress of this. Circulating levels of trimethylamine N-oxide (TMAO) are associated with poor outcomes of cardiovascular disease (2-6). TMAO is generated via hepatic flavin monooxygenase 3 (FMO3) mediated oxidation of trimethylamine (TMA) (7), derived largely from carnitine and choline through gut microbial metabolism. These substrates are found in red meat and eggs which are representative of a Western diet. Therefore, TMAO levels could be used to monitor the effect of dietary intervention, particularly for the consumption of a Western diet. In this study, we examined the effect of acute choline loading on TMAO levels in healthy adult volunteers.

Eighteen healthy omnivorous adult volunteers, with no history of recent use of antibiotics/probiotics, completed this study. Baseline characteristics and blood samples were obtained from participants at baseline (BL). Participants were asked to complete a daily written food diary and returned after 2 weeks (visit 2, V2). The consumption of red meat, fish, and eggs was not permitted 24 hours before BL and V2. Prior to taking 700 mg of choline bitartrate capsules (Solgar, USA), a blood sample was collected to measure TMAO level at time zero (0), then at 2, 4, 6 and 8 hours post choline consumption. During V2, participants were given a standardised low-choline breakfast and lunch (total choline intake <50 mg) (8), between 0-2 and 4-6 hours respectively. This protocol was approved by the local ethics committee and adhered to the Declaration of Helsinki. All participants provided written informed consent. Plasma samples were quantified in duplicate for TMAO, using stable isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a Shimadzu LC-30AD coupled with an 8050 triple quadruple mass spectrometer (TQ-MS), using an optimised version of a previously described method (9), with a run time of 1 minute and limit of quantification (LOQ) of 0.1 μmol/L. Individual responses of circulating TMAO levels in participants are shown in Figure 1a.

Participants showed a range in circulating TMAO levels between 0-20 μmol/L over the 8 hour period, with a few (n=4) showing higher TMAO levels following six hours post-choline consumption (>20 μmol/L). We observed that there were patients that showed raised TMAO levels in response to choline loading ('responders') and those that did not ('non-responders'). To further investigate the different responses to choline loading (response vs non-response), participants were split according to the median baseline TMAO level of 2.0 μmol/L (Figure 1b), into high TMAO (HT) and low TMAO (LT) groups. TMAO levels rapidly and significantly increased from 4 hours after choline loading in the HT group; in contrast, TMAO levels showed no change at any time point after choline loading in the LT group (Figure 1b) (p=0.012). TMAO levels in the HT group were higher than those in the LT group at all times during the acute oral choline challenge (3.1-16.3 μmol/L vs. 1.3-3.3 μmol/L, respectively). Participants in the HT group (n=9) tended to be older and had higher alcohol intake compared to those in the LT group (n=9); however this was not statistically significant (p=0.094 and p=0.063, respectively). Gender, ethnicity, BMI, and weekly physical activity were similar between groups (p>0.05). Dietary records from the two weeks prior to acute choline loading as well as baseline records did not show any differences in TMA rich food intake (red meat, eggs or fish) between groups (Table 1). No side effects (e.g. fishy body odour) were reported during the study.

The present study indicates that acute choline loading in healthy subjects produces a differential response in circulating TMAO levels (i.e. 'responders' and 'non-responders'). These two different response groups in TMAO production following choline challenge were demonstrated for the first time by the present study. To note, individuals with a 'responder' phenotype had higher baseline TMAO levels. Furthermore, food diaries from these individuals, assessed to determine protein intake in the two weeks before choline loading, showed no significant differences in TMA rich foods (e.g. red meat, eggs) compared to those with a 'non-

responder' phenotype. This indicates that omnivorous individuals can have different responses to dietary sources of choline, independently of daily dietary conditions. TMAO levels were also analysed for association with participants' physical activity due to the beneficial effect of physical activity on cardiovascular risk, in particular when combined with dietary intervention (10). We did not observe differences in TMAO levels with respect to physical activity between the low and high TMAO groups. In previous studies, acute dietary supplementation of choline has been shown to increase TMAO levels in a general population (11). However, most studies have focused on carnitine, demonstrating the effect of L-carnitine supplementation with increased TMAO levels correlated to chronic carnitine intake (12). TMAO levels have been attributed more to carnitine rather than choline and from red meat rather than other sources of protein (13). Whilst these studies showed that TMAO levels are significantly increased with L-carnitine and red meat intake, they did not account for baseline differences in the TMAO levels of participants and whether these could affect subsequent TMAO increase, underlying a responder or non-responder phenotype. Further, single TMAO time point cross-sectional studies are unable to ascertain whether there are temporal or individual differences in TMAO production, following dietary/loading challenge (e.g. response). The current study sheds light upon the hypothesis that individuals respond differently to dietary choline intake, which could be related to reduced FMO3 activity or gut bacterial conversion of choline to TMA, resulting in inefficient conversion of choline to TMAO. Further investigation of gut microbial composition and FMO3 activity to identify outstanding factors for response to choline loading is warranted.

In conclusion, our findings suggest the presence of different TMAO responses to acute choline loading. As a current goal of cardiovascular disease prevention is to target lifestyle factors (14), with dietary manipulation (e.g. to reduce red meat consumption) being one of the mainstays for intervention, the finding that not all subjects respond uniformly to dietary choline

intake, and the use of a loading test to identify 'responders/non-responders' might help to further stratify patients into appropriate dietary interventions. Further studies that address whether the 'responder' phenotype affords a higher risk for cardiovascular risk, and can be used to identify patients warranting dietary intervention are needed.

Conflicts of Interest.

The authors declare that they have no conflict of interest.

Author Contribution

TS contributed to the conception, design, data analysis and interpretation of the work and critically revised the manuscript. SC and AS contributed to conduction of the study, data analysis, interpretation of the work, drafted the manuscript and critically revised the manuscript. YY contributed to conduction of the study, data analysis, and critically revised the manuscript. DB, MW, and MZI contributed to the acquisition of data for the work and critically revised the manuscript. LMH contributed to the conception and design of the study, and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work ensuring integrity and accuracy.

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Table 1. Demographics data from the participants assessed for this study.

	Total (n=18)	low TMAO (≤2.0 μmol/L)	high TMAO (>2.0 μmol/L)	p value
Demographics				
Age	28 [23-36]	25 [21-29]	33 [27-49]	0.094
Male	5 (28%)	1 (11%)	4 (44%)	0.294
Caucasian	14 (78%)	7 (78%)	7 (78%)	1.000
BMI	21.7 [20.9-23.8]	20.9 [20.2-23.8]	22.6 [21.5-23.7]	0.258
Current smoker	1 (6%)	0 (0%)	1 (11%)	1.000
FH of CVD	3 (17%)	2 (22%)	1 (11%)	1.000
Systolic blood pressure (mmHg)	117 [110-129]	118 [110-124]	116 [115-133]	0.666
Diastolic blood pressure (mmHg)	78 [74-86]	78 [70-86]	77 [76-82]	0.863
Heart rate (beats/min)	68 [63-78]	65 [65-76]	70 [62-78]	0.605
Physical Activity (days/week)	3.5 [2.5-7]	4.0 [2.5-7]	3.0 [3-7]	0.876
Intake history				
Alcohol (units/week)	1 [0-3]	0 [0-0.1]	2 [1-6]	0.063
Red meat (units/week)	3 [1-4]	2 [1-3]	3 [3-4]	0.258
Fish (units/week)	1 [1-3]	1 [0.1-3]	2 [1-2]	0.489
Eggs (units/week)	1 [1-3]	1 [1-2]	2 [1-4]	0.297
Laboratory				
Cholesterol (g/dL)	4.7 [4.0-5.2]	4.6 [4.4-5.2]	4.7 [3.9-5.2]	0.796
HDL (g/dL)	1.6 [1.4-1.9]	1.5 [1.4-1.7]	1.9 [1.4-2.0]	0.546
TMAO (μmol/L)				
Baseline	2.0 [1.3-4.0]	1.3 [0.7-1.6]	4.0 [3.3-5.9]	<0.001*
After choline loading				
0 hour	2.1 [1.4-3.4]	1.5 [1.3-1.9]	3.4 [2.2-3.5]	0.024*
2 hours	2.0 [1.3-3.1]	1.3 [1.2-2.0]	3.1 [2.0-4.0]	0.021*
4 hours	1.9 [1.2-4.5]	1.3 [1.1-1.8]	4.5 [2.7-6.5]	0.001*
6 hours	4.1 [1.0-10.3]	1.0 [0.8-1.9]	10.3 [7.3-34.0]	0.001*
8 hours	6.6 [4.4-16.3]	3.3 [1.0-6.2]	16.3 [11.6-50.5]	0.001*

Analysed data were split into high and low TMAO groups (HT and LT groups respectively), based on the median of baseline values. Mann-Whitney U test for continuous variables and chi-squared test for categorical variables were used to examine between-group differences. Subsequent analyses used a general linear model with repeated measures and Bonferroni correction to compare group TMAO means during the oral acute choline challenge. P values <0.05 were deemed statistically significant. IBM SPSS Statistics (v25, IBM Corp., Armonk, NY, USA) and Prism (v.7, GraphPad) were used.

BMI: Body mass index; FH: familiar history; CVD: cardiovascular diseases; TMAO: trimethylamine N-oxide; HDL: high density lipoprotein. * P< 0.05

Figure 1. (a) Plasma TMAO levels over an 8 hour period following acute choline loading of 700 mg orally after 0 hours (arrow). **(b)** Participants were split into two groups dependent on the median TMAO levels at baseline (high or low TMAO).
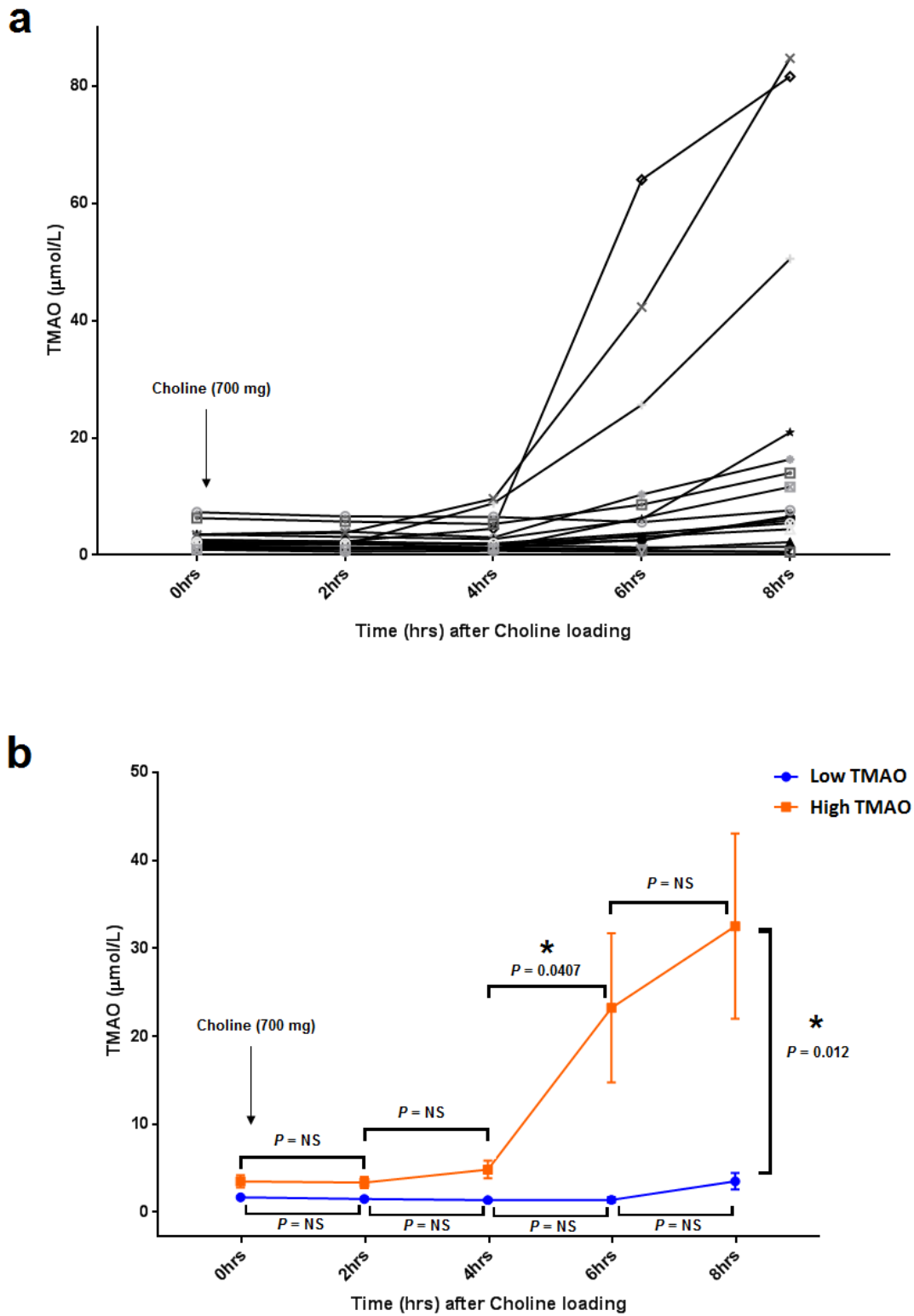
Data are presented as the mean ± SEM. NS: non-significant. * P<0.05