



Association of Serum Trimethylamine-N-Oxide Concentration from Childhood to Early Adulthood with Age and Sex

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BACKGROUND: Primary prevention is the cornerstone of cardiometabolic health. In the randomized, controlled Special Turku Coronary Risk Factor Intervention Project (STRIP), dietary counseling intervention was given to children from infancy to 20 years of age and a follow-up was completed at age 26 years. We investigated the associations of age, sex, gut microbiome, and dietary intervention with the gut metabolite and the cardiac biomarker trimethylamine-N-oxide (TMAO).

METHODS: Overall, 592 healthy participants (females 46%) from STRIP were investigated. Compared to the control group, the intervention group had received dietary counseling between ages 7 months and 20 years focused on low intakes of saturated fat and cholesterol and the promotion of fruit, vegetable, and whole-grain consumption. TMAO serum concentrations were measured by a liquid chromatography-tandem mass spectrometry method at ages 11, 13, 15, 17, 19, and 26 years. Microbiome composition was assessed using 16S rRNA gene sequencing at 26 years of age.

RESULTS: TMAO concentrations increased from age 11 to 26 years in both sexes. At all measurement time points, males showed significantly higher serum TMAO concentrations compared to females, but

concentrations were similar between the intervention and control groups. A direct association between TMAO concentrations and reported fiber intake was found in females. Gut microbiome analysis did not reveal associations with TMAO.

CONCLUSIONS: TMAO concentration increased from childhood to early adulthood but was not affected by the given dietary intervention. In females, TMAO concentrations could be directly associated with higher fiber intake suggesting sex-specific differences in TMAO metabolism.

Introduction

The small organic molecule trimethylamine oxide (TMAO) is generated in a gut microbiota-dependent way and originates from dietary precursors metabolized by specific bacteria (1). Elevated blood TMAO concentrations have been directly associated with a wide range of adverse health outcomes, including all-cause as well as cardiovascular mortality, cardiovascular disease (CVD), type 2 diabetes mellitus, frailty, hypertension, and renal failure (2, 3). In this context, TMAO was found to be an

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important protein stabilizer by preserving protein folding, whereby it acts as an electron acceptor and thereby increases oxidative stress, which ultimately leads to vascular inflammation (4). In a large study cohort with young adults, TMAO was associated with CVD events but not associated with measures of atherosclerosis (e.g., coronary artery calcium, incidence and progression, carotid intima-media thickness) (5, 6). Previous studies also suggest that there are sex-related differences in TMAO serum concentrations (7, 8), whereby the female sex hormone estrogen seems to have an influence on the generation of TMAO (9), although mechanisms are not fully clarified.

Robust evidence shows that serum TMAO can be increased through the intake of TMAO-containing food components, such as saltwater fish and other marine organisms (10). However, more importantly, the frequent consumption of food ingredients high in L-carnitine and choline—such as animal products like red meat and eggs—can lead to higher serum TMAO concentrations (1). Both compounds are metabolized by specific bacterial taxa of the gut microbiome into trimethylamine (TMA) (11), the substrate for hepatic TMAO synthesis, which is catalyzed in humans mostly by flavin monooxygenase-3 in the liver (12).

Focussing on dietary components, fiber is important because high dietary fiber intake is suggested to have several health benefits. It has been shown to promote gastrointestinal function and to help reduce CVD risk by decreasing serum concentrations of total and low-density lipoprotein cholesterol in adults and children (13, 14). Furthermore, it is suggested to reduce the risk for type 2 diabetes, obesity, and even some types of cancer (15). A predominantly plant-based diet characterized, e.g., by low intake of meat and high intake of whole grains, vegetables, and thus fiber, is linked to lower serum TMAO concentrations (2). Additionally, a recent publication by Genoni et al. reported that resistant starch, a dietary fiber that escapes the digestion of the small intestine, can influence elevated blood TMAO concentrations as well as the diversity of the microbiome (16).

While many studies propose TMAO as a risk factor for multiple adverse health outcomes, opposite effects have also been suggested. For example, protective or even beneficial effects have been reported in carcinogenesis, glucose homeostasis, inflammation, steatohepatitis, and even the pathogenesis of atherosclerosis (17–21). Moreover, the associations between TMAO and CVD fade when corrected for renal function (22, 23). This suggests that TMAO may be a marker for renal impairment and chronic kidney diseases (24, 25) and that the association between TMAO and CVD may be due to confounders or reverse causality (23). In summary, it can be assumed that TMAO is involved in several metabolic processes, but the interplay and regulation of these

processes are poorly understood and need to be investigated in more detail.

The longitudinal randomized Special Turku Coronary Risk Factor Intervention Project (STRIP), initiated in 1989, aimed to prevent the development of modifiable adverse CVD risk factors beginning in infancy (26). The intervention consisted of dietary counseling that promoted a heart-healthy diet. In particular, the participants were encouraged to replace saturated fat with unsaturated fat. Starting at 8 months, the counseling was repeated at least biannually until the age of 20 years (27). Previous analyses from STRIP have shown that the intervention resulted in dietary and phenotypic changes pointing to a reduced risk of atherosclerotic CVD and type 2 diabetes (28–31). Serum TMAO, a potential indicator of adverse health outcomes, has not been investigated systematically during early life. Prospective studies exploring the longitudinal impact of age and sex on serum TMAO levels in infants, children, and adolescents are currently lacking. In addition, studies on the effect of long-term dietary interventions on serum TMAO have not been performed yet. Therefore, in the current study, we performed serial measurements of serum TMAO concentrations and investigated associations with age, sex, and the STRIP dietary intervention from 11 to 26 years of age. In addition, we explored longitudinal associations between fiber intake and TMAO and the links between TMAO and composition of the microbiome at the age of 26.

Materials and Methods

STRIP STUDY DESIGN AND PARTICIPANTS

The randomized controlled STRIP study recruited children at age 5 months from well-baby clinics in Turku, Finland, via nurses. The study was approved by the associated university and hospital district ethical authorities (26). Written informed consent was obtained from parents at study entry and from the participants at ages 15, 18, and 26 years. Briefly, at the age of 7 months, 1062 White infants (56.5% of the eligible-age cohort; born between July 1989 and December 1991) were randomly assigned to a dietary intervention ($n = 540$) or control ($n = 522$) group (Supplemental Fig. 1).

The aim of the intervention was to reduce exposure to known environmental cardiovascular risk factors, particularly through diet (26, 27). Intervention families met with the counseling team, including nutritionists, nurses, and physicians at 1- to 3-month intervals until the child was age 2 years and thereafter twice per year until the age of 20 years.

The first post-intervention follow-up with the participants was conducted between April 2015 and January 2018 at the age of 26 years, 6 years after the intervention had ended (32). Of the participating cohort ($n = 1116$),

1072 were invited to participate (Supplemental Fig. 1). Of these, 551 provided follow-up data (51%; intervention $n = 263$, control $n = 288$). Loss to follow-up at the age of 26 years has been previously reported; briefly, those who have stayed in the study have been similar to those who withdrew (32).

SERUM TMAO MEASUREMENTS

After blood collection and centrifugation, serum portions were stored at -80°C until analysis. TMAO concentrations were measured from serum samples collected at the age of 11, 13, 15, 17, 19, and 26 years. TMAO was measured with a liquid chromatography-tandem mass spectrometry method on a SCIEX QTRAP 6500 triple quadrupole instrument (Applied Biosystems) according to Enko et al. (33). All 3242 samples were measured in daily batches over several months, always including quality controls. Within-day CVs in percent for TMAO were 5.5% (2.8 $\mu\text{mol/L}$ mean value) and 2.2% (12.8 $\mu\text{mol/L}$ mean value), and between-day CVs were 9.9% (2.8 $\mu\text{mol/L}$) and 7.6% (12.6 $\mu\text{mol/L}$). Long-term stability was tested over 7 years. Control samples frozen at -20° and -80° in the lower and upper range showed no deviations from the specified target value range during this period.

DIETARY DATA COLLECTION

All participants completed a food record before each study visit (26, 27). The food record data were entered into the Micro-Nutrica[®] food analysis software to calculate food and nutrient intakes (Research Center of the Social Insurance Institution). The software has been regularly updated throughout the study period.

MICROBIOME ANALYSIS

The gut microbiota of the STRIP participants was assessed for the first time in the 26-year follow-up study (34). Fecal samples (approximately 500 mg) were collected by the participants ($n = 370$) in an OMNIgene[®] GUT collection tube (DNA Genotek). Three samples were omitted due to poor sample quality. Fecal microbiota profiles were analyzed by 16S rRNA gene sequencing; variable region V4 of the bacterial 16S rRNA gene was amplified with custom-designed dual-indexed primers and sequenced with an Illumina MiSeq system. The raw 16S rRNA gene sequencing data were demultiplexed, and the sequence adapters, primers, and barcodes were clipped using the Illumina BaseSpace platform. Ten samples were excluded from further analyses due to unsuccessful 16S rRNA gene sequencing, resulting in a final sample cohort of 357 individuals. The raw sequence data were processed into an amplicon sequence variant (ASV) table using the Divisive Amplicon Denoising Algorithm 2 pipeline (35). First,

the demultiplexed fastq files were filtered and trimmed, each sample was dereplicated, and a portion of the data set was used to estimate the error parameters. Then, function data was applied using the inferred error parameters and chimeric sequences were filtered out using function *isBimeraDenovo*. The generated ASV table altogether comprised 6.3×10^7 trimmed and chimera-removed high-quality sequence reads. Taxonomic classification of the sequences was performed using the NCBI RefSeq 16S rRNA database supplemented by the Ribosomal Database Project database (RefSeqRDP16S_v2_May2018). The generated unfiltered phyloseq object altogether included 6591 unique ASVs that corresponded to 20 different bacterial phyla and 291 bacterial genera.

Abundance data, metadata, and taxonomy files were analyzed with R ("phyloseq," "vegan," "tidyverse") and Calypso V8.84. TMAO serum concentrations were included in the metadata file. During the preprocessing, samples with fewer than 200 sequence reads were removed. Sequences from cyanobacteria/chloroplasts were removed. The data were then normalized via total sum scaling and subsequent square root transformation (Hellinger transformation).

CARDIOVASCULAR RISK FACTORS

Cardiovascular risk factors were used to characterize the participants of this study. Standard methods were used for measuring blood pressure and concentrations of serum total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides as well as glucose and insulin (32). Blood pressure, serum total cholesterol, and HDL cholesterol were measured at baseline (age of 7 months) and annually thereafter (except for ages of 6 and 8 years). Non-HDL cholesterol was calculated as total cholesterol – HDL cholesterol concentration. Triglycerides were measured beginning at the age of 5 years (fasted sampling), and fasting serum glucose and insulin were measured annually from the age of 7 to 26 years. At all annual study visits, the participants' weight and height were measured and body mass index was calculated (kg/m^2). Beginning at the age of 7 years, waist circumference was measured using a flexible tape measure. The Friedewald formula was used to determine low-density lipoprotein cholesterol concentration. The Homeostatic Model Assessment for Insulin Resistance was calculated as $[\text{fasting glucose (mmol/L)} \times \text{fasting insulin (mU/mL)}] / 22.5$.

STATISTICAL ANALYSIS

We analyzed the effects of age, sex, STRIP study group (intervention/control), and fiber intake on the longitudinal TMAO measurements using repeated-measures ANOVA or analysis of covariance (age and sex included

in all analyses). Age-specific differences between sexes were assessed using the Mann–Whitney *U*-test.

Beta diversity on genus level was calculated by Redundancy analysis based on BrayCurtis dissimilarity. Core microbiome as well as linear discriminant analysis effect size of genera between both sexes were determined. TMAO serum concentrations were included in the metadata file. Linear regression analysis was done by point-by-point correlation of TMAO concentrations with the corresponding abundances of single genera separately. Significances were calculated using the Spearman correlation coefficient. Analyses were performed with Calypso V8.84.

To investigate the differences between sexes in *Firmicutes/Bacteroidetes* ratio, we calculated the counts of *Bacteroidetes* and *Firmicutes* as a sum of all ASVs related to these phyla. Analysis were performed with R ("phyloseq," "vegan," "tidyverse"). Differences between sexes in this ratio were assessed using the nonparametric Wilcoxon test.

Results

ASSOCIATIONS OF AGE AND SEX WITH TMAO

CONCENTRATIONS FROM CHILDHOOD TO EARLY ADULTHOOD
Table 1 describes CVD risk factor characteristics and TMAO concentrations among females and males at the ages of 11, 13, 15, 17, 19, and 26 years. The age-specific serum TMAO reference ranges (2.5th;97.5th percentiles for the ages of 11, 13, 15, 17, 19, and 26 years) in females were 0.75;7.12, 0.64;6.15, 0.66;7.98, 0.75;7.88, 0.80;7.21, 0.95;9.91 $\mu\text{mol/L}$, and in males 0.82;10.3, 0.76;6.37, 0.85;8.20, 0.86;11.58, 0.95;8.48, 1.18;9.85 $\mu\text{mol/L}$. During the follow-up period, TMAO concentrations increased with age in both females ($P < 0.001$) and males ($P < 0.001$) (Fig. 1). During the follow-up period, TMAO concentrations were higher in males compared to females (mean difference 0.17 $\mu\text{mol/L}$, $P < 0.0001$). Similar results were observed in cross-sectional age-specific analyses (age 11 y: $P = 0.0055$, 13 y: $P = 0.0480$, 15 y: $P = 0.0028$, 17 y: $P = 0.0001$, 19 and 26 y: $P < 0.0001$) (Fig. 1).

In repeated measures of the whole study cohort, total cholesterol, low-density lipoprotein cholesterol, and HDL cholesterol were positively correlated with TMAO concentrations, while triglycerides were negatively correlated (all P values < 0.05). In separate analysis at each age, no significant correlations were found between TMAO and cardiovascular risk factors.

EFFECT OF THE DIETARY INTERVENTION ON TMAO CONCENTRATIONS

In sex-stratified analyses, no differences in serum TMAO concentrations between the intervention and

control groups were found either for males or females (Table 2 and Fig. 2).

REPORTED FIBER INTAKE IS DIRECTLY ASSOCIATED WITH TMAO CONCENTRATIONS IN FEMALES

During the follow-up period, reported fiber intake (g/MJ) was directly associated with the concentrations of TMAO (β 0.038, SE 0.017, $P = 0.0259$; adjusted for age and sex). In the sex-stratified analyses, the association was found for females (β 0.066, SE 0.021, $P = 0.0017$; adjusted for age) but not for males (β 0.0084, SE 0.029, $P = 0.76$; adjusted for age).

DIFFERENCES IN THE MICROBIOME COMPOSITION BETWEEN FEMALES AND MALES AND RELATIONSHIP TO TMAO

The beta diversity measures are estimates of similarity or dissimilarity in the overall taxonomic composition between different samples. A redundancy analysis for beta diversity of the microbiome from the whole cohort revealed a significant variance between taxa from females and males on genus level ($P < 0.001$) (Fig. 3A). Also, a core microbiome analysis was found to show some unique genera for each sex (Fig. 3B). Linear discriminant analysis effect size also identified different high abundant genera between the sexes (Fig. 3C).

Comparison of the phylum level *Firmicutes/Bacteroidetes* ratio between males and females showed a trend for a difference that was not significant ($P = 0.06$). The median count of *Bacteroidetes* was 80.5K in males and 73.7 in females, whereas the median count of *Firmicutes* was 69.8K in females and 62 in males (Supplemental Table 1).

A linear regression analysis between TMAO concentrations and single taxa on genus level revealed a direct association with *Roseburia hominis*, a *Firmicute* from the *Lachnospiraceae* family, but only in males ($\beta = 0.284$, $P < 0.01$). No other associations between the microbiome composition and TMAO concentrations were found.

Discussion

In this longitudinal observational study, we investigated the circulating serum TMAO concentrations in healthy individuals of the STRIP study cohort at 11, 13, 15, 17, 19, and 26 years of age. At all time points, TMAO concentrations were significantly higher in males compared to females. These data are in line with previously published cross-sectional studies, which also indicate an association between sex and serum TMAO levels in healthy individuals (36).

Although it is well known that many factors (e.g., diet, gut microbiome composition, kidney function) may influence serum TMAO concentrations, the

Table 1. Anthropometric and biochemical characteristics of the participants. Data are presented as medians and 25th/75th percentiles.

Age Sex	11 years		13 years		15 years		17 years		19 years		26 years	
	f	m	f	m	f	m	f	m	f	m	f	m
n	286	306	276	298	266	284	259	255	236	232	303	241
BMI Kg/m ²	17.7	17.5	18.7	19.0	19.9	19.9	21.0	21.1	21.8	22.1	23.0	24.4
	16.1;19.9	16.0;19.1	17.5;21.1	17.4;20.5	18.8;22.2	18.0;21.6	19.5;23.3	19.2;22.9	19.7;23.9	20.0;24.2	21.0;25.6	22.5;27.2
Syst.BP mm/Hg	104	106	107	108	112	120	112	123	113	127	115	126
	100;114	99;112	102;113	103;117	105;120	11;129	106;117	114;133	107;122	117;135	110;121	119;133
Diast.BP mm/Hg	59	58	61	60	61	62	61	62	65	66	70	73
	54;62	54;63	56;65	57;65	56;66	57;66	56;65	57;67	61;69	60;71	66;75	68;78
TG mg/dL	66.4	66.4	66.4	57.5	66.4	66.4	79.6	75.2	88.5	88.5	79.6	88.5
	49.6;84.1	46.9;85.9	49.6;85.9	45.1;75.2	49.6;92.9	49.6;92.9	57.5;109.7	57.5;101.8	70.8;123.9	61.9;106.2	53.1;106.2	61.9;115.0
Tot. Chol. mg/dL	179.1	171.3	167.4	159.6	159.6	144.1	163.5	148.0	175.2	155.8	179.1	175.2
	163.5;194.7	155.8;194.7	148.0;179.1	148.0;171.3	144.1;183.0	128.5;159.6	148.0;186.9	132.4;163.5	159.6;194.7	140.2;175.2	159.6;202.5	151.9;198.6
HDL Chol mg/dL	49.5	49.8	46.7	45.6	46.7	41.3	50.6	40.5	56.1	44.4	55.3	45.6
	44.4;56.1	44.4;57.6	40.5;50.6	40.1;52.6	42.1;53.7	35.6;46.8	44.4;56.5	35.6;46.2	48.7;63.0	39.1;50.6	48.7;62.3	39.1;51.8
LDL Chol mg/dL	109.0	105.6	103.6	99.0	97.1	86.6	97.4	88.9	99.0	91.6	105.6	110.6
	98.6;124.9	89.7;121.0	101.3;116.4	89.7;106.3	81.6;113.7	73.5;102.1	83.5;114.1	75.0;102.5	85.8;116.0	79.3;109.8	88.9;122.6	88.5;127.6
HOMA Index	1.46	1.20	1.76	1.50	1.65	1.70	1.59	1.60	1.33	1.42	1.55	1.55
	1.14;1.97	0.90;1.60	1.39;2.23	1.10;1.99	1.25;2.20	1.36;2.23	1.15;2.07	1.21;2.20	1.07;1.85	1.11;2.01	1.10;2.00	1.11;2.23
TMAO μmol/L	2.02	2.20	2.05	2.1	1.84	2.2	1.92	2.40	2.04	2.6	2.46	3.10
	1.49;2.85	1.58;3.29	1.42;2.83	1.53;3.01	1.32;2.69	1.58;3.15	1.41;2.76	1.67;3.62	1.52;2.90	1.76;3.78	1.85;3.28	2.13;4.26

SI conversion factors: TG: mg/dL x 0.0114 = mmol/L; cholesterol: mg/dL x 0.02586 = mmol/L. Abbreviations: f, female; m, male; BMI, body mass index; BP, blood pressure; TG, triglycerides; LDL, low-density lipoprotein; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance.

potential causes of sex-related differences in TMAO metabolism remain unclear. One possible explanation may be a different gut bacteria composition between men and women. Recently, Cho et al. found that higher *Firmicutes* to *Bacteroidetes* enrichment in men leads to higher TMAO production by the gut microbiome (37). In general, the ratio between these 2 phyla has

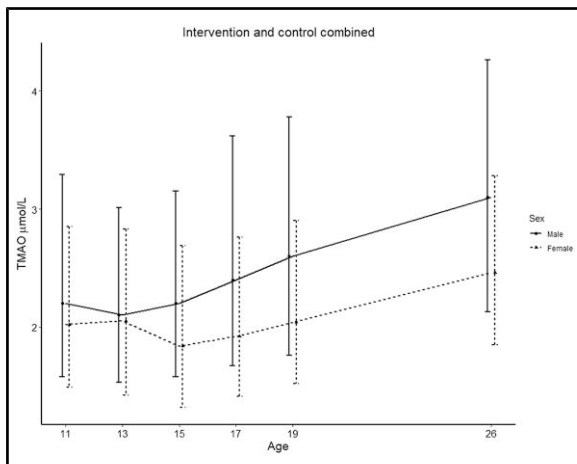


Fig. 1. TMAO concentrations in females and males between the ages of 11 and 26 years. The data are presented as medians and 25th;75th percentiles. TMAO concentrations were higher in males compared to females during the follow-up ($P < 0.0001$) as well as in age-specific analyses (age 11 y: $P = 0.0055$, 13 y: $P = 0.0480$, 15 y: $P = 0.0028$, 17 y: $P = 0.0001$, 19 and 26 y: $P < 0.0001$).

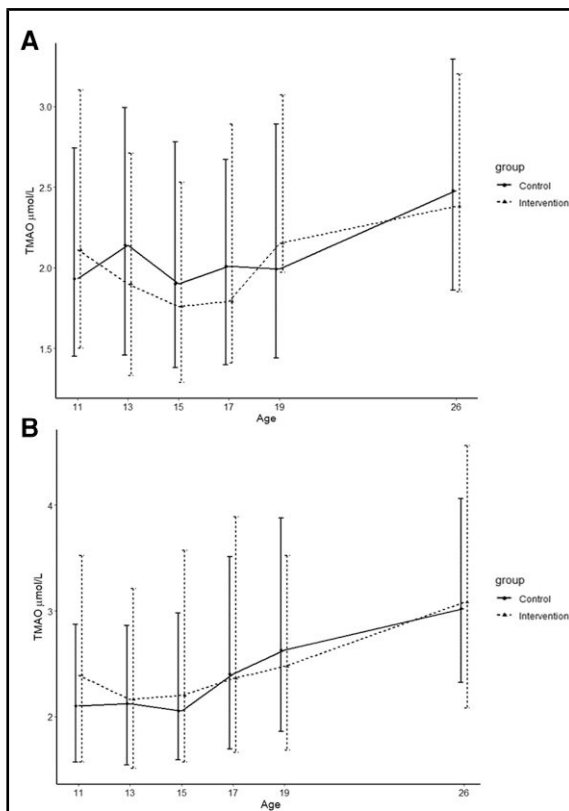


Fig. 2. TMAO concentrations between 11 and 26 years of age (A females, B males) in controls and participants who received dietary counseling. No differences between the intervention and control groups in either females ($P = 0.83$) or males ($P = 0.29$) were found.

Table 2. Impact of dietary counseling on serum TMAO concentrations in female and male participants of the STRIP study between 11 and 26 years of age. Data are presented as medians and 25th;75th PCT.

Age, y	Females				Males					
	n	Intervention		Control		n	Intervention		Control	
		TMAO, μmol/L Median, 25th,75th PCT	n	TMAO, μmol/L Median, 25th,75th PCT	n		TMAO, μmol/L Median, 25th,75th PCT	n	TMAO, μmol/L Median, 25th,75th PCT	
11	132	2.11, 1.50;3.10	154	1.93, 1.45;2.74	142	2.39, 1.57;3.52	164	2.10, 1.57;2.87		
13	129	1.90, 1.33;2.71	147	2.14, 1.46;2.99	142	2.16, 1.51;3.21	156	2.12, 1.54;2.86		
15	120	1.76, 1.29;2.53	146	1.90, 1.38;2.78	136	2.20, 1.57;3.57	148	2.05, 1.59;2.98		
17	114	1.79, 1.41;2.89	145	2.01, 1.40;2.67	117	2.36, 1.66;3.89	138	2.40, 1.69;3.51		
19	102	2.15, 1.97;3.07	134	1.99, 1.44;2.89	107	2.47, 1.68;3.52	125	2.63, 1.86;3.88		
26	134	2.38, 1.85;3.20	169	2.48, 1.86;3.29	125	3.08, 2.08;4.56	116	3.02, 2.32;4.06		

Abbreviations: PCT, percentile.

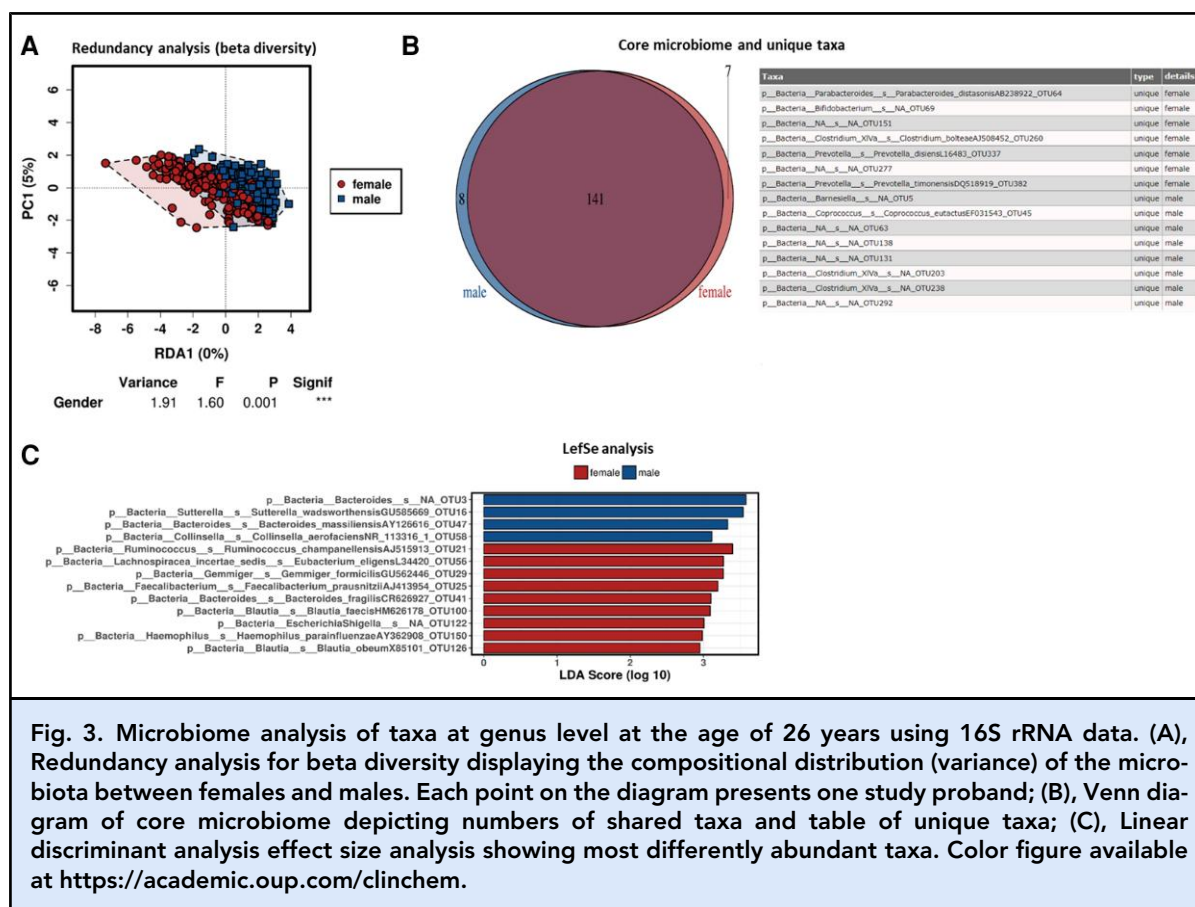


Fig. 3. Microbiome analysis of taxa at genus level at the age of 26 years using 16S rRNA data. (A), Redundancy analysis for beta diversity displaying the compositional distribution (variance) of the microbiota between females and males. Each point on the diagram presents one study proband; (B), Venn diagram of core microbiome depicting numbers of shared taxa and table of unique taxa; (C), Linear discriminant analysis effect size analysis showing most differently abundant taxa. Color figure available at <https://academic.oup.com/clinchem>.

been associated with maintaining homeostasis, and changes in this ratio are associated with various pathologies, such as obesity and inflammatory bowel disease (38, 39). Another possible explanation for sex-related variations on TMAO concentrations could be the impact of the higher estrogen level in females, which was reported to decrease hepatic expression of flavinmonooxygenase-3, the most important enzyme in the conversion of TMA to TMAO (9). Alternatively, it could also be possible that sex hormones influence TMAO and that TMAO has an impact on gut microbiota, an issue that needs further investigation. In line with this, the substantially different sex hormone profiles in males and females could possibly lead to differences in the gut microbiota composition that drive sex-specific alterations of intestinal TMAO production (40).

A recent study with this cohort showed different microbiota beta diversity between males and females but an association between dietary and intervention group with overall gut microbiota profile only in males (34). In-depth analyses of the microbiome metadata in the present study revealed different highly abundant genera between the sexes and single taxa that either occur only in females or males. Higher reported fiber

intake was observed only in the male intervention group compared to male controls (27) (Supplemental Fig. 2). It can be speculated that females make healthy dietary choices earlier in life than males, which could explain the comparable fiber intake and the unchanged *Firmicutes* to *Bacteroidetes* ratio in females with and without dietary counseling.

In this study, serum TMAO concentrations increased with age in males and females. Considering the young age of the participants, age-related mechanisms, which are independent from kidney function, are highly likely to be involved in an age-dependent increase of serum TMAO. It has been shown previously that the gut microbiome composition varies from childhood through puberty and adolescence (40). Continuous changes in the exposure to nutritional items and in the abundance of bacterial strains probably promote age-related adaptations in the gut's bacterial colonialization that impact the production of TMAO. Recently, Brunt et al. reported higher TMAO levels in middle-aged/older (64 ± 7 years) healthy males and females, with a mean of 6.5 μmol/L compared to young adults (22 ± 0.7 years) with 1.6 μmol/L (41). They observed these age-dependent differences without apparent changes in the

intake of dietary TMAO precursors. These data suggest age-dependent alterations in the gut microbiome composition and liver metabolism that could promote the conversion of TMA into TMAO resulting in higher TMAO serum concentrations in older age (41). However, the young adult cohort only contained 22 participants while the older cohort contained 101.

In line with these results, animal experiments also showed that aging increases the abundance of the TMA-producing microbial genus *Desulfovibrio* and the hepatic expression of flavinmonooxygenase-3 enzyme (41, 42). Thus, a potentially increasing colonization with TMA-producing species in elderly individuals could modulate the bioavailability of dietary choline, which could further contribute to the accumulation of TMAO with age (43).

In this study, associations between serum TMAO concentrations and beta diversity or the *Bacteroidetes* to *Firmicutes* ratio at the age of 26 years were not found. However, in males there was a direct association between TMAO and *Roseburia hominis*, a *Firmicute* of the *Lachnospiraceae* family. *Firmicutes* and especially *Lachnospiraceae*s are known to contain TMA(O) high producers (39), although no associations between *Roseburia hominis* and TMAO metabolism are known.

No differences in the serum TMAO concentrations between the dietary intervention and control groups were observed. However, serum TMAO levels were directly associated with reported total fiber intake in females but not in males. One explanation could be that the indigestible dietary fiber stimulates the intestinal peristalsis. As a result, TMA precursors (e.g., carnitine, choline, phosphatidylcholine, betaine) could pass the small intestine more rapidly so that higher amounts reach the large intestine in less time, where they are degraded by resident bacteria. A longer dwelling time of fiber and TMA precursors in the large intestine of females could favor a higher TMA/TMAO production. Also, hormonal influences and age may affect colon peristaltic patterns (44). The possibility of dietary effects on intestinal TMAO production is also supported by a previous animal study from our group. In this study, female rats fed with a high-fat diet surprisingly showed decreased serum TMAO concentrations, probably due to a reduced intestinal abundance of TMA-producing microbiota (45). In summary, more longitudinal prospective studies investigating sex differences in dietary fiber modulation of intestinal passage and microbiome are needed to shed more light on the observation.

Our study has strengths and limitations that should be considered when interpreting the present results. A main strength of this study is the long follow-up of participants from infancy to young adulthood with regular study visits and blood sampling until the age of 26. This longitudinal assessment of serum TMAO throughout large parts of the human growth and developmental

period provides unique metabolic insights that facilitate a better interpretation of this biomarker in the future. Although the TMAO determinations have shown strong intraindividual variations, the differences between women and men are significant as well as the increase of TMAO concentrations over time, which can be attributed to the size of the study cohort, another strength of this study. Regarding limitations, the food record used was not designed to capture specific nutritional sources high or low in TMAO precursors like carnitine or choline, which impeded an investigation of the effects that these compounds have on serum TMAO concentrations. Furthermore, we were not able to differentiate reliably the fiber sources, such as soluble fibers (inulin), resistant starch, or other insoluble fibers. Only the total fiber intake could be considered. Regarding microbiome analysis, stool samples were collected only at 26 years of age, which impeded longitudinal profiling of the intestinal microbiome. The 16sRNA approach was used in which the depth of investigation does not extend to individual bacteria, as is the case with shotgun metagenomics for example. Finally, we did not have enough clinical endpoints by the age of 26. Therefore, we could not study TMAO associations with outcomes.

Conclusion

In summary, this longitudinal study found serum TMAO concentrations to increase from the age of 11 to 26 years in both sexes, with males constantly having higher mean TMAO levels. While TMAO concentrations were not affected by repeated dietary counseling, a direct association between reported fiber intake and TMAO was observed in females, suggesting sex-specific differences in the metabolism and resorption of dietary compounds. Although we did not observe associations between serum TMAO and intestinal microbial patterns at the age of 26, there were differences in the microbiome compositions between females and males. Future research should consider sex-specific associations between the gut microbiome and its metabolites.

Data Availability

Selected variables and their descriptions without personal identification codes are distributed to investigators and collaborators working on specific projects. The rights to the data belong to the STRIP research group. Data sharing outside the STRIP group requires a data-sharing agreement. Investigators can submit an expression of interest to the STRIP Steering Committee.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: TMAO, trimethylamine-N-oxide; CVD, cardiovascular disease; TMA, trimethylamine; STRIP, Special Turku Coronary Risk Factor Intervention Project; ASV, amplicon sequence variant; HDL, high-density lipoprotein.

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