Plasma TMAO increase after healthy diets: Results from 2 randomized controlled trials with dietary fish, polyphenols, and whole-grain cereals

Plasma TMAO increase after healthy diets: results from two randomized controlled trials with dietary fish, polyphenols, and whole grain cereals.

Running head: Plasma TMAO increase after healthy diets.

Giuseppina Costabile, Claudia Vetrani, Lutgarda Bozzetto, Rosalba Giacco, Letizia Bresciani, Daniele Del Rio, Marilena Vitale, Giuseppe Della Pepa, Furio Brighenti, Gabriele Riccardi, Angela A. Rivellese, Giovanni Annuzzi.

Author Affiliations:

Department of Clinical Medicine and Surgery, University of Naples "Federico II", Naples, Italy (GC, CV, LB, MV, GDP, GR, AAR, GA).

Institute of Food Sciences, National Research Council, Avellino, Italy (RG).

Human Nutrition Unit, Department of Veterinary Science, University of Parma, Via Volturno 39, 43125 Parma, Italy (LB, DDR)

Human Nutrition Unit, Department of Food and Drugs, University of Parma, Via Volturno 39, 43125 Parma, Italy (FB)

Corresponding author:

Giovanni Annuzzi, MD

Department of Clinical Medicine and Surgery, Federico II University

Via Pansini 5, 80131, Naples, Italy.

tel. +39 081 7462311

Email: annuzzi@unina.it

Data Share Statement: I confirm that the data described in the manuscript, code book, and analytic code will be made available to editors upon request either before or after publication for checking.

Clinical Trial Registry Number: NCT01154478; NCT00945854 (https://clinicaltrials.gov).

Funding: The Etherpaths trial was funded by the European Community's Seventh Framework Programme FP7/2009-2012 under grant agreement FP7-KBBE-222639, Etherpaths Project, and 'Ministero Istruzione Università e Ricerca' PRIN 2010-2011-2010JCWWKM. The HealthGrain trial was funded by the European Commission in the 6th Framework Programme, Project HEALTHGRAIN (FOOD-CT-2005-514008).

Abbreviations List:

BMI: Body mass index

CHD: Coronary heart disease

CVD: Cardiovascular disease

DHA: Docosahexaenoic acid

EPA: Eicosapentaenoic acid

HOMA-IR: Homeostatic model assessment-insulin resistance

LCn3: long-chain n-3 fatty acids

MUFA: Monounsaturated fatty acids

PUFA: polyunsaturated fatty acids

PP: Polyphenols

RC: Refined cereals

SAFA: Saturated fatty acid

TMA: Trimethylamine

TMAO: Trimethylamine N-oxide

WGC: Whole grain cereals

Abstract

Background: Plasma trimethylamine N-oxide (TMAO) has drawn much attention as a marker of several chronic diseases. Data on the relationship between diet and TMAO are discordant and few human intervention studies assessed causality for this association.

Objective: To evaluate the effects on plasma TMAO of diets based on foods rich in polyphenols (PP) and/or long-chain n-3 fatty acids (LCn3) or whole grain cereals (WGC), in individuals at high cardiometabolic risk.

Design: An ancillary study was performed within two randomized-controlled trials, aimed at evaluating the medium-term effects on cardiometabolic risk factors of diets naturally rich in PP and/or LCn3 (Etherpaths Project) or WGC (HealthGrain Project)

Results: In the Etherpaths study (n=78), the changes in TMAO (8-week minus baseline) were statistically significant for the diets rich in LCn3 (+1.15±11.58 μmol/L) (p=0.007), while not for the diets rich in polyphenols (-0.14±9.66 μmol/L) (p=0.905) or their interaction (p=0.655) (two-factor ANOVA). In the HealthGrain Study (n=48), the TMAO change (12-week *minus* baseline) in the WGC (+0.94±3.58 μmol/L) was significantly different from that in the Refined Cereals group (-1.29±3.09 μmol/L) (p=0.037). Considering the pooled baseline data of the participants in the two studies, TMAO levels directly correlated with LCn3, eicosapentaenoic acid, and protein, but not saturated fatty acids, fiber, monounsaturated fatty acids, and polyphenols intake. Among food groups, TMAO directly correlated with the intake of fish, vegetables, and whole grain products, but not meat, processed meat, and dairy products.

Conclusions: Diets rich in LCn3 of marine origin or WGC significantly increased plasma TMAO concentration. These changes mirrored the direct associations between TMAO levels and intakes of fish and WGC, suggesting that TMAO reflects intakes of these healthy foods,

and, therefore, it is not a universally valid biomarker of cardio-metabolic risk independent of the background diet.

Keywords: TMAO, diet, fish, whole grain cereals, long-chain n-3 fatty acids, dietary polyphenols, cardiometabolic risk factors.

Introduction

Trimethylamine N-oxide (TMAO) is a small organic compound belonging to the class of amine oxides with chemical formula $(CH_3)_3NO$ (1,2). In recent years TMAO has drawn much attention as a marker or mediator of several chronic diseases, including cardiovascular disease (CVD), obesity, colorectal cancer, diabetes, and kidney disease (3-8). In different meta-analyses of epidemiological studies, TMAO has been related to the risk of major adverse cardiovascular events including myocardial infarction (MI) and coronary heart disease (CHD) (9–11). In contrast, other studies have not shown significant associations between circulating levels of TMAO and cardiovascular outcomes (12-16).

TMAO is naturally found in the diet in a preformed state, as for fish, or it can be generated in the human gut from dietary precursors, mainly L-carnitine, choline, and other choline-containing compounds, particularly abundant in eggs, red meat, poultry, and some dairy products, or, to a lesser extent, betaine, present in wheat bran, wheat germ, and spinach (17,18). The impact of diet on TMAO levels has been examined in several studies leading to conflicting results. A low-carbohydrate, high-starch diet was able to slightly increase plasma TMAO levels (19). On the contrary, a Mediterranean diet lasting 6 months did not influence

fasting TMAO concentrations in people at increased risk of developing colon cancer (20). Wang et al. showed that chronic dietary red meat consumption increased systemic TMAO levels (21). On the contrary, in a cross-sectional analysis in healthy people, TMAO was not associated with meat, egg, or fish consumption, while a slightly positive association was observed between TMAO concentration and consumption of dairy products (22). In addition, a metabolomics study on biomarkers of fish and meat intake showed that plasma TMAO was highly increased by fish and vegetable rich-diets than by red meat and egg rich-diets (23). This makes the fish issue challenging and intriguing, since fish intake and its high n-3 fatty acids content have been often associated with cardioprotective effects. To this regard, results from recent meta-analyses are controversial (24,25). Beneficial cardiovascular effects were shown for a high-dose pharmaceutical modification of fish oil (26), while evidence from studies on unmodified fish oil or fish intake were less clear, also considering the adverse outcomes shown in the DART study (27).

To date, no human intervention studies evaluated the medium-long term effects on plasma TMAO levels of diets containing different amount of n-3 fatty acids from marine sources, reflecting reliable intakes in the context of a balanced diet. Beside fish, little is known about the effect of other potentially "healthy" foods, as cereals and other polyphenol-rich foods, on plasma TMAO levels. Whole grains are an important source of fiber and bioactive compounds, but they also contain betaine, a precursor of TMAO. A cross-sectional study showed that TMAO was inversely associated with intake of whole grains in healthy subjects (28), but no human intervention studies assessed the possible cause-effect relation.

Polyphenols have shown beneficial effects on several cardiovascular risk factors (29), but evidence on their impact on TMAO levels comes mainly from animal, *in vitro* or clinical studies, evaluating single classes of polyphenols (30-31) or supplements (32).

The aim of the present study was to evaluate, in controlled nutritional intervention studies, the effects on plasma TMAO levels of diets characterized by the consumption of "healthy foods" – i.e. naturally rich in polyphenols and/or n-3 fatty acids or whole grains- in individuals at high cardio-metabolic risk. To pursue this aim, an ancillary study was performed within two nutritional randomized controlled trials (33,34).

SUBJECTS AND METHODS

Subjects and study design.

Samples were collected within two randomized-controlled trials previously conducted aimed at evaluating the medium-term effects on several cardiometabolic risk factors of *(a)* diets naturally rich in different sources of polyphenols (PP) and/or marine long-chain n-3 fatty acids (LCn3) (Etherpaths Project, NCT01154478) or *(b)* diets rich in whole grain cereals (WGC) (HealthGrain Project, NCT00945854). Both studies involved individuals with features of metabolic syndrome, and, therefore, at high risk of type 2 diabetes and CVD development. Both trials were conducted at the Department of Clinical Medicine and Surgery of Naples (Italy), and approved by the Ethics Committee of Federico II University (Naples, Italy). All study participants gave informed consent for participation. Full details, including the study design, characteristics of subjects, and diets were published elsewhere (33, 34).

Etherpaths study. Seventy-eight high-cardiometabolic risk individuals (33 males and 45 females) with high waist circumference (above 102 cm for men and 88 cm for women), and at least one or more features of the metabolic syndrome (ATPIII), completed the study (Supplemental Figure 1). According to a 2×2 factorial design, they were randomly assigned to one of four nutritional isoenergetic intervention arms for the duration of 8 weeks.

The assigned diets differed in LCn3 and PP contents and were similar in macronutrient composition and micronutrient content (Supplemental Table 1). Four diets were assigned: (a)

control diet, low in LCn3 (1.5 g/day) and low in PP (365 mg/day); (b) high in LCn3 (4 g/day) and low in PP (363 mg/day); (c) high in PP (2903 mg/day) and low in LCn3 (1.4 g/day); and (d) high in PP (2861 mg/day) and high in LCn3 (4 g/day). The difference in LCn3 and/or PP amount was obtained through the selection of specific foods and beverages. The main dietary sources of LCn3 were salmon (330 g twice a week), dentex or anchovies (350 g once a week). Dietary PP were provided by daily intake of decaffeinated green tea (400 ml, 4 bags) and coffee (4 cups), dark chocolate (25 g), blueberry jam (40 g), extra-virgin olive oil (60 g), and polyphenol-rich vegetables (88 g rocket salad, 200 g fennels, 200 g onions). Meals and beverages were provided to the participants for the whole study period in amounts sufficient to cover their household consumption. Meals were prepared in a qualified catering service under the surveillance of the dietitians.

HealthGrain Study. According to a randomized controlled, parallel group design, 61 overweight/obese subjects (27 men and 34 women) were assigned to an isoenergetic diet based on either whole grain cereals (WGC group) or refined cereals (RC group) for 12 weeks (Supplemental Figure 2).

Participants were encouraged to not change their habitual intake of meat, dairy products, eggs, fish, fruits, vegetables, and fats, during the whole study period. The only difference between the WGC and the RC groups was the amount and the quality (whole or refined) of grain and cereal foods as the main dietary carbohydrate source. Therefore, the two diets were designed to have the same energy intake and nutrient composition (1800 kcal/day, 18% protein, 30% fat, 52% carbohydrate), but different cereal fiber intake (Supplemental Table 2). To improve adherence to the diets, all test products were provided to participants in both study arms free of charge, in amounts sufficient to cover their household consumption for the whole study period.

Experimental procedures.

In both studies, anthropometric and metabolic data were collected before and after each dietary intervention period. Anthropometric parameters, including body weight, height, and waist circumference, were measured according to standardized procedures. Blood samples were drawn from an antecubital vein after a 12-h overnight fasting period for the measurement of metabolic parameters and TMAO concentrations. Plasma TMAO measurements were available for 48 subjects within *HealthGrain* Study (n=27 in the WGC group and n=21 in the RC group) (Table 2). In the Etherpaths study (Table 1), fecal samples were also collected before and after the dietary intervention for the microbiota analysis (35). Fecal samples were available for 70 subjects.

Dietary assessment

In both studies, participants were asked to complete a 7-day dietary record at baseline and at the end of intervention to evaluate their usual diet before the start of the dietary interventions and improve the adherence to the diets assigned during the study. Dietary compliance was reinforced through dietary counselling during weekly clinic visits and through phone calls every 2–3 days. Food intakes were calculated from the mean of the 7-day food records. Energy and nutrient composition of the diets were calculated according to the food composition tables of the Italian Institute of Nutrition, with the aid of the MetaDieta software (Meteda s.r.l., Ascoli-Piceno, Italy). The USDA (36) and Phenol-Explorer databases (37) were used to assess dietary PP content of the foods consumed.

In the *Etherpaths study*, participants allocated to the diets rich in PP and/or LCn3 were considered compliant with the treatment if the intake of PP or LCn3, respectively, was ≥80% of that assigned; participants allocated to the diets low in PP or LCn3 were considered compliant with the treatment, if the corresponding intake did not exceed the assigned one by more than 20%. Moreover, phenolic metabolites in 24 h-urine collection were also assessed to

148 evaluate compliance to polyphenols assignment (38), while plasma long-chain PUFA-
149 containing triglycerides were used to evaluate compliance to LCn3 diets (39).
150 In the *HealthGrain study*, participants were considered compliant if, for each dietary
151 component, the intake was within ±20% of that assigned. Moreover, plasma total
152 alkylresorcinol (AR) concentration, a biomarker of whole-wheat intake (40), was measured at
153 baseline and after 12 weeks, in both the WGC and RC groups, to assess compliance with the
154 assigned dietary treatments (34).
155
156 Table 1. Baseline anthropometric and fasting plasma metabolic parameters of the participants
157 in the Etherpaths trial (n=78) assigned to four diets differing for Long-chain n-3
158 polyunsaturated fatty acid (LCn3) and polyphenol (PP) content.

	Low LCn3 & Low PP	**High LCn3 & Low PP**	**Low LCn3 & High PP**	**High LCn3 & High PP**	*p value* (between groups)
Gender (M/F)	8/12	8/11	9/11	8/11	
Age (years)	54 ±9	56 ±8	53 ±9	55 ±9	*0.645*
Body Mass Index (kg/m^2)	32.6 ±3.0	31.8 ±3.7	31.9 ±2.8	30.2 ±3.1	*0.126*
Waist Circumference (cm)	104 ±7	105 ±10	104 ±9	101 ±8	*0.601*
Glucose (mg/dL)	104 ±12	104 ±12	100 ±9	103 ± 1	*0.498*
Insulin (μU/mL)	17 ±5	20 ±7	21 ±6	17 ±6	*0.220*
HOMA-IR	4.45 ±5.2	5.24 ±7.1	5.09 ±6.0	4.50 ±6.0	*0.351*
Triglycerides (mg/dL)	120 ±47	138 ±68	120 ±60	125 ±78	*0.787*
Total cholesterol (mg/dL)	194 ±38	191 ±26	194 ±34	193 ±27	*0.992*

HDL cholesterol (mg/dL)	43 ±10	41 ±11	43 ±9	44 ±14	*0.855*
LDL cholesterol (mg/dL)	118 ±30	114 ±22	115 ±25	112 ±30	*0.874*
TMAO (μmol/L)	4.97 ±3.62	7.33 ±13.9	6.66 ±10.54	7.59 ±7.98	*0.832*

All values are mean ± SD; HOMA-IR, homeostasis model assessment of insulin resistance. TMAO, Trimethylamine N-oxide. Comparisons made by one-way ANOVA.

Laboratory methods

Plasma glucose, triglyceride and cholesterol concentrations were assayed by enzymatic colorimetric methods (ABX Diagnostics, Montpellier, France) on an ABX Pentra 400 Autoanalyzer (ABX Diagnostics, Montpellier, France). Plasma insulin was measured by sandwich enzyme-linked immunosorbent assay method (ELISA; DIAsource ImmunoAssays S.A., Nivelles, Belgium) on Triturus Analyser (Diagnostics Grifols, S.A., Barcelona, Spain). After extraction with acidified acetonitrile, plasma TMAO levels were analyzed by a UHPLC DIONEX Ultimate 3000 equipped with a triple quadrupole TSQ Vantage (Thermo Fisher Scientific Inc., San Josè, CA, USA) fitted with a heated-ESI (H-ESI) (Thermo Fisher Scientific Inc., San Jose, CA, USA) probe. Separations were carried out by means of an XBridge BEH HILIC XP (100 mm × 2.1 mm) column, with a 2.5 μm particle size (Waters, Milford, MA, USA), as previously reported (41,42).

The analysis of microbiota was performed on 0.2 g of feces, DNA was extracted, partial 16S rRNA gene was amplified, and a quantitative real-time PCR (35) was performed for the analysis of bacterial groups (*Eubacterium rectale-Blautia coccoides (*EREC*), Clostridium leptum (*CLEPT)), representing families that account for 60–80% of the fecal microbiota of healthy adults. B*ifidobacteria* and *Lactobacillus* were also analyzed for their known beneficial association with human health (43).

Table 2. Baseline anthropometric and fasting plasma metabolic parameters of the participants in the HealthGrain trial (n=48) assigned to diets differing for refined and whole grain cereals.

	Refined Cereals	**Whole grain Cereals**	***p* value**
Gender (M/F)	10/11	12/15	
Age (years)	57±8	56±9	0.766
Body Mass Index (kg/m^2)	31.6±5.6	32.2±5.9	0.728
Waist Circumference (cm)	105±12	108±15	0.437
Glucose (mg/dL)	104 ± 9	102 ± 10	0.494
Insulin (μU/mL)	14 ± 7	16 ± 9	0.444
HOMA-IR	3.05 ± 1.35	3.78 ± 2.20	0.178
Triglycerides (mg/dL)	147 ± 63	153 ± 48	0.869
Total cholesterol (mg/dL)	198 ± 36	202 ± 48	0.736
HDL cholesterol (mg/dL)	36 ± 6	42 ± 11	0.089
LDL cholesterol (mg/dL)	132±32	129±46	0.775
TMAO (μmol/L)	4.49±3.78	3.68±2.03	0.351

All values are mean ± SD; HOMA-IR, homeostasis model assessment of insulin resistance; TMAO, Trimethylamine N-oxide. Comparisons made by Independent-samples t-test.

Statistical analysis

Data are expressed as mean ± standard deviation (M±SD), unless otherwise stated.

In *the Etherpaths study*, the differences in baseline characteristics between the four groups were analyzed by one-way ANOVA. According to a 2x2 factorial design, the effects of

dietary PP and LCn3 and their interaction were evaluated by two-factor ANOVA analysis. In the General Linear Model (GLM)-Univariate Analysis, the absolute change of TMAO (8-week *minus* baseline) was added as "dependent variable", and PP group (with two levels: low PP and high PP) and LCn3 group (with two levels: low LCn3 and high LCn3) as "independent variables/ fixed factors"; sex, age, baseline TMAO, and BMI were added as covariates.

In the *HealthGrain study*, the differences in baseline characteristics between the two groups were analyzed by independent *t*-test. Differences between the effects of the two experimental diets were evaluated by GLM-Univariate Analysis of absolute change of TMAO (12-week *minus* baseline) adjusted for sex, age, baseline TMAO and, BMI. The associations between plasma TMAO concentrations, metabolic parameters, nutrient intake, and food items were explored by partial correlation analysis and linear regression analysis also controlling for potential confounders, i.e., sex, age, BMI, and study (Etherpaths/HealthGrain).

For all analyses, the level of statistical significance was set at $p<0.05$ (two tails). Statistical analysis was performed according to standard methods using the Statistical Package for Social Sciences software version 21.0 (SPSS, Chicago, IL, USA).

RESULTS

Etherpaths Study. At baseline, the clinical characteristics of the participants were not different between the 4 dietary groups (Table 1). In all groups, the compliance to the experimental diets was optimal (33), the reported PP or LCn3 intakes being close to the assigned ones, with no differences in macronutrients, fiber, and vitamin intakes (Supplemental Table 1).

As previously reported, High-PP diets significantly decreased fasting and postprandial triglyceride concentrations in whole plasma and large very-low-density lipoproteins (VLDLs) (33), reduced the urinary 8-isoprostane concentrations (33), improved glucose tolerance and

early insulin secretion during an oral glucose tolerance test (44), and increased fecal amount of CLEPT and EREC groups (35). High LCn3 diets reduced postprandial triglyceride-rich lipoproteins (33) and increased the number of *Bifidobacteria* (35).

After the 8-week interventions, plasma TMAO did not change significantly from baseline in each group: High LCn3 & Low PP (7.33±13.93 *vs.* 8.86±7.28 μmol/L, baseline *vs.* 8-week, respectively, p=0.640); High LCn3 & High PP (7.59 ±7.98 *vs.* 8.37±4.40 μmol/L, p=0.713); Low LCn3 & High PP (6.65±10.54 *vs.* 5.60±4.00 μmol/L, p=0.664); and Low LCn3 & Low PP (4.97±3.62 *vs.* 4.86 ±2.91 μmol/L, p=0.886). By two-factor ANOVA, the changes in TMAO (8-week minus baseline) were statistically significant for the diets rich in LCn3 (p=0.007), while not for the diets rich in polyphenols (p=0.905) or their interaction (p=0.655) (Figure 1, Panel A).

HealthGrain Study. At baseline, the clinical characteristics of the participants were not different between the WGC and RC groups (Table 2). In both groups, the compliance to the experimental diets was optimal, the reported total and cereal fiber intakes being close to the assigned ones, with no differences in energy and macronutrient contents (Supplemental table 2) (34). As previously reported, WGC diet reduced postprandial serum insulin and triglyceride responses (34).

Plasma TMAO levels did not change significantly after the WGC (3.68±2.03 and 4.63 ± 3.00 μmol/L, baseline and 12-week, respectively; p=0.133) or the RC diet (4.48±3.78 and 3.20±1.85 μmol/L; p=0.114). However, the difference in absolute change (12-week *minus* baseline values) in plasma TMAO between the WGC and the RC diet group was statistically significant (p=0.037; GLM- Univariate Analysis, corrected for sex, age, baseline TMAO and BMI) (Figure 1, Panel B).

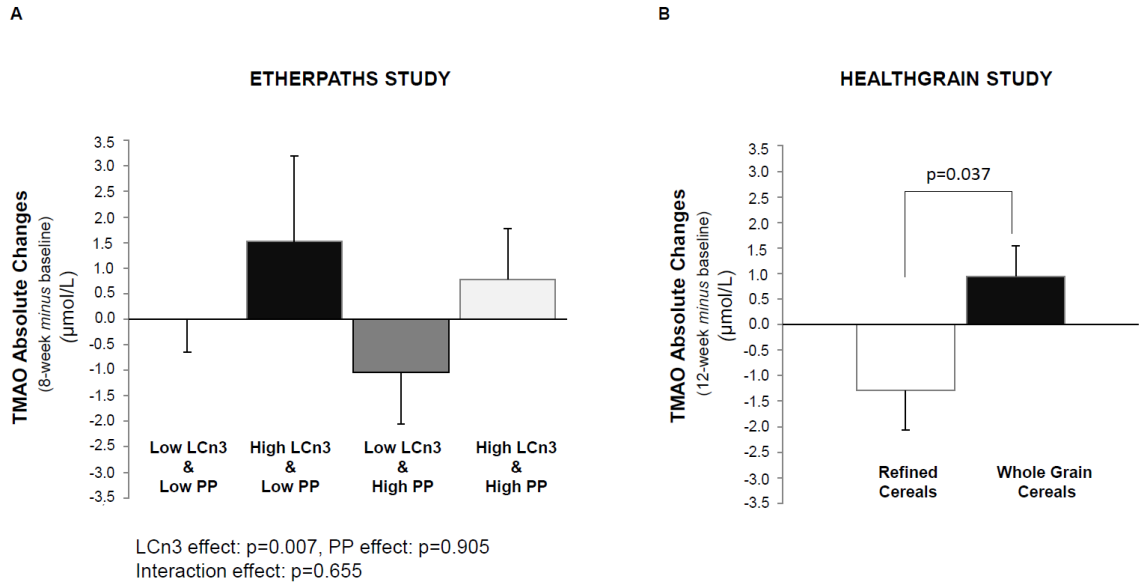


FIGURE 1. Absolute changes (8-week minus baseline) in fasting plasma TMAO concentrations in the four experimental groups in the Etherpaths Study (Low LCn3 & Low PP group, n= 20; High LCn3 & Low PP group, n=19; Low LCn3 & High PP group, n=20; High LCn3 & High PP group, n=19) (Panel A). Absolute changes (12-week minus baseline) in fasting plasma TMAO concentrations after the Refined or Whole Grain Cereals diets in the HealthGrain Study (Refined Cereals group, n=21; Whole Grain Cereals group, n=27) (Panel B). LCn3, long-chain n-3 fatty acids; PP, polyphenols. Mean ± SEM. Comparisons made by GLM- Univariate analysis of absolute changes in plasma TMAO adjusted for sex, age, baseline TMAO and BMI.

Association analyses.

Partial correlation analyses were performed on the pooled baseline data of the participants in the Etherpaths and HealthGrain studies (n=126), adjusting for age, sex, BMI, and study (Etherpaths/HealthGrain). No significant correlation was observed between TMAO and metabolic parameters (Supplemental Table 3).

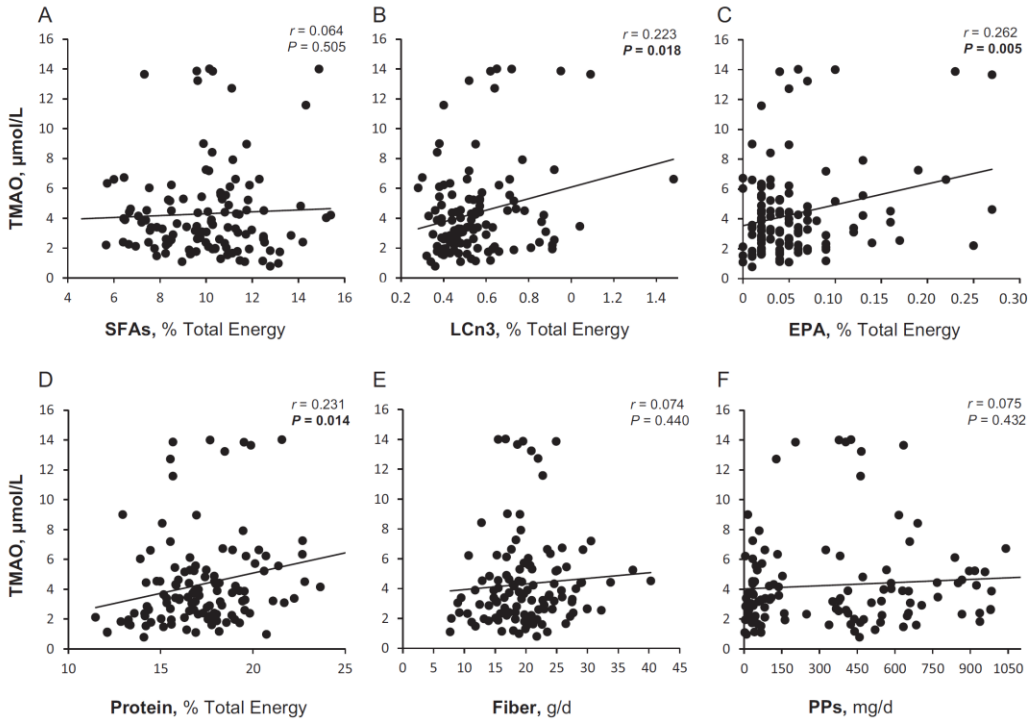
Regarding dietary components, TMAO concentrations directly significantly correlated with the intake of LCn3 (r=0.223, p=0.018), EPA (r=0.262, p=0.005), and protein (r=0.231,

p=0.014) (Figure 2). A correlation trend was observed with the intake of DHA (r=0.184, p=0.053) and carbohydrates (r=-0.179, p=0.058). No statistically significant correlation was observed between TMAO and intake of SAFA (r=0.064, p=0.505), fiber (r=0.074, p=0.440), polyphenols (r=0.075, p=0.432) (Figure 2), and MUFA (r=0.081, p=0.398). In linear regression analyses, entering the baseline plasma TMAO as "dependent variable" and nutritional factors (proteins, carbohydrates, total fat, MUFA, SFA, PUFA, EPA, DHA, cholesterol, fiber, polyphenols) as "independent variables", EPA resulted the only variable associated with TMAO levels (Beta=0.501, p=0.015). The results were similar in the model including age, sex, BMI, and Etherpaths/HealthGrain study (Beta=0.500, p=0.015), with an increase of 1 μmol/L TMAO by each 0.2% increase in EPA intake.

Regarding food groups, TMAO concentrations directly significantly correlated with the intake of fish (r=0.215, p=0.040), vegetables (r=0.277, p=0.007), and whole grain products (r=0.204, p=0.049) (Figure 3). On the contrary, no significant correlations were found between TMAO levels and the intake of meat (r=-0.071, p=0.502), processed meat (r= -0.164, p=0.118), and dairy products (r= -0.142, p=0.176) (Figure 3).

No significant correlations were observed between the changes (final minus baseline, in the Etherpaths and HealthGrain studies) in plasma TMAO levels and changes in the main fasting metabolic parameters.

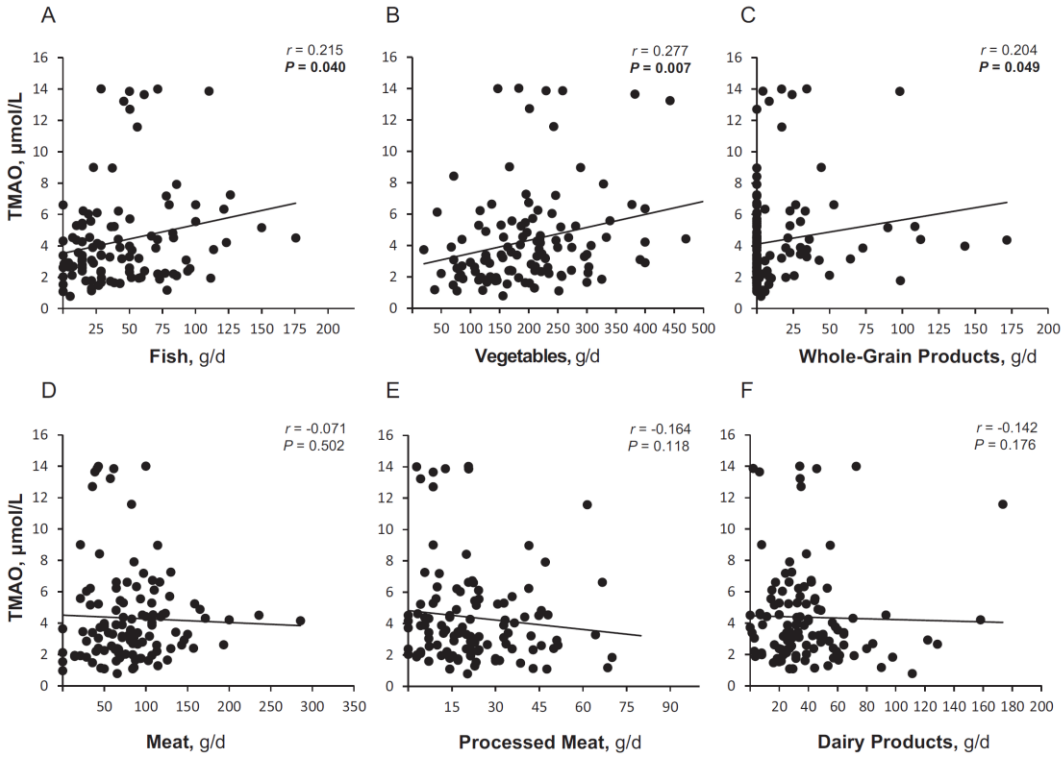
In the Etherpaths study, TMAO concentrations inversely significantly correlated with fecal *Bifidobacterium* (r= -0.356, p=0.003) (Figure 4), no significant correlations were found with *Lactobacillus* (r= -0.136, p=0.265) and *Clostridium leptum* (CLEPT, r= -0.097, p=0.430) whereas a correlation trend was observed with *Eubacterium rectale-Blautia coccoides* (EREC, r= -0.215, p=0.079) (Figure 4).



277

278 **FIGURE 2.** Relationships between fasting plasma TMAO and dietary daily intakes of long-
279 chain n-3 fatty acids (LCn3), eicosapentaenoic acid (EPA), saturated fatty acid (SAFA),
280 protein, fiber, and polyphenols in the habitual diet of the participants in the Etherpaths and
281 HealthGrain studies (n=126) as calculated through the 7-day food records obtained before the
282 dietary interventions. Partial correlation analysis adjusted for sex, age, BMI, and study.

283



284

285

286 **FIGURE 3.** Relationships between fasting plasma TMAO and dietary daily intakes of main
287 foods, including fish, meat, processed meat, vegetables, whole grain products, and dairy
288 products, in the habitual diet of the participants in the Etherpaths and HealthGrain studies
289 (n=126) as calculated through the 7-day food records obtained before the dietary
290 interventions. Partial correlation analysis adjusted for sex, age, BMI, and study**.**

291

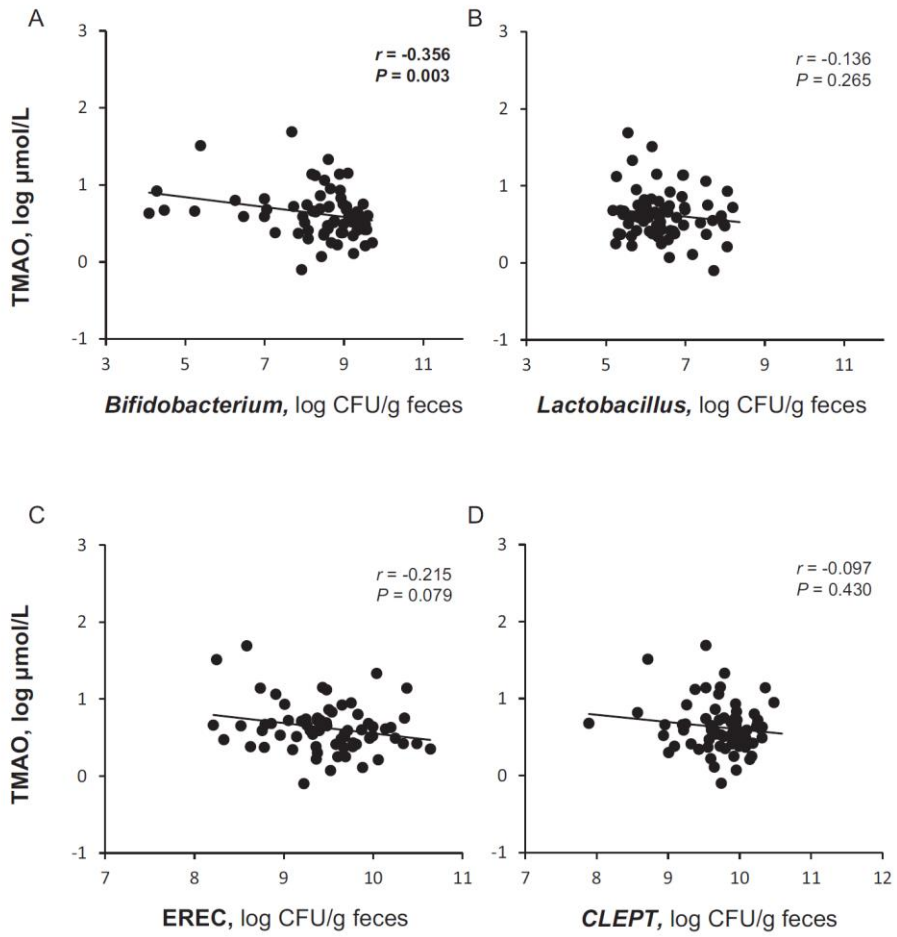


FIGURE 4. Correlation between fasting plasma TMAO and fecal concentrations of Bifidobacterium, Lactobacillus, Eubacterium rectale-Blautia coccoides (EREC), and Clostridium leptum (CLEPT) groups in the Etherpaths study (n=70). Pearson's correlation analysis. CFU, Colony Forming Units.

DISCUSSION

To our knowledge, this is the first study aimed at evaluating the medium-term effects of dietary interventions, characterized by different LCn3, PP, and whole grain cereal content, on fasting plasma TMAO concentration in randomized controlled trials involving individuals at high cardio-metabolic risk.

Since a strong interest has recently grown up in TMAO as a possible risk factor/biomarker of CVD and other relevant chronic diseases (3-8), it is important to define the relations between dietary factors, which represent the main contributors of TMAO levels, and cardiovascular risk. To this regard, an intriguing finding from cross-sectional studies was that TMAO levels were higher not only in association with meat intake - repeatedly shown to be associated with higher cardiovascular and cancer risk (45-46)- but also with some foods, including fish, whole grain cereals, and vegetables, generally associated with a reduced risk for these diseases (23,28). Our study reinforces the relationship of TMAO with intakes of wholegrain, fish and vegetables, but does not confirm the association with meat consumption. Moreover, we have shown in two randomized controlled dietary interventions using natural foodstuffs, that diets rich in LCn3 of marine origin and diets rich in whole grain cereals significantly increase TMAO levels.

The finding that these “healthy” diets increase TMAO levels does not support the hypothesis that TMAO represents an independent cardiovascular risk factor. This hypothesis is backed by a recent meta-analysis showing that the risk of both major adverse cardiovascular events and death from all causes is more than 60% higher in people with elevated plasma TMAO concentration (11). Therefore, we face a paradox between this evidence and findings of our intervention studies, that reinforce previous research, demonstrating that foods generally associated with significant benefits in relation to cardiovascular risk are major contributors of TMAO.

This paradox might be partially justified hypothesizing that the presence of healthful components in fish, namely LCn3, and in whole grain, namely dietary fiber, would be able to offset or even overcome the negative effects of TMAO on the cardiovascular risk. On the other hand, in the presence of high intake of meat and dairy products, the role of TMAO might be negligible and the harmful cardiovascular effects would possibly be due to other

329 compounds present in these foods able to increase the cardiovascular risk, i.e. SAFA,
330 polycyclic aromatic hydrocarbons, heterocyclic aromatic amines, advanced glycation end-
331 products, salt/sodium and N-nitroso compounds (47).
332 This hypothesis is supported by findings from the INTERMAP study, in which TMAO
333 directly correlated with blood pressure and BMI in Western populations on relatively low fish
334 intake, while in a population sample on a relatively elevated level of fish consumption,
335 TMAO was significantly associated with higher fish intake, but not with blood pressure and
336 BMI (48). Overall, we believe that the role of TMAO depends on the background diet of a
337 population and that TMAO *per se* cannot be considered an independent risk factor for
338 cardiovascular disease.
339 In our study population, the baseline diet was characterized by a limited consumption of meat
340 and dairy products and regular fish and vegetable intake, according to the features of the
341 Mediterranean Diet. The lack of correlations between TMAO and metabolic parameters at
342 baseline in our study population agrees with the results reported by Gibson and colleagues in
343 relation to the population group consuming a healthier diet (48).
344 In the Etherpaths study, we evaluated gut microbiota for its known role in influencing TMAO
345 production in the host (16,49,50). We found an inverse association between *Bifidobacteria*
346 abundance and plasma TMAO levels. Interestingly, *in vitro* and animal studies have
347 demonstrated that some microbial species –including *Bifidobacteria*- can reconvert TMAO to
348 TMA, thus reducing TMAO levels (51). Unfortunately, we did not measure TMA levels to
349 test this hypothesis. It must be considered that the analyses of microbiota by real-time PCR is
350 accurate, but it only allows the quantification of bacterial groups for which specific primers
351 were constructed. The assessment of global microbial composition would have added more
352 comprehensive information about the relation between microbiota composition and TMAO
353 concentrations.

The present study obviously has strengths and weaknesses. The major strength is the study design since intervention studies had the greatest validity to support a cause-effect relationship. Our study demonstrated for the first time that healthy diets, consistently associated with a reduced cardiovascular risk, induced significant increase of circulating TMAO. On the other side, an important limitation is the lack of information on hard endpoints, due to a small sample size and a relatively short follow-up period. However, in order to get this type of information, a completely different study design should have been employed, with much larger investments in time and resources.

In conclusion, this study demonstrated that diets rich in fish or in whole grain cereals significantly increased plasma TMAO. These changes mirrored the baseline associations between higher TMAO levels and higher intakes of fish and whole grain products, suggesting that plasma TMAO mainly reflects dietary intakes of these "healthy foods" and, therefore, it is not a universally valid biomarker of cardio-metabolic risk independent of the background diet. Future intervention studies with hard endpoints would be useful to finally disproving the role of TMAO as an independent cardiovascular risk factor.

Acknowledgments

The authors' responsibilities were as follows: GA, GR, and AAR: designed the research; RG, GC, LuB, GDP, MV conducted the research; GC, CV analyzed the data or performed the statistical analysis; LB, DDR, FB provided the essential materials; GC and GA wrote the manuscript and had primary responsibility for the final content. All authors read and approved the final manuscript.

Conflict of interest: The authors declare that they have no conflict of interest.

References

1. Ufnal M, Zadlo A, Ostaszewski R. TMAO: A small molecule of great expectations. Nutrition 2015;31:1317-23.
2. Subramaniam S, Fletcher C. Trimethylamine N-oxide: breathe new life. Br J Pharmacol 2018;175:1344-53.
3. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, Feldstein AE, Britt EB, Fu X, Chung YM, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature. 2011;472:57-63.
4. Zeisel SH, Warrier M. Trimethylamine N-oxide, the microbiome, and heart and kidney disease. Annu Rev Nutr 2017;37:157-81.
5. Tang WH, Wang Z, Fan Y, Levison B, Hazen JE, Donahue LM, Wu Y, Hazen SL. Prognostic value of elevated levels of intestinal microbe-generated metabolite trimethylamine-N-oxide in patients with heart failure: refining the gut hypothesis. J Am Coll Cardiol 2014;64:1908-14.
6. Tang WH, Wang Z, Kennedy DJ, Wu Y, Buffa JA, Agatisa-Boyle B, Li XS, Levison BS, Hazen SL. Gut microbiota-dependent trimethylamine N-oxide (TMAO) pathway contributes to both development of renal insufficiency and mortality risk in chronic kidney disease. Circ Res 2015;116:448-55.
7. Tang WH, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, Wu Y, Hazen SL. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med 2013;368:1575–1584.
8. Dehghan P, Farhangi MA, Nikniaz L, Nikniaz Z, Asghari-Jafarabadi M. Gut microbiota-derived metabolite trimethylamine N-oxide (TMAO) potentially increases the risk of obesity in adults: An exploratory systematic review and dose-response meta- analysis. Obes Rev 2020: e12993 (doi: 10.1111/obr.12993).

9. Schiattarella GG, Sannino A, Toscano E, Giugliano G, Gargiulo G, Franzone A, Trimarco B, Esposito G, Perrino C. Gut microbe-generated metabolite trimethylamine-N-oxide as cardiovascular risk biomarker: a systematic review and dose-response meta-analysis. Eur Heart J 2017;38:2948-2956.
10. Qi J, You T, Li J, Pan T, Xiang L, Han Y, Zhu L. Circulating trimethylamine N-oxide and the risk of cardiovascular diseases: a systematic review and meta-analysis of 11 prospective cohort studies. J Cell Mol Med 2018;22:185-194.
11. Heianza Y, Ma W, Manson JE, Rexrode KM, Qi L. Gut Microbiota Metabolites and Risk of Major Adverse Cardiovascular Disease Events and Death: A Systematic Review and Meta-Analysis of Prospective Studies. J Am Heart Assoc 2017;6:e004947.
12. Guasch-Ferré M, Hu FB, Ruiz-Canela M, Bulló M, Toledo E, Wang DD, Corella D, Gómez-Gracia E, Fiol M, Estruch R, et al. Plasma Metabolites From Choline Pathway and Risk of Cardiovascular Disease in the PREDIMED (Prevention With Mediterranean Diet) Study. J Am Heart Assoc 2017;6:e006524.
13. Meyer KA, Benton TZ, Bennett BJ, Jacobs DR Jr, Lloyd-Jones DM, Gross MD, Carr JJ, Gordon-Larsen P, Zeisel SH. Microbiota-Dependent Metabolite Trimethylamine N-Oxide and Coronary Artery Calcium in the Coronary Artery Risk Development in Young Adults Study (CARDIA). J Am Heart Assoc 2016;5:e003970.
14. Paynter NP, Balasubramanian R, Giulianini F, Wang DD, Tinker LF, Gopal S, Deik AA, Bullock K, Pierce KA, Scott J, et al. Metabolic Predictors of Incident Coronary Heart Disease in Women. Circulation 2018;137:841-53.
15. Yin J, Liao SX, He Y, Wang S, Xia GH, Liu FT, Zhu JJ, You C, Chen Q, Zhou L, et al. Dysbiosis of Gut Microbiota With Reduced Trimethylamine-N-Oxide

Level in Patients With Large-Artery Atherosclerotic Stroke or Transient Ischemic Attack. J Am Heart Assoc 2015;4:e002699.

16. Stubbs JR, Stedman MR, Liu S, Long J, Franchetti Y, West RE, Prokopienko AJ, Mahnken JD, Chertow JM, Nolin TD. Trimethylamine N-Oxide and Cardiovascular Outcomes in Patients with ESKD Receiving Maintenance Hemodialysis. Clin J Am Soc Nephrol 2019;14:261-67.
17. Cho CE, Taesuwan S, Malysheva OV, Bender E, Tulchinsky NF, Yan J, Sutter JL, Caudill MA. Trimethylamine-N-oxide (TMAO) response to animal source foods varies among healthy young men and is influenced by their gut microbiota composition: A randomized controlled trial. Mol Nutr Food Res 2017;61(1).
18. Wang Z, Tang WH, Buffa JA, Fu X, Britt EB, Koeth RA, Levison BS, Fan Y, Wu Y, Hazen SL. Prognostic value of choline and betaine depends on intestinal microbiota-generated metabolite trimethylamine-N-oxide. Eur Heart J 2014;35:904-10.
19. Bergeron N, Williams PT, Lamendella R, Faghihnia N, Grube A, Li X, Wang Z, Knight R, Jansson JK, Hazen SL, et al. Diets high in resistant starch increase plasma levels of trimethylamine-N-oxide, a gut microbiome metabolite associated with CVD risk. Br J Nutr 2016;116:2020-2029.
20. Griffin LE, Djuric Z, Angiletta CJ, Mitchell CM, Baugh ME, Davy KP, Neilson AP. A Mediterranean diet does not alter plasma trimethylamine N-oxide concentrations in healthy adults at risk for colon cancer. Food Funct 2019;10:2138-47.
21. Wang Z, Bergeron N, Levison BS, Li XS, Chiu S, Jia X, Koeth RA, Li L, Wu Y, Tang WHW, et al. Impact of chronic dietary red meat, white meat, or non-meat

protein on trimethylamine N-oxide metabolism and renal excretion in healthy men and women. Eur Heart J 2019;40:583-594.

22. Rohrmann S, Linseisen J, Allenspach M, von Eckardstein A, Muller D. Plasma Concentrations of Trimethylamine-N-oxide Are Directly Associated with Dairy Food Consumption and Low-Grade Inflammation in a German Adult Population. J. Nutr 2016;146:283-89.
23. Cheung W, Keski-Rahkonen P, Assi N, Ferrari P, Freisling H, Rinaldi S, Slimani N, Zamora-Ros R, Rundle M, Frost G, et al. A Metabolomic Study of Biomarkers of Meat and Fish Intake. Am J Clin Nutr 2017;105:600-608.
24. Hu Y, Hu FB, Manson JE. Marine Omega-3 Supplementation and Cardiovascular Disease: An Updated Meta-Analysis of 13 Randomized Controlled Trials Involving 127 477 Participants. J Am Heart Assoc. 2019 Oct;8(19):e013543.
25. Abdelhamid AS, Brown TJ, Brainard JS, Biswas P, Thorpe GC, Moore HJ, Deane KH, Summerbell CD, Worthington HV, Song F, Hooper L. Omega-3 fatty acids for the primary and secondary prevention of cardiovascular disease. Cochrane Database Syst Rev. 2020 Feb 29;3(2):CD003177.
26. Bhatt DL, Steg PG, Miller M, Brinton EA, Jacobson TA, Ketchum SB, Doyle RT Jr, Juliano RA, Jiao L, Granowitz C, et al; REDUCE-IT Investigators. Cardiovascular Risk Reduction with Icosapent Ethyl for Hypertriglyceridemia. N Engl J Med. 2019 Jan 3;380(1):11-22.
27. Ness AR, Hughes J, Elwood PC, Whitley E, Smith GD, Burr ML. The long-term effect of dietary advice in men with coronary disease: follow-up of the Diet and Reinfarction trial (DART). Eur J Clin Nutr. 2002 Jun;56(6):512-8.
28. Genoni A, Christophersen CT, Lo J, Coghlan M, Boyce MC, Bird AR, Lyons-Wall P, Devine A. Long-term Paleolithic diet is associated with lower resistant

starch intake, different gut microbiota composition and increased serum TMAO concentrations. Eur J Nutr 2020;59:1845-58.

29. Giacco R, Costabile G, Fatati G, Frittitta L, Maiorino MI, Marelli G, Parillo M, Pistis D, Tubili C, Vetrani C, et al. Effects of polyphenols on cardio-metabolic risk factors and risk of type 2 diabetes. A joint position statement of the Diabetes and Nutrition Study Group of the Italian Society of Diabetology (SID), the Italian Association of Dietetics and Clinical Nutrition (ADI) and the Italian Association of Medical Diabetologists (AMD). Nutr Metab Cardiovasc Dis 2020;30:355-367.
30. Chen ML, Yi L, Zhang Y, Zhou X, Ran L, Yang J, Zhu JD, Zhang QY, Mi MT. Resveratrol Attenuates Trimethylamine-N-Oxide (TMAO)-Induced Atherosclerosis by Regulating TMAO Synthesis and Bile Acid Metabolism via Remodeling of the Gut Microbiota. mBio 2016;7:e02210-15.
31. Savi M, Bocchi L, Bresciani L, Falco A, Quaini F, Mena P, Brighenti F, Crozier A, Stilli D, Del Rio D. Trimethylamine-N-Oxide (TMAO)-Induced Impairment of Cardiomyocyte Function and the Protective Role of Urolithin B-Glucuronide. Molecules 2018;23:549.
32. Annunziata G, Maisto M, Schisano C, Ciampaglia R, Narciso V, Tenore GC, Novellino E. Effects of Grape Pomace Polyphenolic Extract (Taurisolo(®) in Reducing TMAO Serum Levels in Humans: Preliminary Results from a Randomized, Placebo-Controlled, Cross-Over Study. Nutrients 2019;11:139.
33. Annuzzi G, Bozzetto L, Costabile G, Giacco R, Mangione A, Anniballi G, Vitale M, Vetrani C, Cipriano P, Della Corte G, et al. Diets naturally rich in polyphenols improve fasting and postprandial dyslipidemia and reduce oxidative stress: a randomized controlled trial. Am J Clin Nutr 2014;99:463-67.

34. Giacco R, Costabile G, Della Pepa G, Anniballi G, Griffo E, Mangione A, Cipriano P, Viscovo D, Clemente G, Landberg R, et al. A whole-grain cereal-based diet lowers postprandial plasma insulin and triglyceride levels in individuals with metabolic syndrome. Nutr Metab Cardiovasc Dis 2014;24:837-44.
35. Vetrani C, Maukonen J, Bozzetto L, Della Pepa G, Vitale M, Costabile G, Riccardi G, Rivellese AA, Saarela M, Annuzzi G. Diets naturally rich in polyphenols and/or long-chain n-3 polyunsaturated fatty acids differently affect microbiota composition in high-cardiometabolic-risk individuals. Acta Diabetol. 2020;57:853-60.
36. USDA database for the flavonoid content of selected foods. Release 2.1. Washington, DC: USDA, 2007.
37. Phenol-Explorer: database on polyphenol content in foods. http://phenolexplorer.eu/. Accessed 13 Sept 2018.
38. Vetrani C, Rivellese AA, Annuzzi G, Mattila I, Meudec E, Hyötyläinen T, Orešič M, Aura AM. Phenolic metabolites as compliance biomarker for polyphenol intake in a randomized controlled human intervention. Food Research International 2014;63:233-38.
39. Bondia-Pons I, Pöhö P, Bozzetto L, Vetrani C, Patti L, Aura AM, Annuzzi G, Hyötyläinen T, Rivellese AA, Orešič M. Isoenergetic diets differing in their n-3 fatty acidand polyphenol content reflect different plasmaand HDL-fraction lipidomic profiles in subjectsat high cardiovascular risk. Mol Nutr Food Res 2014;58:1873-82.

40. Landberg R, Kamal Eldin A, Andersson A, Vessby B, Aman P. Alkylresorcinols as biomarkers of whole grain wheat and rye intake: plasma concentration and intake estimated from dietary records. Am J Clin Nutr 2008;87:832-38.
41. Bresciani L, Dall'Asta M, Favari C, Calani L, Del Rio D, Brighenti F. An in vitro exploratory study of dietary strategies based on polyphenol-rich beverages, fruit juices and oils to control trimethylamine production in the colon. Food Funct 2018;9:6470-83.
42. Miller CA, Corbin KD, da Costa KA, Zhang S, Zhao X, Galanko JA, Blevins T, Bennett BJ, O'Connor A, Zeisel SH. Effect of egg ingestion on trimethylamine-N-oxide production in humans: a randomized, controlled, dose-response study. Am J Clin Nutr 2014;100:778-86.
43. Maukonen J, Saarela M. Human gut microbiota: does diet matter? Proc Nutr Soc. 2015 Feb;74(1):23-36. doi: 10.1017/S0029665114000688. Epub 2014 Aug 26. PMID: 25156389.
44. Bozzetto L, Annuzzi G, Pacini G, Costabile G, Vetrani C, Vitale M, Griffo E, Giacco A, De Natale C, Cocozza S, et al. Polyphenol-rich diets improve glucose metabolism in people at high cardiometabolic risk: a controlled randomised intervention trial. Diabetologia 2015;58:1551-60.
45. Schwingshackl L, Schwedhelm C, Hoffmann G, Lampousi AM, Knuppel S, Iqbal K, Bechthold A, Schlesinger S, Boeing H. Food groups and risk of all-cause mortality: a systematic review and meta-analysis of prospective studies. Am J Clin Nutr 2017;105:1462-73.
46. Kim K, Hyeon J, Lee SA, Kwon SO, Lee H, Keum N, Lee JK, Park SM. Role of Total, Red, Processed, and White Meat Consumption in Stroke Incidence and

Mortality: A Systematic Review and Meta-Analysis of Prospective Cohort Studies. J Am Heart Assoc 2017;6:e005983.

47. Rohrmann S, Linseisen J. Processed meat: the real villain? Proc Nutr Soc 2016;75:233-41.
48. Gibson R, Lau CE, Loo RL, Ebbels TMD, Chekmeneva E, Dyer AR, Miura K, Ueshima H, Zhao L, Daviglus ML, et al. The association of fish consumption and its urinary metabolites with cardiovascular risk factors: the International Study of Macro-/Micronutrients and Blood Pressure (INTERMAP). Am J Clin Nutr 2020;111:280-290.
49. Falony G, Vieira-Silva S, Raes J. Microbiology meets big data: the case of gut microbiota-derived trimethylamine. Annu Rev Microbiol 2015;69:305-21.
50. Brugère JF, Borrel G, Gaci N, Tottey W, O'Toole PW, Malpuech-Brugère C. Archaebiotics: proposed therapeutic use of archaea to prevent trimethylaminuria and cardiovascular disease. Gut Microbes. 2014;5:5-10.
51. Hoyles L, Jiménez-Pranteda ML, Chilloux J, Brial F, Myridakis A, Aranias T, Magnan C, Gibson GR, Sanderson JD, Nicholson JK, et al. Metabolic retroconversion of trimethylamine N-oxide and the gut microbiota. Microbiome 2018;6:73.