Prognostic value of choline and betaine depends on intestinal microbiota-generated metabolite trimethylamine-N-oxide

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Aims

Recent metabolomics and animal model studies show trimethylamine-N-oxide (TMAO), an intestinal microbiota-dependent metabolite formed from dietary trimethylamine-containing nutrients such as phosphatidylcholine (PC), choline, and carnitine, is linked to coronary artery disease pathogenesis. Our aim was to examine the prognostic value of systemic choline and betaine levels in stable cardiac patients.

Methods and results

We examined the relationship between fasting plasma choline and betaine levels and risk of major adverse cardiac events (MACE = death, myocardial infraction, stroke) in relation to TMAO over 3 years of follow-up in 3903 sequential stable subjects undergoing elective diagnostic coronary angiography. In our study cohort, median (IQR) TMAO, choline, and betaine levels were 3.7 (2.4–6.2) μM, 9.8 (7.9–12.2) μM, and 41.1 (32.5–52.1) μM, respectively. Modest but statistically significant correlations were noted between TMAO and choline ($r = 0.33$, $P < 0.001$) and less between TMAO and betaine ($r = 0.09$, $P < 0.001$). Higher plasma choline and betaine levels were associated with a 1.9-fold and 1.4-fold increased risk of MACE, respectively (Quartiles 4 vs. 1; $P < 0.01$, each). Following adjustments for traditional cardiovascular risk factors and high-sensitivity C-reactive protein, elevated choline [1.34 (1.03–1.74), $P = 0.05$], and betaine levels [1.33 (1.03–1.73), $P < 0.05$] each predicted increased MACE risk. Neither choline nor betaine predicted MACE risk when TMAO was added to the adjustment model, and choline and betaine predicted future risk for MACE only when TMAO was elevated.

Conclusion

Elevated plasma levels of choline and betaine are each associated with incident MACE risk independent of traditional risk factors. However, high choline and betaine levels are only associated with higher risk of future MACE with concomitant increase in TMAO.

Keywords

Gut microbiota • Cardiovascular disease • Nutrition • Choline • Myocardial infarction

Introduction

Choline is a semi-essential nutrient for humans, meaning that while it can be endogenously synthesized, additional dietary intake of choline is also required or else a deficiency state will eventually occur. A common nutrient in many animal and some plant products, choline is found in dietary sources such as egg yolk, meats, liver, fish, dairy products, nuts, and soybean.1–3 Choline is a component of phosphatidylcholine (PC, also known as lecithin), the major phospholipid in cell membranes. It also is a precursor of the neurotransmitter acetylcholine, and following oxidation to betaine, choline functions as a methyl group donor in pathways that produce S-adenosylmethionine.4 As a methyl donor, choline and betaine can influence DNA and histone methylation.5 Choline deficiency has been linked to neurological impairment,6 and targeting enzymes related to choline metabolism has been postulated to provide promising therapeutic opportunities for tumour growth arrest.7 The connection between dietary choline and betaine and heart disease in subjects is unclear. While high intake of dietary choline and betaine was reportedly associated with diminished

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inflammation in one study,8 epidemiological studies have reported a limited association between high choline and betaine intake and cardiovascular morbidity that was attenuated following adjustments for other cardiovascular risk markers.9,10

There is a growing appreciation that intestinal microbiota can participate in metabolic phenotypes such as obesity and insulin resistance.11–13 Recently, through a series of animal and human studies, our group has identified a direct mechanistic link between intestinal microbiota-dependent metabolism of trimethylamine (TMA)-containing dietary nutrients such as choline, PC, and carnitine and production of trimethylamine-N-oxide (TMAO), a metabolite shown to both alter cholesterol and sterol metabolism in multiple compartments and to directly enhanced atherosclerosis in animal models.14–16 Gut microbiota have been shown to play an obligate role in TMAO formation from ingested choline, PC, and carnitine in both animal models and humans.14,16 Moreover, elevated levels of TMAO have been shown to predict increased prevalent cardiovascular disease (CVD) risk, and increased incident risk of major adverse cardiovascular events (MACE = myocardial infarction, stroke, and death) in large-scale human clinical studies.14–16 Betaine, an oxidation product of choline, is generally regarded as safe for dietary ingestion. Surprisingly, although a TMA-containing species, whether orally ingested betaine produces TMAO or not is unknown. Moreover, while elevated whole-blood choline levels have been reported to predict increased cardiovascular risk,18–21 the relationship between TMAO and the clinical prognostic value of choline or betaine is unknown. Herein, we show that oral ingestion of d9-betaine results in generation of circulating levels of d9-TMAO in a gut microbiota-dependent fashion. We further examine the relationship between circulating levels of choline and betaine and development of cardiovascular morbidity and mortality, and the impact of TMAO on this relationship.

Methods

Study population

We prospectively recruited sequential stable subjects (n = 3903) undergoing elective, non-urgent coronary angiography at the Cleveland Clinic without any evidence of acute coronary syndrome (cardiac troponin I ≤0.03 ng/mL). All subjects were followed over a period of 3 years from time of enrollment including adjudication of MACE (which includes the future development of all-cause mortality, non-fatal myocardial infarction, or non-fatal stroke). Fasting blood glucose, high-sensitivity (hs) C-reactive protein, and lipid profiles were measured on the Abbott Architect c8200 platform (Abbott Diagnostics, Abbott Park, IL, USA).

Mass spectrometry analyses of trimethylamine-N-oxide, choline, and betaine

Plasma was immediately prepared from venous blood drawn into ethylenediaminetetraacetic acid tubes and then stored at −80°C until analysis. Trimethylamine-N-oxide, choline, and betaine levels in plasma were determined using stable isotope dilution high-performance liquid chromatography with on line electrospray ionization tandem mass spectrometry (LC/MS/MS) on either an AB SCIEX 5000 or AB SCIEX 5500 triple quadrupole mass spectrometer using d9-(trimethyl)-labelled internal standards as described previously.14

Metabolic challenges in mice

Whole blood (50 μL) was collected via the saphenous vein from C57BL/6j mice prior to gavage for the measurement of baseline analyte levels. Mice were administered an oral dose of stable isotope-labelled betaine or choline. The gavage consisted of 150 μL of either 150 mM betaine-NNN-trimethyl-d₉ (d₉-betaine, CDN Isotopes) or 150 mM choline-NNN-trimethyl-d₉ (d₉-choline, Cambridge Isotope Lab) followed by post-gavage blood collection at the indicated times. Where indicated, gut microbiota were suppressed in mice as previously described14 by placement of a cocktail of antibiotics in drinking water for 3 weeks prior to the d₉-betaine challenge. Ethylenediaminetetraacetic acid plasma was generated following centrifugation at 1000 g for 20 min at 4°C, and d₉-labelled parent compounds and metabolites were measured by LC/MS/MS using choline-1,1,2,2-d₄ (d₄-choline, Cambridge Isotope Lab) as an internal standard. The chromatographic gradient employed was as previously reported14 and specific parent to daughter transitions in positive ion multiple reaction monitoring mode were 85→66 for d₉-TMAO, 113→69 for d₉-choline, 127→68 for d₉-betaine, and 108→60 for d₄-choline. In additional studies, the role of gut flora in TMAO formation from dietary betaine was determined in C57BL/6j mice by performance of oral (gavage) d₉-betaine challenge. Mice were housed in conventional cages in the absence or presence of a cocktail of broad spectrum antibiotics added to the drinking water previously shown to suppress gut flora.14

Statistical analysis

Student’s t-test or Wilcoxon-rank sum test for continuous variables and χ² test for categorical variables were used to examine the difference between groups. Kaplan–Meier analysis with Cox proportional hazards regression was used for time-to-event analysis to determine hazard ratio (HR) and 95% confidence intervals (95% CI) for MACE. Stratification between median levels of choline, betaine, and TMAO levels was performed. Adjustments were made for individual traditional risk factors including age, sex, systolic blood pressure, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, smoking, diabetes mellitus, and hs C-reactive protein to predict incident 3-year MACE risks. All analyses performed used R 2.8.0 (Vienna, Austria). P < 0.05 was considered statistically significant.

Results

Baseline characteristics and relation of plasma choline and betaine levels and cardiovascular risks

Table 1 shows the baseline characteristics of the study population. The median (inter-quartile ranges) for choline, betaine, and TMAO plasma levels was 9.8 (7.9–12.2) μM, 41.1 (32.5–52.1) μM, and 3.7 (2.4–6.2) μM, respectively. Modest but statistically significant correlations were noted between TMAO and choline (r = 0.33, P < 0.001) and between TMAO and betaine (r = 0.09, P < 0.001). As illustrated in the Kaplan–Meier analyses shown in Figures 1 and 2, a graded increase in risk for MACE was observed with increasing plasma levels of either choline or betaine. Higher plasma choline or betaine levels were each associated with increased risk for MACE (1.9-fold and 1.4-fold, respectively; Quartile 4 vs. 1, P < 0.01, each; Table 2). This is lower than the 2.5-fold increased risk for MACE observed with increasing TMAO levels observed in the cohort (Quartile 4 vs. 1, P < 0.01), similar to previous reports.17 Following
adjustments for traditional risk factors and hs C-reactive protein, elevated choline [hazard ratio, 1.34 (95% CI, 1.03–1.74, \( P = 0.05 \)], and betaine levels [hazard ratio, 1.33 (95% CI: 1.03–1.73, \( P < 0.05 \)] each predicted increased MACE risk (Table 2).

Interaction between choline, betaine, and trimethylamine-N-oxide in predicting future major adverse cardiac event risk

Choline, betaine, and TMAO were previously shown to be metabolites of dietary PC and are each independently associated with the prevalence of CVD.\(^{14}\) In addition, dietary choline serves as a substrate to ultimately produce TMAO.\(^{14}\) To directly test whether dietary betaine can serve as a substrate for generation of TMAO similar to choline, we performed oral (gavage) challenges in mice with synthetic isotope-labelled choline or betaine. First, C57BL/6J mice were fed isotope-labelled choline (d9-trimethyl-choline) via gavage. After an initial lag period, a rapid increase in plasma levels of the d9-isotopologue of TMAO was observed that peaks at \( \approx 4 \) h following ingestion, and then disappears rapidly within the subsequent 4 h period. In comparison, plasma d9-choline levels peaked earlier (within 1 h) following ingestion (Figure 3A and D). Although there are reports that some bacterial species can cleave betaine to produce TMA,\(^{22}\) to date, there have been no demonstrations that TMAO can be produced from oral ingestion of betaine in animals. We next examined whether oral betaine ingestion can produce TMAO. C57BL/6J mice were similarly challenged with isotope-labelled betaine (d9-trimethyl-betaine) via gavage, and plasma levels of analytes monitored before vs. following ingestion. Similar to the time course observed with d9-choline ingestion, time dependent increases in the plasma levels of the anticipated d9-isotopologue of TMAO were observed following oral ingestion of d9-betaine, with peak level observed within 4 h. Further, following oral ingestion, d9-betaine levels increased rapidly (peak level within 1 h), and then disappeared gradually (Figure 3E). These results directly demonstrate for the first time that oral betaine can serve as a precursor for TMAO formation. Of note, however, compared with comparable oral challenge of d9-choline,\(^{14}\) betaine was a far poorer precursor for TMAO, as it produced 100-fold less d9-TMAO (Figure 3A and B). To assess the requirement of gut flora in dietary betaine-dependent formation of TMAO, parallel studies were performed in mice following suppression of gut flora with an oral cocktail of poorly absorbed antibiotics (3 weeks). Notably, no detectable d9-TMAO was observed in plasma following oral d9-betaine challenge (Figure 3C), consistent with gut flora involvement in oral betaine metabolism into TMAO.

To further investigated the interaction between plasma levels of either choline or betaine and TMAO vs. incident risk for MACE, we constructed a model that included plasma levels of all three PC

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**Table 1** Baseline characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tbody>
<tr>
<td>Age (years)</td>
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<tr>
<td>Male sex (%)</td>
<td>64</td>
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<tr>
<td>History of cardiovascular disease (%)</td>
<td>65</td>
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<tr>
<td>History of coronary artery disease (%)</td>
<td>61</td>
</tr>
<tr>
<td>History of myocardial infarction (%)</td>
<td>41</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>72</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>31</td>
</tr>
<tr>
<td>Cigarette smoking (%)</td>
<td>65</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>96 (78–116)</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>34 (28–41)</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>118 (85–169)</td>
</tr>
<tr>
<td>High-sensitivity C-reactive protein (mg/L)</td>
<td>2.4 (1.1–5.9)</td>
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<tr>
<td>Betaine ((\mu)M)</td>
<td>9.8 (7.9–12.2)</td>
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<tr>
<td>Choline ((\mu)M)</td>
<td>41.1 (32.5–52.1)</td>
</tr>
<tr>
<td>TMAO ((\mu)M)</td>
<td>3.7 (2.4–6.2)</td>
</tr>
</tbody>
</table>

Values expressed in means ± standard deviation or median (inter-quartile range).

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**Figure 1** Kaplan–Meier estimates of freedom from major adverse cardiac events (death, myocardial infarction, or stroke) and plasma levels of choline. Data are shown for 3903 participants in the clinical-outcome study according to quartiles of plasma levels of choline. The \( P \)-value is for all comparisons.

**Figure 2** Kaplan–Meier estimates of freedom from major adverse cardiac events (death, myocardial infarction, or stroke) and plasma levels of betaine. Data are shown for 3903 participants in the clinical-outcome study according to quartiles of plasma levels of betaine. The \( P \)-value is for all comparisons.

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**Figure 3A** Graph showing the time course of plasma levels of choline following oral ingestion of d9-choline. The figure illustrates the rapid increase in plasma levels of the d9-isotopologue of TMAO that peaks at \( \approx 4 \) h following ingestion, and then disappears rapidly within the subsequent 4 h period.

**Figure 3B** Graph showing the time course of plasma levels of betaine following oral ingestion of d9-betaine. The figure illustrates the rapid increase in plasma levels of the d9-isotopologue of TMAO that peaks at \( \approx 4 \) h following ingestion, and then disappears rapidly within the subsequent 4 h period.

**Figure 3C** Graph showing the time course of plasma levels of TMAO following oral ingestion of d9-betaine. The figure illustrates the rapid increase in plasma levels of the d9-isotopologue of TMAO that peaks at \( \approx 4 \) h following ingestion, and then disappears rapidly within the subsequent 4 h period.

**Figure 3D** Graph showing the time course of plasma levels of choline following oral ingestion of d9-choline. The figure illustrates the rapid increase in plasma levels of the d9-isotopologue of TMAO that peaks at \( \approx 4 \) h following ingestion, and then disappears rapidly within the subsequent 4 h period.

**Figure 3E** Graph showing the time course of plasma levels of betaine following oral ingestion of d9-betaine. The figure illustrates the rapid increase in plasma levels of the d9-isotopologue of TMAO that peaks at \( \approx 4 \) h following ingestion, and then disappears rapidly within the subsequent 4 h period.
metabolites within the cohort of sequential subjects presenting for diagnostic cardiac evaluation (n = 3903; Table 1). Remarkably, only TMAO predicted MACE risk when all three metabolites were included in the model [HR: 1.73 (1.3–2.31), P < 0.01]. Further, while choline and betaine each predicted future risk for MACE within 3 years following adjustments for traditional cardiovascular risk factors, addition of TMAO to the model completely attenuated the association for choline and significantly attenuate the association of betaine for MACE risk (Figure 4A and B). Interestingly, if we separated plasma levels of choline, betaine, and TMAO into two ranges, within 3 years following adjustments for traditional cardiovascular risk factors, addition of TMAO to the model completely attenuated the association for choline and significantly attenuate the association of betaine for MACE risk (Figure 4A and B). Interestingly, if we separated plasma levels of choline, betaine, and TMAO into two ranges,
below median value as low and equal to or above median value as high, cox regression analysis adjusted for multiple traditional risk factors reveals that choline and betaine predict increased MACE risk, but only among those with concomitant elevated TMAO levels and not in those with low TMAO levels (Figures 5 and 6).

**Discussion**

Choline, betaine, and TMAO are all metabolites formed following ingestion of dietary PC. Moreover, systemic levels of choline, betaine, and TMAO each are independently associated with the prevalence of CVD. However, recent animal model studies demonstrate that the mechanism through which dietary PC or choline enhances atherosclerosis requires the presence of intact intestinal microbiota and formation of TMAO. In the present animal study, we demonstrated for the first time the production of TMAO from dietary betaine administration, albeit at a significantly less efficient rate (~100-fold) than that from dietary choline. Importantly, we show that the TMAO formed from oral betaine has an obligatory requirement for intact gut flora, similar to previous observations with
dietary choline. Prior mechanistic studies in mice reveal that TMAO itself inhibits reverse cholesterol transport, and also alters cholesterol and sterol metabolism in multiple different compartments, including suppression of bile acid pool size and altered composition.

Given (i) the direct pro-atherogenic effects observed with direct dietary administration of TMAO; (ii) the precursor product metabolite relationships between both PC and choline and betaine, as well as either choline or betaine (proved for first time in this study) and TMAO formation; and (iii) the observation in animal models that the pro-atherogenic effects of dietary choline is only observed with intact gut microbiota where TMAO could be formed, we sought in the present clinical studies to test several hypotheses. First, we tested the hypothesis that circulating choline and betaine levels were associated with an incident risk for MACE. Second, we sought to test whether any associations between either choline or betaine and CVD risks would be attenuated by inclusion of TMAO into the models since it is TMAO that appears to mechanistically serve as the culprit in enhancing atherosclerosis.

As we continue to explore the intricate relationship between various PC metabolites and atherogenesis, the role of intestinal microbiota has emerged as a key regulator. Recent animal studies have explored the contributions of distinct flavin monooxygenases, a family of hepatic enzymes, in converting TMA to TMAO. We have also established the obligatory role of intestinal microbiota in the production of TMAO from dietary choline and other TMA nutrients in humans, and the association between elevated fasting levels of TMAO and higher incident risks of long-term MACEs. Interestingly, the administration of antibiotics in humans in our prior studies had limited effects on circulating choline and betaine levels, despite abolition of TMAO levels, upon ingestion of stable-isotope-labelled d9-PC. Hence, the presence of elevated

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**Figure 6** Relationship between plasma betaine concentration and risk for major adverse cardiac events in the context of plasma trimethylamine-N-oxide levels. Kaplan–Meier plot and hazard ratio with 95% confidence intervals for unadjusted model, or following adjustments for traditional risk factors and C-reactive protein as in Figure 4B. Median plasma concentration of betaine (41.1 μM) and trimethylamine-N-oxide (3.7 μM) within the cohorts were used to stratify subjects as ‘high’ (≥median) or ‘low’ (<median) values.
plasma levels of choline and betaine, which individually are associated with poorer prognosis, only confer this added risk when associated with concomitant TMAO elevation. In other words, subjects in whom one observes the absence of high TMAO levels despite high choline and betaine levels (i.e. low TMAO producers) are significantly less likely to have an adverse cardiovascular event than those with intestinal microbiota that readily produces TMA and TMAO (i.e. high TMAO producers).

**Conclusion**

Elevated plasma levels of choline and betaine are each associated with poor prognosis even after adjusting for traditional cardiovascular risk factors. However, high choline and betaine levels are associated with higher risk of future MACE only with concomitant increased level of TMAO.

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**Conflict of interest:** Z.W. and S.L.H. are named as co-inventors on pending patents held by the Cleveland Clinic relating to cardiovascular diagnostics. Z.W. reports that he has the right to receive royalty payment for inventions or discoveries related to cardiovascular diagnostics from Liposcience Inc., W.H.W.T. has no relationships to disclose. S.L.H. reports having been paid as a consultant for the following companies: Cleveland Heart Lab, Esperion, Lilly, Liposcience Inc., Merck and Co., Inc., Pfizer Inc. and Proctor and Gamble. S.L.H. reports receiving research funds from Abbott, Cleveland Heart Lab, Liposcience Inc., Pfizer Inc., Proctor and Gamble and Takeda. Dr. S.L.H. reports having the right to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics or therapeutics from the companies shown below: Cleveland Heart Lab., Esperion, Frantz Biomarkers, LLC, Liposcience Inc.

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