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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.

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Giovanni Annuzzi, MD Department of Clinical Medicine and Surgery, Federico II University Via Pansini 5, 80131, Naples, Italy. tel. +39 081 7462311 Email: <u>annuzzi@unina.it</u> **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.

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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

1 Abstract

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2 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 3 4 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 5 polyphenols (PP) and/or long-chain n-3 fatty acids (LCn3) or whole grain cereals (WGC), in 6 7 individuals at high cardiometabolic risk. Design: An ancillary study was performed within two randomized-controlled trials, aimed at 8 evaluating the medium-term effects on cardiometabolic risk factors of diets naturally rich in 9 10 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 11 statistically significant for the diets rich in LCn3 ($+1.15\pm11.58 \mu$ mol/L) (p=0.007), while not 12 for the diets rich in polyphenols ($-0.14\pm9.66 \,\mu mol/L$) (p=0.905) or their interaction (p=0.655) 13 (two-factor ANOVA). In the HealthGrain Study (n=48), the TMAO change (12-week minus 14 15 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 16 of the participants in the two studies, TMAO levels directly correlated with LCn3, 17 eicosapentaenoic acid, and protein, but not saturated fatty acids, fiber, monounsaturated fatty 18 acids, and polyphenols intake. Among food groups, TMAO directly correlated with the intake 19 of fish, vegetables, and whole grain products, but not meat, processed meat, and dairy 20 21 products. Conclusions: Diets rich in LCn3 of marine origin or WGC significantly increased plasma 22 23 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,

26 the background diet. 27 Keywords: TMAO, diet, fish, whole grain cereals, long-chain n-3 fatty acids, dietary 28 29 polyphenols, cardiometabolic risk factors. 30 31 32 Introduction 33 Trimethylamine N-oxide (TMAO) is a small organic compound belonging to the class of 34 amine oxides with chemical formula (CH₃)₃NO (1,2). In recent years TMAO has drawn much 35 36 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-37 analyses of epidemiological studies, TMAO has been related to the risk of major adverse 38 cardiovascular events including myocardial infarction (MI) and coronary heart disease (CHD) 39 (9–11). In contrast, other studies have not shown significant associations between circulating 40 41 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 42 43 the human gut from dietary precursors, mainly L-carnitine, choline, and other choline-44 containing compounds, particularly abundant in eggs, red meat, poultry, and some dairy 45 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 46 47 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 48

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

fasting TMAO concentrations in people at increased risk of developing colon cancer (20). 49 Wang et al. showed that chronic dietary red meat consumption increased systemic TMAO 50 levels (21). On the contrary, in a cross-sectional analysis in healthy people, TMAO was not 51 associated with meat, egg, or fish consumption, while a slightly positive association was 52 observed between TMAO concentration and consumption of dairy products (22). In addition, 53 a metabolomics study on biomarkers of fish and meat intake showed that plasma TMAO was 54 highly increased by fish and vegetable rich-diets than by red meat and egg rich-diets (23). 55 This makes the fish issue challenging and intriguing, since fish intake and its high n-3 fatty 56 acids content have been often associated with cardioprotective effects. To this regard, results 57 from recent meta-analyses are controversial (24,25). Beneficial cardiovascular effects were 58 59 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 60 outcomes shown in the DART study (27). 61

To date, no human intervention studies evaluated the medium-long term effects on plasma 62 TMAO levels of diets containing different amount of n-3 fatty acids from marine sources, 63 reflecting reliable intakes in the context of a balanced diet. Beside fish, little is known about 64 the effect of other potentially "healthy" foods, as cereals and other polyphenol-rich foods, on 65 66 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 67 showed that TMAO was inversely associated with intake of whole grains in healthy subjects 68 69 (28), but no human intervention studies assessed the possible cause-effect relation. Polyphenols have shown beneficial effects on several cardiovascular risk factors (29), but 70 evidence on their impact on TMAO levels comes mainly from animal, in vitro or clinical 71 studies, evaluating single classes of polyphenols (30-31) or supplements (32). 72

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).

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79 SUBJECTS AND METHODS

80 Subjects and study design.

81 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 82 naturally rich in different sources of polyphenols (PP) and/or marine long-chain n-3 fatty 83 acids (LCn3) (Etherpaths Project, NCT01154478) or (b) diets rich in whole grain cereals 84 (WGC) (HealthGrain Project, NCT00945854). Both studies involved individuals with 85 features of metabolic syndrome, and, therefore, at high risk of type 2 diabetes and CVD 86 development. Both trials were conducted at the Department of Clinical Medicine and Surgery 87 of Naples (Italy), and approved by the Ethics Committee of Federico II University (Naples, 88 89 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). 90 Etherpaths study. Seventy-eight high-cardiometabolic risk individuals (33 males and 45 91 92 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 93 94 (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. 95 The assigned diets differed in LCn3 and PP contents and were similar in macronutrient 96 composition and micronutrient content (Supplemental Table 1). Four diets were assigned: (a) 97

control diet, low in LCn3 (1.5 g/day) and low in PP (365 mg/day); (b) high in LCn3 (4 g/day) 98 and low in PP (363 mg/day); (c) high in PP (2903 mg/day) and low in LCn3 (1.4 g/day); and 99 (d) high in PP (2861 mg/day) and high in LCn3 (4 g/day). The difference in LCn3 and/or PP 100 amount was obtained through the selection of specific foods and beverages. The main dietary 101 102 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 103 104 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 105 beverages were provided to the participants for the whole study period in amounts sufficient 106 107 to cover their household consumption. Meals were prepared in a qualified catering service 108 under the surveillance of the dietitians.

109 *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, 113 fish, fruits, vegetables, and fats, during the whole study period. The only difference between 114 115 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 116 to have the same energy intake and nutrient composition (1800 kcal/day, 18% protein, 30% 117 118 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 119 of charge, in amounts sufficient to cover their household consumption for the whole study 120 period. 121

123 Experimental procedures.

147

In both studies, anthropometric and metabolic data were collected before and after each 124 dietary intervention period. Anthropometric parameters, including body weight, height, and 125 waist circumference, were measured according to standardized procedures. Blood samples 126 were drawn from an antecubital vein after a 12-h overnight fasting period for the 127 measurement of metabolic parameters and TMAO concentrations. Plasma TMAO 128 measurements were available for 48 subjects within *HealthGrain* Study (n=27 in the WGC 129 group and n=21 in the RC group) (Table 2). In the Etherpaths study (Table 1), fecal samples 130 were also collected before and after the dietary intervention for the microbiota analysis (35). 131 132 Fecal samples were available for 70 subjects. 133 Dietary assessment In both studies, participants were asked to complete a 7-day dietary record at baseline and at 134 the end of intervention to evaluate their usual diet before the start of the dietary interventions 135 and improve the adherence to the diets assigned during the study. Dietary compliance was 136 reinforced through dietary counselling during weekly clinic visits and through phone calls 137 every 2–3 days. Food intakes were calculated from the mean of the 7-day food records. 138 Energy and nutrient composition of the diets were calculated according to the food 139 140 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) 141 were used to assess dietary PP content of the foods consumed. 142 143 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 $\geq 80\%$ 144 of that assigned; participants allocated to the diets low in PP or LCn3 were considered 145 compliant with the treatment, if the corresponding intake did not exceed the assigned one by 146

more than 20%. Moreover, phenolic metabolites in 24 h-urine collection were also assessed to

- 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 $\pm 20\%$ of that assigned. Moreover, plasma total
- alkylresorcinol (AR) concentration, a biomarker of whole-wheat intake (40), was measured at
- baseline and after 12 weeks, in both the WGC and RC groups, to assess compliance with the
- assigned dietary treatments (34).
- 155
- 156 Table 1. Baseline anthropometric and fasting plasma metabolic parameters of the participants
- 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	<i>p value</i> (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	$105\pm\!10$	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	$4.45\pm\!\!5.2$	5.24 ± 7.1	$5.09 \pm \! 6.0$	$4.50\pm\!\!6.0$	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
ΤΜΑΟ (μmol/L)	4.97 ± 3.62	$7.33 \pm \! 13.9$	6.66 ± 10.54	7.59 ± 7.98	0.832

All values are mean \pm SD; HOMA-IR, homeostasis model assessment of insulin resistance.

160 TMAO, Trimethylamine N-oxide. Comparisons made by one-way ANOVA.

161

162 *Laboratory methods*

163 Plasma glucose, triglyceride and cholesterol concentrations were assayed by enzymatic

164 colorimetric methods (ABX Diagnostics, Montpellier, France) on an ABX Pentra 400

165 Autoanalyzer (ABX Diagnostics, Montpellier, France). Plasma insulin was measured by

sandwich enzyme-linked immunosorbent assay method (ELISA; DIAsource ImmunoAssays

167 S.A., Nivelles, Belgium) on Triturus Analyser (Diagnostics Grifols, S.A., Barcelona, Spain).

168 After extraction with acidified acetonitrile, plasma TMAO levels were analyzed by a UHPLC

169 DIONEX Ultimate 3000 equipped with a triple quadrupole TSQ Vantage (Thermo Fisher

170 Scientific Inc., San Josè, CA, USA) fitted with a heated-ESI (H-ESI) (Thermo Fisher

171 Scientific Inc., San Jose, CA, USA) probe. Separations were carried out by means of an

172 XBridge BEH HILIC XP (100 mm \times 2.1 mm) column, with a 2.5 μ m particle size (Waters,

173 Milford, MA, USA), as previously reported (41,42).

174 The analysis of microbiota was performed on 0.2 g of feces, DNA was extracted, partial 16S

175 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

178 healthy adults. Bifidobacteria and Lactobacillus were also analyzed for their known

179 beneficial association with human health (43).

	Refined Cereals	Whole grain Cereals	<i>p</i> 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

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.

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

181

182

183 Statistical analysis

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

185 In *the Etherpaths study*, the differences in baseline characteristics between the four groups

186 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 187 the General Linear Model (GLM)-Univariate Analysis, the absolute change of TMAO (8-188 week minus baseline) was added as "dependent variable", and PP group (with two levels: low 189 PP and high PP) and LCn3 group (with two levels: low LCn3 and high LCn3) as 190 "independent variables/ fixed factors"; sex, age, baseline TMAO, and BMI were added as 191 covariates. 192 In the *HealthGrain study*, the differences in baseline characteristics between the two groups 193 were analyzed by independent *t*-test. Differences between the effects of the two experimental 194 diets were evaluated by GLM-Univariate Analysis of absolute change of TMAO (12-week 195 196 minus baseline) adjusted for sex, age, baseline TMAO and, BMI. The associations between 197 plasma TMAO concentrations, metabolic parameters, nutrient intake, and food items were explored by partial correlation analysis and linear regression analysis also controlling for 198 potential confounders, i.e., sex, age, BMI, and study (Etherpaths/HealthGrain). 199

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

202 Sciences software version 21.0 (SPSS, Chicago, IL, USA).

203

204 **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

- of CLEPT and EREC groups (35). High LCn3 diets reduced postprandial triglyceride-rich
- 214 lipoproteins (33) and increased the number of *Bifidobacteria* (35).
- After the 8-week interventions, plasma TMAO did not change significantly from baseline in
- 216 each group: High LCn3 & Low PP (7.33±13.93 vs. 8.86±7.28 μmol/L, baseline vs. 8-week,
- 217 respectively, p=0.640); High LCn3 & High PP (7.59 ±7.98 vs. 8.37±4.40 μmol/L, p=0.713);
- 218 Low LCn3 & High PP (6.65±10.54 *vs*. 5.60±4.00 μmol/L, p=0.664); and Low LCn3 & Low
- 219 PP (4.97 ± 3.62 vs. 4.86 ± 2.91 µmol/L, p=0.886). By two-factor ANOVA, the changes in
- 220 TMAO (8-week minus baseline) were statistically significant for the diets rich in LCn3
- 221 (p=0.007), while not for the diets rich in polyphenols (p=0.905) or their interaction (p=0.655)
- 222 (Figure 1, Panel A).
- 223 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
- 227 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 $(3.68\pm2.03 \text{ and } 4.63\pm3.00 \text{ m})$
- μ mol/L, baseline and 12-week, respectively; p=0.133) or the RC diet (4.48±3.78 and
- 231 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).



235

FIGURE 1. Absolute changes (8-week minus baseline) in fasting plasma TMAO

237 concentrations in the four experimental groups in the Etherpaths Study (Low LCn3

238 & Low PP group, n= 20; High LCn3 & Low PP group, n=19; Low LCn3 & High PP group,

239 n=20; High LCn3 & High PP group, n=19) (Panel A).

240 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;

242 Whole Grain Cereals group, n=27) (Panel B). LCn3, long-chain n-3 fatty acids; PP,

- 243 polyphenols. Mean ± SEM. Comparisons made by GLM- Univariate analysis of absolute
- changes in plasma TMAO adjusted for sex, age, baseline TMAO and BMI.
- 245

246 Association analyses.

- 247 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
- 249 (Etherpaths/HealthGrain). No significant correlation was observed between TMAO and
- 250 metabolic parameters (Supplemental Table 3).
- 251 Regarding dietary components, TMAO concentrations directly significantly correlated with
- 252 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
- 255 observed between TMAO and intake of SAFA (r=0.064, p=0.505), fiber (r=0.074, p=0.440),
- 256 polyphenols (r=0.075, p=0.432) (Figure 2), and MUFA (r=0.081, p=0.398). In linear
- 257 regression analyses, entering the baseline plasma TMAO as "dependent variable" and
- 258 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 μ mol/L TMAO by each 0.2% increase in EPA intake.
- 263 Regarding food groups, TMAO concentrations directly significantly correlated with the intake
- 264 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
- 267 dairy products (r= -0.142, p=0.176) (Figure 3).
- 268 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 fastingmetabolic parameters.
- 271 In the Etherpaths study, TMAO concentrations inversely significantly correlated with fecal
- 272 *Bifidobacterium* (r= -0.356, p=0.003) (Figure 4), no significant correlations were found with
- 273 *Lactobacillus* (r= -0.136, p=0.265) and *Clostridium leptum* (CLEPT, r= -0.097, p=0.430)
- 274 whereas a correlation trend was observed with *Eubacterium rectale-Blautia coccoides*
- 275 (EREC, r= -0.215, p=0.079) (Figure 4).
- 276



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





FIGURE 3. Relationships between fasting plasma TMAO and dietary daily intakes of main 286 287 foods, including fish, meat, processed meat, vegetables, whole grain products, and dairy products, in the habitual diet of the participants in the Etherpaths and HealthGrain studies 288 (n=126) as calculated through the 7-day food records obtained before the dietary 289 290 interventions. Partial correlation analysis adjusted for sex, age, BMI, and study. 291





292

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.

298

299 DISCUSSION

300 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
- 302 fasting plasma TMAO concentration in randomized controlled trials involving individuals at
- 303 high cardio-metabolic risk.

Since a strong interest has recently grown up in TMAO as a possible risk factor/biomarker of 304 CVD and other relevant chronic diseases (3-8), it is important to define the relations between 305 dietary factors, which represent the main contributors of TMAO levels, and cardiovascular 306 risk. To this regard, an intriguing finding from cross-sectional studies was that TMAO levels 307 were higher not only in association with meat intake - repeatedly shown to be associated with 308 higher cardiovascular and cancer risk (45-46)- but also with some foods, including fish, whole 309 grain cereals, and vegetables, generally associated with a reduced risk for these diseases 310 (23,28). Our study reinforces the relationship of TMAO with intakes of wholegrain, fish and 311 vegetables, but does not confirm the association with meat consumption. Moreover, we have 312 313 shown in two randomized controlled dietary interventions using natural foodstuffs, that diets 314 rich in LCn3 of marine origin and diets rich in whole grain cereals significantly increase TMAO levels. 315

The finding that these "healthy" diets increase TMAO levels does not support the hypothesis 316 that TMAO represents an independent cardiovascular risk factor. This hypothesis is backed 317 by a recent meta-analysis showing that the risk of both major adverse cardiovascular events 318 and death from all causes is more than 60% higher in people with elevated plasma TMAO 319 320 concentration (11). Therefore, we face a paradox between this evidence and findings of our 321 intervention studies, that reinforce previous research, demonstrating that foods generally 322 associated with significant benefits in relation to cardiovascular risk are major contributors of TMAO. 323

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

directly correlated with blood pressure and BMI in Western populations on relatively low fish

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

BMI (48). Overall, we believe that the role of TMAO depends on the background diet of a

population and that TMAO *per se* cannot be considered an independent risk factor for

338 cardiovascular disease.

In our study population, the baseline diet was characterized by a limited consumption of meat and dairy products and regular fish and vegetable intake, according to the features of the Mediterranean Diet. The lack of correlations between TMAO and metabolic parameters at baseline in our study population agrees with the results reported by Gibson and colleagues in relation to the population group consuming a healthier diet (48).

In the Etherpaths study, we evaluated gut microbiota for its known role in influencing TMAO
production in the host (16,49,50). We found an inverse association between *Bifidobacteria*

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

test this hypothesis. It must be considered that the analyses of microbiota by real-time PCR is

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 354 design since intervention studies had the greatest validity to support a cause-effect 355 relationship. Our study demonstrated for the first time that healthy diets, consistently 356 associated with a reduced cardiovascular risk, induced significant increase of circulating 357 TMAO. On the other side, an important limitation is the lack of information on hard 358 endpoints, due to a small sample size and a relatively short follow-up period. However, in 359 order to get this type of information, a completely different study design should have been 360 employed, with much larger investments in time and resources. 361 In conclusion, this study demonstrated that diets rich in fish or in whole grain cereals 362 significantly increased plasma TMAO. These changes mirrored the baseline associations 363 364 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 365 is not a universally valid biomarker of cardio-metabolic risk independent of the background 366 diet. Future intervention studies with hard endpoints would be useful to finally disproving the 367 role of TMAO as an independent cardiovascular risk factor. 368

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