



Plasma Trimethylamine N-Oxide and Its Precursors: Population Epidemiology, Parent–Child Concordance, and Associations with Reported Dietary Intake in 11- to 12-Year-Old Children and Their Parents

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ABSTRACT

Background: Trimethylamine N-oxide (TMAO) is a microbiome- and diet-derived metabolite implicated in adverse cardiovascular outcomes. To date, studies of plasma TMAO concentrations have largely focused on individuals with metabolic disease. As such, data on TMAO concentrations in population settings and parent–child dyads are lacking.

Objectives: This study aimed to investigate parent–child concordance, age, and sex effects on plasma concentrations of TMAO and its precursors [l-carnitine, choline, betaine, and dimethylglycine (DMG)]. Associations between concentrations of TMAO and its precursors and self-reported dietary intakes of animal protein (i.e., red meat, meat products, chicken, fish, milk products, and cheese) and fast-food meals were also investigated.

Methods: A total of 1166 children (mean \pm SD age: 11 \pm 0.5 y, 51% female) and 1324 parents (mean \pm SD age: 44 \pm 5.1 y, 87% female) had a biomedical assessment as part of Growing Up in Australia’s Child Health Checkpoint. Plasma TMAO and precursor concentrations were quantified using ultra-high-pressure LC coupled with tandem MS.

Results: Familial dyads significantly contributed to plasma TMAO and precursor concentrations ($P < 0.0001$), explaining 37% of variance for TMAO concentrations. Least-square mean \pm SE plasma TMAO was lower in children (0.79 \pm 0.02 μ M on the log-scale) than in adults (1.22 \pm 0.02 μ M). By contrast, children’s betaine (40.30 \pm 0.34 μ M) and DMG concentrations (1.02 \pm 0.01 μ M on the log-scale) were higher than adults’ betaine (37.50 \pm 0.32 μ M) and DMG concentrations (0.80 \pm 0.01 μ M) ($P < 0.0001$). Mean values of all metabolites, except adult TMAO, were higher in males than in females ($P < 0.001$). Greater reported intake of red meat and fish was associated with higher TMAO concentrations in both children [estimates (95% CIs) for red meat: 0.06 (0.01, 0.10); fish: 0.11 (0.06, 0.17)] and adults [red meat: 0.13 (0.08, 0.17); meat products: 0.07 (0.03, 0.12); and fish: 0.09 (0.04, 0.14)].

Conclusions: Age, sex, and shared family factors, including diet, contribute to variation in plasma concentrations of TMAO and its precursors.

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Keywords: trimethylamine N-oxide, l-carnitine, choline, betaine, dimethylglycine, epidemiology, children, adults, Growing Up in Australia, Longitudinal Study of Australian Children

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Supplemental Figures 1 and 2 and Supplemental Tables 1–7 are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents at <https://academic.oup.com/cdn/>.

Data described in the article will be made available upon request after application and approval by our teams.

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Abbreviations used: CheckPoint, Child Health CheckPoint; DMG, dimethylglycine; FMO3, flavin monooxygenase 3; HFBA, heptafluorobutyric acid; MS-MS, tandem mass spectrometry; QC, quality control; TMA, trimethylamine; TMAO, trimethylamine N-oxide; UHPLC, ultra-high-performance liquid chromatography.

Introduction

Trimethylamine N-oxide (TMAO) has gained increasing attention over the past decade owing to its potential links to metabolic syndrome and cardiovascular disease (1–8). TMAO concentrations, and those of its precursors, have prognostic utility in predicting cardiovascular events (4). Despite this, links between TMAO concentrations and cardiovascular disease have been inconsistent (9, 2, 3–7, 10–16), with stronger relations found in diseased patients (4) than in “healthy” population settings (11). Varying results may also, in part, be due to baseline concentrations of TMAO, with a U-shaped curve of association with cardiovascular outcomes being reported (17). Studies on TMAO and related metabolites have generally focused on individuals with existing metabolic conditions (2, 3, 9, 18, 19), with few population-wide studies (11, 13, 15, 20).

TMAO concentrations vary with diet and pharmacological interventions (5, 21, 22). TMAO can be directly ingested in its preformed state from fish (1, 12, 22–26). This ingested form of TMAO is more rapidly metabolized. TMAO can also be synthesized directly or indirectly from L-carnitine, choline, betaine, dimethylglycine (DMG), and their precursors (e.g., phosphatidylcholine, crono-betaine, γ -butyrobetaine) (1, 12, 22–26) (Figure 1). These are obtained from a variety of dietary or supplemental sources, endogenous biological fluids (e.g., bile), or by synthesis from other amino acids (1, 12, 22–26). Precursors are metabolized by trimethylamine (TMA)-producing bacteria in the gut microbiota to form TMA, which is subsequently oxidized by hepatic flavin-containing monooxygenase 3 (FMO3) enzymes into TMAO (1, 12, 22–26). TMAO circulates in the blood, is absorbed by peripheral tissues, and is almost fully (>90%) excreted in the urine in healthy subjects (22, 23, 24, 27–29). However, the dietary contributions to plasma TMAO concentrations have been inconsistent in the literature (11, 22, 23, 24, 29, 30, 31, 32).

Cardiovascular disease is one of the leading causes of death worldwide (33). Measures of TMAO and of its precursors have shown utility in the prediction and prognosis of cardiovascular disease (2, 4), and these metabolites’ concentrations can be altered by the diet (5, 21, 22). Although cardiometabolic conditions have their origins in early life (34), baseline reference values of TMAO concentrations in healthy populations of children, and across generations, are generally lacking (11, 35, 36). Therefore, we aimed at characterizing homeostatic plasma concentrations of TMAO and of its precursors L-carnitine, choline, betaine, and DMG in 11- to 12-y-olds and their parents from a population-derived cohort study. We analyzed familial concordance (i.e., differences between parent–child dyads), absolute differences between generations (i.e., children compared with adults) and sexes, as well as associations with self-reported dietary animal protein intake in children and adults.

Methods

Ethical approval, consent, and sample collection

The Child Health CheckPoint study was approved by The Royal Children’s Hospital (Melbourne, Australia) Human Research Ethics Committee (33225D) and the Australian Institute of Family Studies Ethics Committee. A total of 1874 parent–child dyads participated in a

biomedical assessment, Child Health CheckPoint (CheckPoint), nested between waves 6 and 7 of the B-cohort of the Longitudinal Study of Australian Children (LSAC) (37, 38) (Supplemental Figure 1). Parents or caregivers provided consent for themselves and their child to participate in CheckPoint and for the collection of their blood samples (39).

Procedures

Venous blood was collected from parents and children after on average a 4-h fast (39). Venous blood was collected into EDTA-coated tubes, processed within 1 h into plasma, and frozen at -80°C . For the current analysis, a total of 2490 plasma samples (1166 children and 1324 parents) were shipped, on dry ice in thermally monitored boxes, to the Liggins Institute, New Zealand. Samples were received in plates containing 96 FluidX™ tubes in no specific order except for keeping parent–child pairs on the same plate. For the purpose of our assay, only 74 samples could be run per plate because the remaining positions were required for blanks, quality controls (QCs), and the calibration curve. All 96 samples were firstly sorted by the family barcode to ensure that parent–child pairs were kept on the same plate after randomization. After filtering by family, the last 22 samples were moved to the following plate. The RAND() formula was then used in Microsoft Excel to assign a random number for the remaining 74 samples on each plate. The list of numbers generated by the RAND() formula was then ascendingly sorted to randomly distribute all 74 samples on the randomization plate. Samples were randomly distributed onto 34 different 96-well plates on dry ice, keeping all parent–child pairs (1121 pairs) on the same plate, and stored at -80°C before both ultra-high-performance liquid chromatography (UHPLC)/tandem mass spectrometry (MS-MS) assays.

Sample preparation and UHPLC/MS-MS analysis

All compounds were measured from a single plasma aliquot (100 μL , thawed to room temperature) using a Vanquish UHPLC+ system coupled with a TSQ Quantiva triple quadrupole mass spectrometer (Thermo Scientific). The protein precipitation was conducted using an Eppendorf robot fitted with a thermal mixer and vacuum manifold (EpMotion 5075vt).

Two analytical methods were used. The assay measuring precursor concentrations (L-carnitine, choline, betaine, and DMG) is described in detail elsewhere (40). TMAO concentrations were profiled as part of an analytical panel quantifying B vitamins, the results of which were analyzed separately (under review). Briefly, 300 μL of the protein precipitation mixture 0.3% (vol:vol) acetic acid and 2.5% (vol:vol) MilliQ® H₂O in methanol was added to either 1) 100 μL calibration curve standards (Supplemental Table 1); 2) 100 μL plasma sample; 3) 100 μL of a 4% (wt:vol) solution of BSA in PBS; or 4) triplicate QC samples [located at 3 different positions of a 96-well IMPACT protein precipitation plate (Phenomenex®)]. Then, 10 μL of an internal standard solution (detailed in Supplemental Table 2) was added to all wells, and the plate was agitated on the epMotion 5075 robot thermomixer (room temperature; 800 rpm; 5 min). The protein precipitate eluent was then vacuum filtrated into the collection plate (250 mbar, 5 min). The filtrate was dried (40°C , 3 h, 0.03 bars of pressure) in a SpeedVac concentrator (Savant SC250EXP, Thermo Scientific) coupled to a refrigerated vapor trap (Savant RVT4104, Thermo Scientific). The dried residue was suspended in a reconstitution solution [200 μL ; 1% (wt:vol) ascorbic acid]

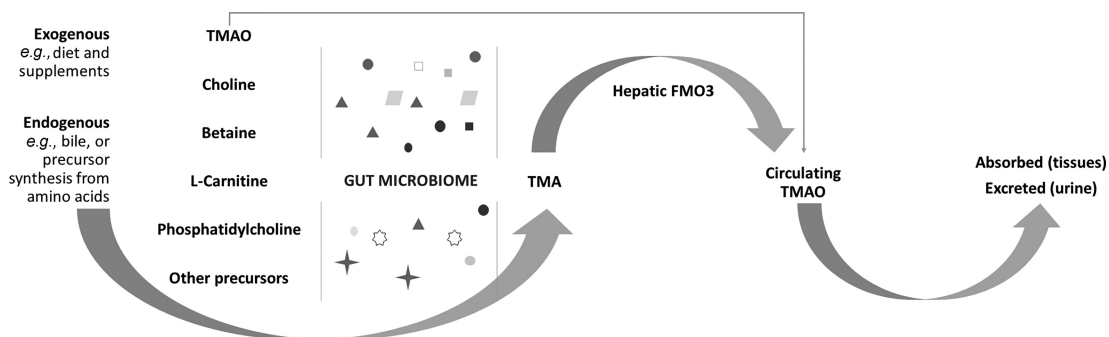


FIGURE 1 Simplified graphical representation of TMAO metabolism. Preformed TMAO and its dietary precursors provided from exogenous or endogenous sources are metabolized by TMA-producing bacteria in the gut microbiota to form TMA, which is then oxygenated by hepatic enzymes to form TMAO. FMO3, flavin monooxygenase 3; TMA, trimethylamine; TMAO, trimethylamine N-oxide.

mixed with the mobile phase [5% acetic acid, 0.2% (wt:vol) heptafluorobutyric acid (HFBA) in deionized Millipore H₂O (MilliQ®)]. The plate was agitated (epMotion 5075 robot thermomixer; room temperature; 800 rpm; 5 min) and placed in the UHPLC autosampler for analysis. Chromatographic separation was achieved using a Kinetex® 2.6 µm F5 100 Å 150 × 2.1 mm column (Phenomenex®) coupled with a Krud-katcher (Phenomenex®) precolumn filter, with a mobile phase consisting of 5% acetic acid, 0.2% (wt:vol) HFBA in Millipore H₂O (MilliQ®). The source consisted of a heated electrospray ionization in positive ionization mode. A 200 µL/min flow was applied starting at 4% acetonitrile and 96% mobile phase. The analytical run time was 14 min/sample. QCs were used to monitor inter- and intra-assay reproducibility (with ≤20% variation between days being accepted). One plate failed the 20% QC cutoff and was reanalyzed.

Dietary data

Adults and children filled out a 26-item brief food-frequency survey of usual intakes of several foods, adapted from the Australian National Secondary Students' Diet and Activity survey, as well as the ISCOLE (International Survey of Childhood Obesity, Lifestyle and Environment) study (39, 41, 42). The food groups analyzed in relation to TMAO concentrations and its precursors included habitual intakes of red meat (e.g., beef, lamb, steaks, chops, roasts, mince, stir fries, and casserole, meat products (e.g., hot dogs, sausages, ham, sausage rolls, salami, meat pies, chicken nuggets, or bacon), chicken, fish and canned fish, fast-food meals and snacks (e.g., pizzas, burgers, chips), cheese, and dairy products (e.g., yogurt, chocolate milk, pudding). Intake was reported as follows: never, less than once a week, ~1–2 times/wk, ~3–4 times/wk, ~5–6 times/wk, or every day.

Statistical analysis

All statistical analyses were performed in the R programming environment, version 3.6.1 (43). TMAO and DMG concentrations were positively skewed after accounting for plate effects and were therefore log transformed before use in mixed modeling. Choline, betaine, and L-carnitine were normally distributed after adjustment for plate effects, and therefore were not transformed (Supplemental Figure 2).

Two sets of mixed models were used to identify the effects of generation (parents compared with children) and within-family concordance

using the *lme4* package on R (44), after controlling for plate effects. Likelihood ratio tests were conducted between models to compare the fit (i.e., log likelihood) of models with and without the factor tested (i.e., family or generation). Log likelihoods were compared between models that contained both family (as a random effect) and generation (as a fixed effect), and those excluding one or the other. Family effect sizes were calculated as the ratio of family variance divided by the total variance of each adjusted variable. Pearson's correlations adjusted for multiple testing using the *Holm* method in R were also conducted within parent–child dyads to confirm familial correlations.

Two sets of linear models were fitted for each plate-adjusted/log-transformed variable to determine the effect of 1) sex in children and adults; and 2) age within the adult subgroup (28–71 y). Given that females exhibit fluctuations in TMA oxidation (and therefore TMAO production) throughout their menstrual cycle, a sensitivity analysis, using *t* tests, was conducted to assess differences in TMAO concentrations between menstruating (on the day of blood collection) and non-menstruating females in the child and adult groups separately. ANOVAs were computed to evaluate associations between the reported intakes of the listed foods and the concentrations of metabolites of interest in both children and adults (45).

Results

Population characteristics

The sample analyzed in this study consisted of 2490 individuals (1121 parent–child pairs), including 1166 children (601 girls; 565 boys; mean ± SD age: 11 ± 0.5 y) and 1324 adults (1150 women; 174 men; mean ± SD age: 44 ± 5.1 y) (Table 1).

Measures of TMAO and precursor concentrations were concordant between children and parents

Both likelihood ratio tests ($P < 0.0001$) and Pearson's correlations between dyads highlighted the strongest family effect for TMAO (correlation coefficient: 0.39; CI adjusted for multiple testing: 0.34, 0.44), followed by DMG (0.38; CI: 0.33, 0.43), betaine (0.22; CI: 0.17, 0.28), and L-carnitine (0.21; CI: 0.15, 0.26), with the weakest correlation for choline (0.13; CI: 0.08, 0.19). Family effects explained 37% of the vari-

TABLE 1 Sample characteristics¹

Characteristic	Children			Adults		
	All	Boys	Girls	All	Men	Women
<i>n</i>	1166	565	601	1324	174	1150
Age, y	11.4 ± 0.5	11.4 ± 0.5	11.5 ± 0.5	43.9 ± 5.1	46.2 ± 6.4	43.6 ± 4.8
BMI rounded, kg/m ²						
Median [IQR]	18.4 [16.8, 20.6]	18.1 [16.7, 20.2]	18.8 [17.0, 21.1]	26.5 [23.4, 31.0]	27.4 [25.2, 31.1]	26.3 [23.1, 31.0]
BMI z scores	0.3 ± 0.9	0.3 ± 0.9	0.3 ± 0.9	N/A	N/A	N/A
Biological parent of child, <i>n</i>	N/A	N/A	N/A	1313	172	1141
Australian state of current residence, <i>n</i>	New South Wales (359); Victoria (261); Queensland (221); South Australia (92); West Australia (139); Tasmania (40); Northern Territory (17); Australian Capital Territory (38)			New South Wales (391); Victoria (311); Queensland (240); South Australia (108); West Australia (164); Tasmania (46); Northern Territory (18); Australian Capital Territory (47)		
Socio-Economic Indexes for Areas (SEIFA) disadvantage quintile, <i>n</i>	Most disadvantaged (83); second most (171); middle (199); second least (272); least disadvantaged (442)			Most disadvantaged (94); second most (193); middle (233); second least (304); least disadvantaged (501)		

¹Values are medians [IQRs] for skewed variables and means ± SDs for normally distributed variables.

ability for TMAO, 36% for DMG, 26% for L-carnitine, 22% for betaine, and finally 13% for choline (Table 2).

Plasma TMAO, betaine, and DMG concentrations were age-dependent

Plasma TMAO concentrations were higher ($P < 0.0001$) in adults (least-square mean ± SE: $1.22 \pm 0.02 \mu\text{M}$ on the log scale) than in children ($0.79 \pm 0.02 \mu\text{M}$) (Table 3). By contrast, adult betaine ($37.50 \pm 0.32 \mu\text{M}$) and DMG concentrations ($0.80 \pm 0.01 \mu\text{M}$ on the log scale) were lower than children's betaine ($40.30 \pm 0.34 \mu\text{M}$) and DMG concentrations ($1.02 \pm 0.01 \mu\text{M}$ on the log scale) ($P < 0.0001$ for both). No intergenerational differences were identified for plasma choline and L-carnitine (Table 3).

Given the narrow age distribution in children (11–12 y), we characterized age-specific differences within the adults exclusively (28–71 y as a continuous scale). Only TMAO and betaine plasma concentrations showed weak positive increases with ascending age (Figure 2, Supplemental Table 3).

Plasma concentrations of TMAO and its precursors were higher in males

Sex-specific differences were identified in the plasma concentrations of all tested compounds. Plasma concentrations were higher in boys for TMAO (least-square means ± SEs: 0.86 ± 0.03 in boys; $0.72 \pm 0.03 \mu\text{M}$ in girls on the log-scale; $P = 0.003$), L-carnitine ($50.30 \pm 0.65 \mu\text{M}$ in boys; $46.50 \pm 0.63 \mu\text{M}$ in girls; $P < 0.0001$), DMG ($1.09 \pm 0.01 \mu\text{M}$ in boys; $0.96 \pm 0.01 \mu\text{M}$ in girls on the log scale; $P < 0.0001$), betaine ($42.00 \pm 0.46 \mu\text{M}$ in boys; $38.70 \pm 0.44 \mu\text{M}$ in girls; $P = 0.02$), and choline ($11.20 \pm 0.11 \mu\text{M}$ in boys; $10.50 \pm 0.11 \mu\text{M}$ in girls; $P < 0.0001$). Similarly, the concentrations of L-carnitine ($53.10 \pm 1.20 \mu\text{M}$ in men; $46.70 \pm 0.47 \mu\text{M}$ in women; $P < 0.0001$), DMG ($0.98 \pm 0.03 \mu\text{M}$ in men; $0.78 \pm 0.01 \mu\text{M}$ in women on the log-scale; $P < 0.0001$), betaine ($45.10 \pm 0.89 \mu\text{M}$ in men; $36.40 \pm 0.35 \mu\text{M}$ in women; $P < 0.0001$), and choline ($12.00 \pm 0.20 \mu\text{M}$ in men; $10.80 \pm 0.08 \mu\text{M}$ in women; $P < 0.0001$) were higher in men than women in the adult subgroup. However, TMAO concentrations did not differ by sex in adults (Table 4).

A sensitivity analysis did not identify marked differences in the plasma concentrations of TMAO and of its precursors ($P > 0.05$ for all metabolites) between females who reported that they were menstruat-

ing (20 children and 175 women) and those who reported that they were not (95 postpubertal children and 953 women) on the day of blood collection (Supplemental Table 4). By contrast, within the adults, plasma concentrations of both betaine and L-carnitine were present at higher concentrations in women who were menstruating on the day of blood collection.

TMAO correlated with L-carnitine and choline but not with DMG or betaine

L-carnitine ($r = 0.053$, $P = 0.03$) and choline ($r = 0.076$, $P = 0.0006$) showed small correlations with TMAO concentrations. However, DMG and betaine were not correlated with TMAO concentrations ($r = -0.004$, $P = 0.9$; and $r = 0.02$, $P = 0.6$, respectively) (Supplemental Table 5).

Fish and red meat consumption was positively associated with plasma TMAO

In children, consumption of fish (estimate: 0.11; 95% CI: 0.06, 0.17; $P < 0.0001$) and red meat (0.06; 95% CI: 0.01, 0.10; $P = 0.01$) was positively associated with TMAO concentrations (Figure 3, Supplemental Table 6). The consumption of fast-food meals and snacks (e.g., pizzas, chips, burgers) (-0.08 ; 95% CI: -0.15 , -0.01 ; $P = 0.02$) was negatively associated with TMAO concentrations in children. Most children ($n = 1106$, 96%) consumed fast-food meals ≤ 3 times/wk with only 3 children reportedly consuming fast-food meals 5–7 d/wk (Supplemental Table 7). TMAO concentrations were also positively associated with regular intakes of fish (0.09; 95% CI: 0.04, 0.14; $P = 0.001$), red meat (0.13; 95% CI: 0.08, 0.17; $P < 0.0001$), meat products (0.07; 95% CI: 0.03, 0.12; $P = 0.001$), and chicken (0.05; 95% CI: 0.002, 0.11; $P = 0.04$) in adults. Choline and DMG concentrations were not associated with any of the foods assessed in children ($P > 0.05$ for all). In adults, choline concentrations were positively associated with meat products (0.25; 95% CI: 0.10, 0.41; $P = 0.001$) and fast-food meals and snacks (0.26; 95% CI: 0.03, 0.49; $P = 0.03$), yet DMG concentrations were weakly associated with red meat products and fast-food meals and snacks (Figure 3). L-carnitine concentrations were positively associated with habitual intakes of chicken (1.21; 95% CI: 0.15, 2.28; $P = 0.03$) and fast-food meals and snacks (1.42; 95% CI: 0.12, 2.72; $P = 0.03$) in children only, and positively associated with red meat intake (1.07; 95% CI: 0.11, 2.03; $P = 0.03$) and meat product intake (1.44; 95% CI: 0.52, 2.36; $P = 0.002$)

TABLE 2 Variances and effect sizes of family on compound concentrations¹

Compound, μM	Effect of dyad (or family) on mixed model ²	Family (dyad) effect variance	Plate-adjusted compound variance	Effect size of family on compound concentrations, ³ %
TMAO ⁴	$P < 0.0001$ <ul style="list-style-type: none"> Log likelihood with family = -2859 Log likelihood without family = -2953 	0.25	0.67	37
L-Carnitine	$P < 0.0001$ <ul style="list-style-type: none"> Log likelihood with family = -10,378 Log likelihood without family = -10,404 	63.92	249.62	26
DMG ⁴	$P < 0.0001$ <ul style="list-style-type: none"> Log likelihood with family = -747 Log likelihood without family = -837 	0.05	0.12	36
Betaine	$P < 0.0001$ <ul style="list-style-type: none"> Log likelihood with family = -9603 Log likelihood without family = -9632 	29.86	136.01	22
Choline	$P < 0.0001$ <ul style="list-style-type: none"> Log likelihood with family = -5957 Log likelihood without family = -5967 	0.90	7.07	13

¹DMG, dimethylglycine; TMAO, trimethylamine N-oxide.

²Log likelihoods were derived from mixed models including generation as a fixed effect, with or without family as a random effect.

³Calculated as the family (dyad) effect variance divided by the plate-adjusted compound variance $\times 100$.

⁴Log-transformed variable.

in adults only. Plasma betaine concentrations were not associated with any of the food groups tested in either generation. The CIs for both L-carnitine and betaine were very large (Figure 3), therefore assertions about these associations must be made cautiously. Finally, reported intakes of milk products and cheese were not associated with any of the measured metabolites in either generation (Figure 3, Supplemental Table 6).

Discussion

This is the first study that we know of to report plasma concentrations of TMAO and its precursors in a population-based sample of children and parents. The proportion of variability explained by familial relationships (i.e., a shared gene-environment setting) ranged from 13% for choline concentrations to 37% for TMAO concentrations. Intergenerational dif-

TABLE 3 LS-means, SEs, and mixed models' results for TMAO and its precursors in children and parents¹

Compound, μM	Parent LS-mean \pm SE	Child LS-mean \pm SE	Effect of generation on mixed model ² (children, adults)
TMAO ³	1.22 \pm 0.02	0.79 \pm 0.02	$P < 0.0001$ <ul style="list-style-type: none"> Log likelihood with generation = -2859.2 Log likelihood without generation = -2993.7
L-Carnitine	47.60 \pm 0.43	48.30 \pm 0.46	$P = 0.16$ <ul style="list-style-type: none"> Log likelihood with generation = -10,378 Log likelihood without generation = -10,379
DMG ³	0.80 \pm 0.01	1.02 \pm 0.01	$P < 0.0001$ <ul style="list-style-type: none"> Log likelihood with generation = -747.36 Log likelihood without generation = -922.05
Trimethylglycine (betaine)	37.50 \pm 0.32	40.30 \pm 0.34	* $P < 0.0001$ <ul style="list-style-type: none"> Log likelihood with generation = -9603.2 Log likelihood without generation = -9624.8
Choline	11.00 \pm 0.07	10.80 \pm 0.08	$P = 0.14$ <ul style="list-style-type: none"> Log likelihood with generation = -5956.7 Log likelihood without generation = -5957.8

¹DMG, dimethylglycine; LS-mean, least-square mean; TMAO, trimethylamine N-oxide.

²Log likelihoods were derived from mixed models including family as a random effect, with or without generation as a fixed effect.

³LS-means \pm SEs are computed from log-transformed variables.

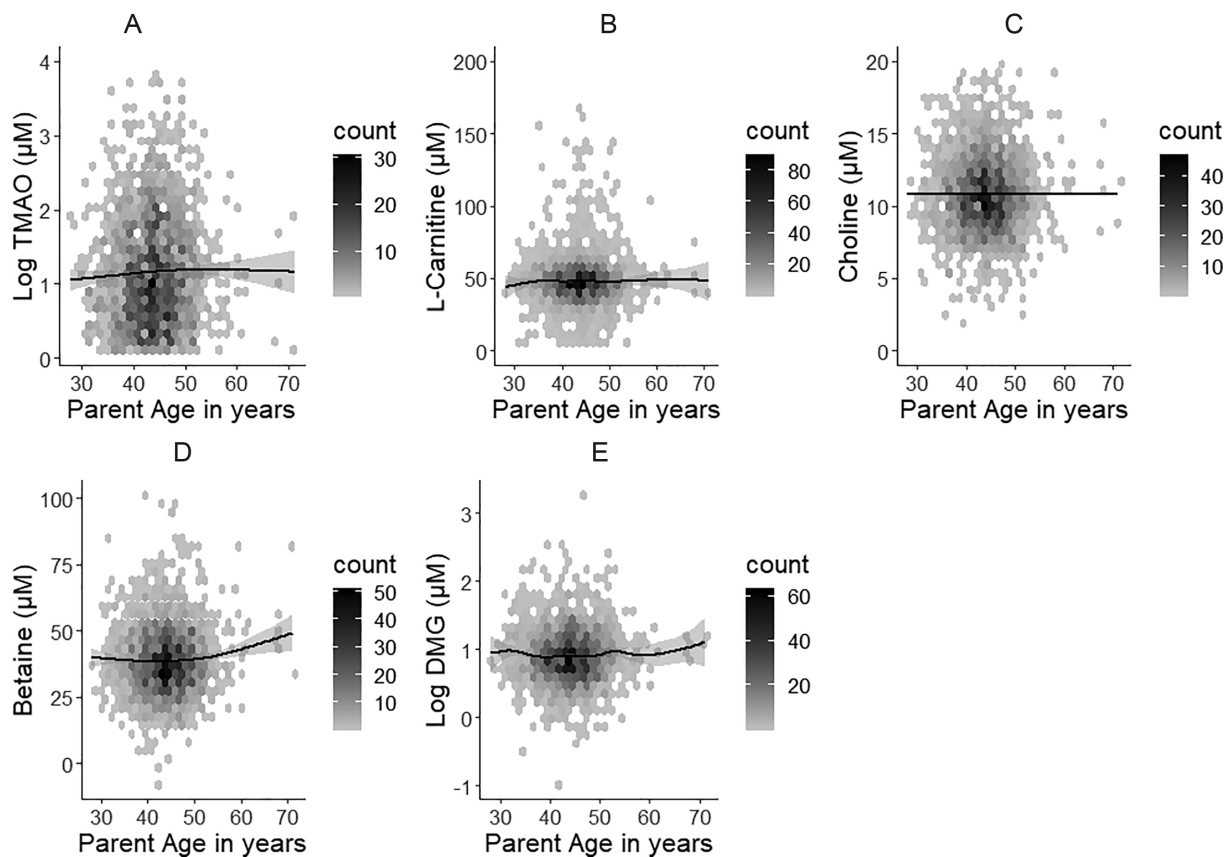


FIGURE 2 Hexagonal plots of TMAO (A), l-carnitine (B), choline (C), betaine (D), and DMG (E) across the adult age range ($n = 1324$). Plasma concentrations of TMAO and betaine are weakly positively associated with age ($P \leq 0.05$). DMG, dimethylglycine; TMAO, trimethylamine N-oxide.

ferences were evident for plasma TMAO, betaine, and DMG concentrations, with higher concentrations of TMAO and lower concentrations for betaine and DMG in adults than in children. Finally, all compounds were higher in males in both children and adults, except for TMAO in adults. We identified family, age, and sex as important factors potentially characterizing plasma concentrations of TMAO and its precursors.

Strong family effects were expected because genetic and dietary factors are known to be important in TMAO synthesis (1, 23, 24, 46, 47, 48–50). Reported heritability estimates based on family studies for TMAO have identified a moderate genetic contribution to its circulating con-

centrations (~27%) (50). The proportion of variability explained by familial relationships for TMAO was higher in our study (37%) but was not calculated from genetic data. Familial concordance in nutritional metabolites is supported by previous work on our cohort, with marked concordance reported for amino acid and fatty acid profiles between children and their parents (51).

Mutations and polymorphisms that inactivate or change the activity of the hepatic FMO3 enzyme have been associated with the accumulation of TMA (known as trimethylaminuria or “fish odor” syndrome) and concomitantly lower plasma TMAO concentrations (1). The hep-

TABLE 4 LS-means, SEs, and linear model results of TMAO concentrations by sex and generation¹

Compound	Children			P value	Adults			P value
	Girls, LS-means \pm SEs	Boys, LS-means \pm SEs	Adjusted R^2 of linear model		Women, LS-means \pm SEs	Men, LS-means \pm SEs	Adjusted R^2 of linear model	
TMAO, ² μ M	0.72 \pm 0.03	0.86 \pm 0.03	0.01	0.003	1.21 \pm 0.02	1.32 \pm 0.06	0.001	0.09
l-Carnitine, μ M	46.50 \pm 0.63	50.30 \pm 0.65	0.01	<0.0001	46.70 \pm 0.47	53.10 \pm 1.20	0.02	<0.0001
DMG, ² μ M	0.96 \pm 0.01	1.09 \pm 0.01	0.04	<0.0001	0.78 \pm 0.01	0.98 \pm 0.03	0.04	<0.0001
Betaine, μ M	38.70 \pm 0.44	42.00 \pm 0.46	0.02	<0.0001	36.40 \pm 0.35	45.10 \pm 0.89	0.06	<0.0001
Choline, μ M	10.50 \pm 0.11	11.20 \pm 0.11	0.02	<0.0001	10.80 \pm 0.08	12.00 \pm 0.20	0.02	<0.0001

¹DMG, dimethylglycine; LS-mean, least-square mean; TMAO, trimethylamine N-oxide.

²LS-means and SEs are computed from log-transformed variables.

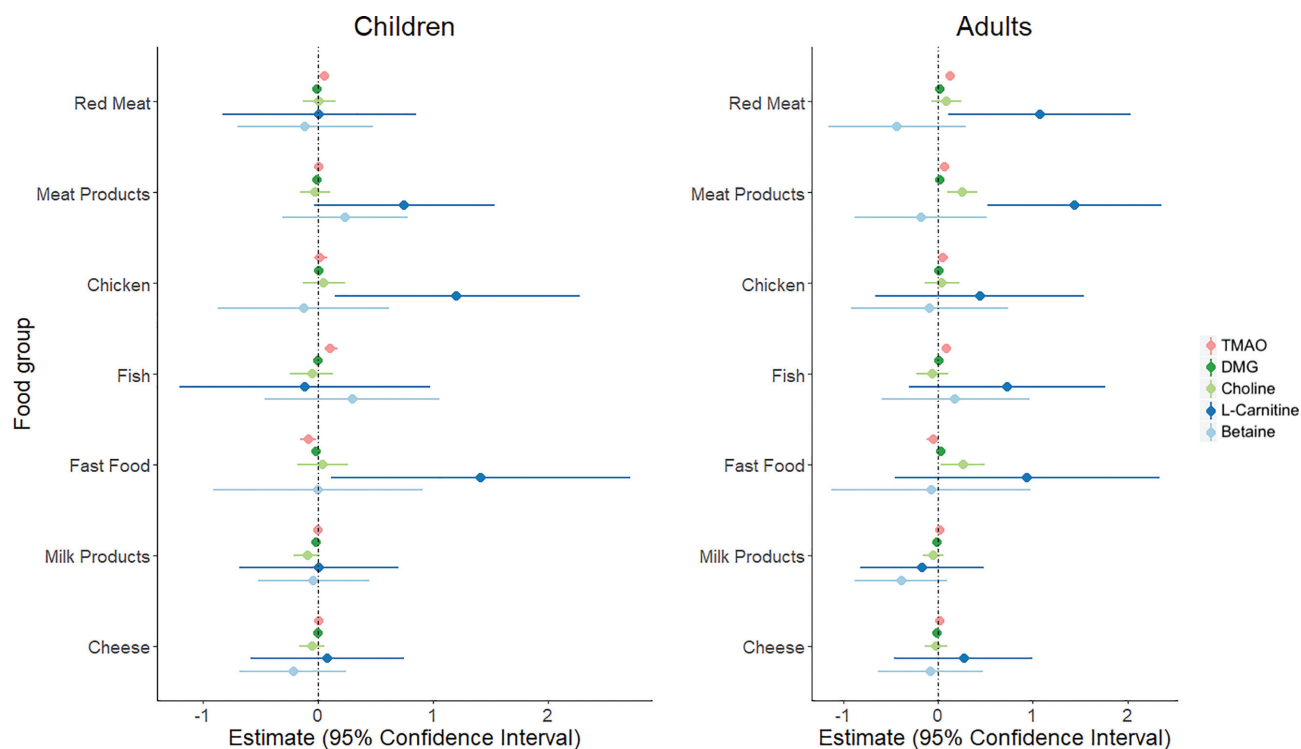


FIGURE 3 Forest plots of associations between reported intakes of animal protein sources and fast food and the concentrations of TMAO and its precursors in children ($n = 1166$) and adults ($n = 1324$). Plasma TMAO concentrations are positively associated with the reported consumption of fish (estimate: 0.11; 95% CI: 0.06, 0.17; $P < 0.0001$) and red meat (0.06; 95% CI: 0.01, 0.10; $P = 0.01$), and negatively associated with the reported consumption of fast-food meals and snacks (-0.08 ; 95% CI: -0.15 , -0.01 ; $P = 0.02$) in children. TMAO concentrations were positively associated with reported regular intakes of fish (0.09; 95% CI: 0.04, 0.14; $P = 0.001$), red meat (0.13; 95% CI: 0.08, 0.17; $P < 0.0001$), meat products (0.07; 95% CI: 0.03, 0.12; $P = 0.001$), and chicken (0.05; 95% CI: 0.001, 0.11; $P = 0.04$) in adults. DMG, dimethylglycine; TMAO, trimethylamine N-oxide.

atic expression of *FMO3* becomes evident between birth and 2 y of age, gradually increasing to intermediate expression around 11–12 y of age and peaking in adulthood (47). Age-dependent changes in *FMO3* expression may explain the intergenerational differences in TMAO concentrations observed here between middle-aged adults and their 11- to 12-y-old children. Consistent with this and previous studies (11, 29, 52), we identified a positive association for TMAO with increased age within the adult subgroup (28–71 y), although lower and upper age ranges were underrepresented (mean \pm SD age: 44 ± 5.1 y). Although age-specific increases in *FMO3* expression have been reported (47), the exact adult age at which *FMO3* expression peaks is unknown. Therefore, studies looking at age-dependent changes of *FMO3* gene expression, enzymatic activity of *FMO3*, and their impact on circulating and urinary concentrations of TMAO in a large cohort of healthy adults with a more consistent age distribution as well as a balanced ratio of males to females are needed.

Although concentrations of TMAO were higher in adults than in children, those of its precursors DMG and betaine were lower, which may reflect increased rates of TMAO synthesis. However, the concentrations of betaine and DMG were not correlated with those of TMAO, consistent (for betaine) with previous reports (29). The oral administration of betaine (a DMG precursor) has been reported to result in only a modest increase in urinary TMAO compared with administration of

L-carnitine, choline, or preformed TMAO (53), which may explain the lack of a strong correlation. Nevertheless, the cross-sectional design of our study coupled with a single plasma measurement may have limited our ability to detect strong correlations between these compounds at 1 specific time point. Therefore, our results should be regarded as preliminary and further longitudinal studies are needed to determine the relations between lower plasma concentrations of betaine and DMG and higher TMAO concentrations in healthy populations.

In both generations, L-carnitine, DMG, betaine, and choline concentrations were markedly higher in males than in their female counterparts. TMAO concentrations were also higher in boys with weak evidence for higher concentrations in men. Previous reports on sex-specific differences in plasma concentrations of these metabolites have been inconsistent (9, 11, 13, 15, 20, 54, 55), which may be due in part to modest sample sizes in some studies. For example, TMAO serum concentrations were similar between adult males ($n = 90$) and females ($n = 130$) in a German cohort with a sample size 10 times smaller than ours (56). Sex differences in TMAO and precursor concentrations have been proposed to be mediated by sex-specific hormonal effects on TMAO metabolism (57–59). For example, females exhibit fluctuations in TMA oxidation throughout their menstrual cycles, with higher TMA and concomitantly lower urinary TMAO concentrations around the time of menstruation (60), explaining the exacerbation

of malodor symptoms of females with trimethylaminuria during that phase of the cycle (61). Theoretically, these differences can be mediated by hormonal suppression of *FMO3* expression and enzymatic activity (62, 57–60). Notably, our results did not identify marked differences for TMAO concentrations by menstruation in either adult or young females. Conversely, both betaine and L-carnitine exhibited differences between menstruating and nonmenstruating adult women. Previous work identified menstrual metabolic rhythmicity for several metabolites (e.g., acylcarnitines, amino acids) involved in key metabolic pathways and proposed that these differences reflect fluctuations in anabolic requirements in response to menstrual hormonal changes (e.g., variations in demands for β -oxidation and energy utilization) (63). Nevertheless, we had limited numbers of females reporting menstruation status and larger studies are needed to determine the effect of menstruation on TMAO concentrations. Moreover, given the reported hormonal influences on TMAO concentrations, it may be important to account for states of hormonal fluctuations (e.g., menstruation, menopause) when characterizing the concentrations of TMAO and of its precursors in future studies.

Higher plasma concentrations of TMAO and its metabolites in males may be associated with higher protein intake (22, 23, 64, 65). Notably, protein intakes of twice the RDAs have been shown to increase circulating TMAO concentrations in an intervention study of healthy older adult males (65). However, associations between the diet and TMAO concentrations have been largely inconsistent (11, 22, 23, 29–32). These differences may partly be attributed to disparities in dietary assessment methods between studies, and/or differences between observational and interventional study designs (11, 22, 23, 29–32). For example, meat, fish, eggs, and dairy products have all been reported to increase plasma and/or urinary TMAO concentrations, although the strength of association varies (11, 22, 23, 29–32). Moreover, although vegetarians generally have lower plasma TMAO concentrations, the strictness of a vegetarian diet (i.e., vegan compared with lacto-ovo-vegetarian) does not affect TMAO concentrations (32). Interestingly, increases in TMAO concentrations in response to a L-carnitine challenge have been shown to vary according to whether individuals were omnivores or vegetarians (21). In addition, sources of fish (e.g., freshwater compared with saltwater) and cooking methods (e.g., deep frying compared with stir frying) correlate differently with urinary TMAO concentrations (15). Collectively, these observations highlight that TMAO concentrations can be altered by the diet, which may be important in cases where TMAO concentrations are considered cardiotoxic (66).

Consistent with previous reports, we found that the frequency of consumption of animal protein sources in our Australian cohort was associated with TMAO and L-carnitine concentrations in both generations. In addition, reported animal protein intake frequency was associated with DMG and choline concentrations in the parent subgroup exclusively. By contrast, the reported habitual intake of dairy products was not associated with any of the precursors, in contrast to observations from a German dietary study (29). Finally, betaine did not show evidence of association with the diet in either generation, consistent with previous reports (30). Differences in dietary associations between studies may be confounded by country-specific dietary habits, health status of the population studied, gut microbial composition and diversity, diet misreporting, timing of blood collection, as well as general lifestyle and dietary patterns. Therefore, diet–metabolite associations must be con-

textualized for each population/individual and extrapolations must be made cautiously.

TMAO originating from different dietary sources (diet, microbiome, etc.) has the same structure and therefore cannot be resolved analytically (29). This knowledge gap is an obstacle to understanding whether TMAO contributes to cardiovascular disease risk. However, preformed TMAO (found in fish) is more readily metabolized than TMAO that is synthesized from its precursors, thus fish-based TMAO is more readily available, and urinary TMAO itself has been identified as a good biomarker of fish intake (23, 67). Given the reported beneficial health effects of fish consumption (68, 69), time-dependent analyses of TMAO concentrations in both urine and plasma linked to robust dietary and metabolic data may be important to stratify individuals with high TMAO concentrations/high TMAO-production capacity by differential metabolic/dietary status, as has been previously performed (21, 46).

Limitations of this study

There are a number of limitations to our study. Despite the large sample size ($n = 2490$ individuals), our study was cross-sectional and semi-fasted plasma was only collected from individuals at 1 time point, and with different fasting times which would have affected our metabolite measures as well as their associations with reported dietary intakes. In addition, dietary intakes were self-reported, which is subject to bias (70, 71), and did not include detailed information on portion size or energy content. Moreover, the ratio of adult males to females in our study was unbalanced (1:10), which limited our power to identify some sex-specific differences, or to examine the effect of age in males. Furthermore, our cohort is relatively wealthier than the Australian population (>78% of our population scored in the middle to least disadvantaged Socio-Economic Indexes for Areas score quintiles compared with ~62% in the general Australian population) (72, 73). Finally, the collection and analysis of our participants' stool samples would have strengthened our conclusions given that the gut microbiome is important in TMAO formation (23), but was not feasible as part of the biomedical assessment.

Future directions

Identifying the drivers of TMAO concentrations (e.g., fish, red meat, diseases, medication, gut dysbiosis, stress, exercise) and the metabolic profiles (e.g., inflammatory markers, lipid and glycemic profiles) of individuals with high TMAO concentrations/increased TMAO-production capacity will improve the stratification of these individuals and their responses to interventions that target metabolic health outcomes. Therefore, future studies should be designed to combine measurements of fasting TMAO concentrations (in plasma and urine) with 1) TMAO concentrations in response to dietary/precursor (e.g., L-carnitine) challenges at different time points; and 2) measures of context-specific regulation of the genetic, transcriptional, gut microbial, and metabolic machinery involved in the metabolism of TMAO (21, 46). Given the emerging putative link between TMAO concentrations and cardiovascular and metabolic outcomes, we plan to analyze some of these associations.

Conclusion

Plasma TMAO concentrations are subject to familial, age, and sex-specific influences. Therefore, given the well-documented differences

in cardiovascular disease risk in association with sex, age, and environmental influences, the relation between TMAO concentrations and cardiovascular preclinical phenotypes warrants further investigation.

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