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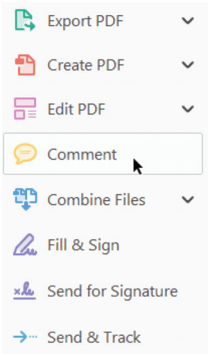

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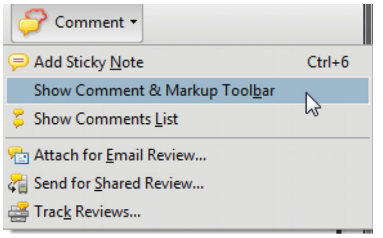

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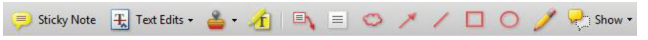

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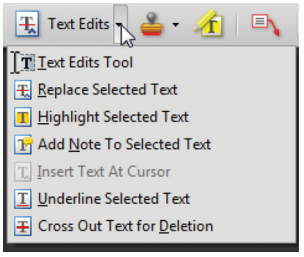

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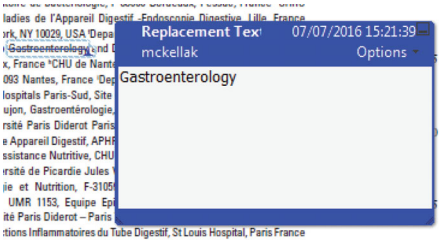

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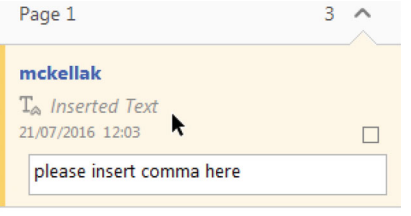

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Plasma trimethylamine-N-oxide and related metabolites are associated with type 2 diabetes risk in the Prevención con Dieta Mediterránea (PREDIMED) trial

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ABSTRACT

Background: The role of trimethylamine-N-oxide (TMAO) in type 2 diabetes (T2D) is currently partially understood and controversial.

Objective: The aim was to investigate associations between TMAO and related metabolites with T2D risk in subjects at high risk of cardiovascular disease.

Design: This is a case-cohort design study within the Prevención con Dieta Mediterránea (PREDIMED) study, with 251 incident T2D cases and a random sample of 694 participants (641 noncases and 53 overlapping cases) without T2D at baseline (median follow-up: 3.8 y). We used liquid chromatography–tandem mass spectrometry to measure plasma TMAO, L-carnitine, betaine, lyso-phosphatidylcholine (LPC) and lyso-phosphatidylethanolamine (LPE) species, phosphocholine, α-glycerophosphocholine, and choline at baseline and after 1 y. We examined associations with the use of weighted Cox proportional hazard models, accounting for the weighted case-cohort design by the Barlow method.

Results: After adjustment for recognized T2D risk factors and multiple testing, individuals in the highest quartile of baseline TMAO and α-glycerophosphocholine had a lower risk of T2D [HR (95% CI): 0.52 (0.29, 0.89) and 0.46 (0.24, 0.89), respectively]. The HR (95% CI) comparing the extreme quartiles of betaine was 0.41 (0.23, 0.74). Similar trends were observed for C16:0 LPC, C18:1 LPC, C18:0 LPC, C20:4 LPC, C22:6 LPC, C18:1 LPC plasmalogen, and C16:0 LPE. After correcting for multiple comparisons, participants in the highest quartile of 1-y changes in ~~C18:1~~ LPC plasmalogen concentrations had a lower T2D risk than the reference quartile.

Conclusion: Whether the associations between plasma TMAO and certain metabolite ~~concentrations~~ with T2D risk reflect its pathophysiology or represent an epiphenomenon need to be elucidated. This trial is registered at http://www.controlled-trials.com as ISRCTN35739639. *Am J Clin Nutr* 2018;0:1–10.

Keywords: trimethylamine-N-oxide, metabolites, type 2 diabetes, case-cohort, Mediterranean diet, PREDIMED

INTRODUCTION

Plasma concentrations of trimethylamine-N-oxide (TMAO) are determined by diet, the gut microbiome, and liver flavin-containing monooxygenase 3 (FMO3) activities (1). They are directly associated with the consumption of animal-derived foods (2, 3) containing choline, phosphatidylcholine, and L-carnitine, which are processed by gut bacteria resulting in the release of various metabolites including trimethylamine (TMA), into the blood. TMA is then transported to the liver where it is converted to TMAO, which is involved in various physiologic and pathophysiologic processes such as the deposition and removal of cholesterol from the artery endothelium (4, 5).

Supported by research grant R01-DK-102896 from the NIH. The Prevención con Dieta Mediterránea (PREDIMED) trial was supported by the official funding agency for biomedical research of the Spanish government,

TMAO has been proposed as a molecule mediating the development of type 2 diabetes (T2D) (6). Higher plasma TMAO concentrations and alterations in interrelated pathways, such as phospholipid modification and methylation, have been associated with T2D (7). In this context, plasma choline concentrations have been found to be positively related, whereas betaine concentrations are inversely related to glucose concentrations (8) and lowered in insulin-resistant subjects (9). Betaine can be derived from choline or from L-carnitine metabolism (10), with L-carnitine associated with better insulin sensitivity in diabetics and with insulin-mediated glucose uptake in normoglycemic subjects (11).

Substantial inverse associations between dietary patterns consisting of healthy foods and the risk of T2D have been reported (12, 13). Recently, in a secondary outcome analysis of the Prevention of Disease with Mediterranean Diet [Prevención con Dieta Mediterránea (PREDIMED)] trial, a Mediterranean diet (MedDiet) reduced the risk of T2D by 30% compared with the control group (14). Although the benefits of this dietary pattern for T2D prevention is clearly observed in the PREDIMED trial, the biological mechanisms underlying these benefits are not completely understood. Consequently, an approach through metabolite-profiling technology (metabolomics) (15) can strengthen existing pathophysiologic evidence, providing further support for dietary prevention of T2D.

To our knowledge, no prospective study has assessed the association between TMAO with T2D risk. Thus, the role of TMAO in T2D development is not completely understood. Taking this into account, the primary aim of the present prospective study, nested in the framework of the PREDIMED trial, was to examine possible associations between baseline and 1-y changes in the ~~concentrations~~ of TMAO and several metabolites involved in relevant pathways [L-carnitine, betaine, lyso-phosphatidylcholine (LPC) and lyso-phosphatidylethanolamine (LPE) species, phosphocholine, α-glycerophosphocholine, and choline], with the risk of incident T2D. In addition, we aimed to examine whether a MedDiet modified these associations.

METHODS

Study design and participants

This study used a case-cohort design nested within the PREDIMED trial (ISRCTN35739639), a multicenter, single-blinded, controlled trial, conducted in Spanish primary health care centers. The design of the PREDIMED trial has been described in detail elsewhere (16, 17). In brief, 7447 participants at high cardiovascular disease (CVD) risk were allocated to a MedDiet supplemented with extra-virgin olive oil, a MedDiet supplemented with mixed nuts, or a control diet consisting of advice to reduce fat intake. For the present study, we considered the 3541 participants who were free of T2D at study inception. The present case-cohort study comprises a random selection of 694 nondiabetic participants (~~?~~20%) from the eligible subjects of the PREDIMED cohort without T2D at study inception and with available blood samples, together with all incident cases of T2D that occurred during the follow-up with available plasma samples (251 out of the 273 cases). Of the 892 participants included in our analyses, 641 were in the subcohort (including 53 overlapping cases between the subcohort and the total cases) and 198 comprised the rest of the T2D cases, which gave a total of 251 cases (**Supplemental Figure 1**). Of these, 686 out of the 892 participants had available samples after 1 y of follow-up and were included in the 1-y change analyses (Supplemental Figure 1). The institutional review boards of the recruitment centers approved the study protocol, and participants provided written informed consent.

Study samples and metabolomics profiling

Fasting (for $\geq$ 8 h) plasma EDTA samples were collected from subjects and stored at $-$ 80°C. In June 2015, pairs of samples for each participant (baseline and at the end of the 1-y follow-up) were randomly ordered and shipped on dry ice to the Broad Institute, Inc., Boston, Massachusetts, for metabolomics assays. Liquid chromatography–tandem mass spectrometry techniques were used to perform semiquantitative profiling of several metabolites in blood plasma (TMAO, L-carnitine, betaine, LPC and LPE species, phosphocholine, α-glycerophosphocholine, and choline) and identify relations between them. A system composed of a Shimadzu Nexera X2 U-HPLC (Shimadzu Corp.) coupled to a Q Exactive hybrid quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific) was used (18–24). Metabolite identities were confirmed by using authentic reference standards. Raw data were

the Instituto de Salud Carlos III, through grants provided to research networks specifically developed for the trial [grant RTIC G03/140 (to RE); Q8 ~~grant RTIC RD 06/0045 (to MAM-G)~~] and through the Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y Nutrición, and by grants from Centro Nacional de Investigaciones Cardiovasculares (grant CNIC 06/2007), the Fondo de Investigación Sanitaria Fondo Europeo de Desarrollo Regional (grants PI04-2239, PI 05/2584, CP06/00100, PI07/0240, PI07/1138, PI07/0954, PI 07/0473, PI10/01407, PI10/02658, PI11/01647, P11/02505, and PI13/00462), the Ministerio de Ciencia e Innovación (grants AGL-2009–13906-C02 and AGL2010–22319-C03), the Fundación Mapfre 2010, Consejería de Salud de la Junta de Andalucía (grant PI0105/2007), the Public Health Division of the Department of Health of the Autonomous Government of Catalonia, Generalitat Valenciana (grants ACOMP06109, GVACOMP2010–181, GVACOMP2011–151, CS2010-AP-111, and CS2011-AP-042), and the Regional Government of Navarra (grant P27/2011). CP was supported by a postdoctoral fellowship granted by the Autonomous Government of Catalonia (PERIS 2016-2020; Incorporació de Científics I Tecnòlegs, SLT002/0016/00428). MG-F was supported by a postdoctoral fellowship granted by the Lilly Foundation European Association of Diabetes (EASD) through the Institut d'Investigacions Sanitàries Pere i Virgili (IISPV), Tarragona, Spain. The Fundación Patrimonio Comunal Olivarero and Hojiblanca (Malaga, Spain), the California Walnut Commission (Sacramento, California), Borges (Reus, Spain), and Morella Nuts (Reus, Spain) donated the olive oil, walnuts, almonds, and hazelnuts, respectively, used in the study; Q9 however, none of these funding sources played a role in the design, collection, analysis, interpretation or publication of the data.

Supplemental Figure 1 and Supplemental Tables 1–6 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: Ach, acetylcholine; CVD, cardiovascular disease; LPC, lyso-phosphatidylcholine; LPE, lyso-phosphatidylethanolamine; MedDiet, Mediterranean diet; PREDIMED, Prevención con Dieta Mediterránea; T2D, type 2 diabetes; TMA, trimethylamine; TMAO, trimethylamine-N-oxide.

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processed with the use of TraceFinder software (Thermo Fisher Scientific) and Progenesis QI (Nonlinear Dynamics).

Ascertainment of T2D cases

Information was collected through contact with participants and primary health care physicians, annual follow-up visits, yearly ad hoc reviews of medical charts, and annual consultation of the National Death Index. Because T2D was a prespecified secondary outcome of the PREDIMED trial, it was identified at baseline by clinical diagnosis or use of antidiabetic medication. The diagnosis of new-onset T2D during follow-up has been described elsewhere (14) and briefly followed the American Diabetes Association criteria (25), namely 2 confirmations of fasting plasma glucose $\geq$ 7.0 mmol/L or 2-h plasma glucose $\geq$ 11.1 mmol/L after a 75-g oral-glucose load.

Assessment of covariates and other variables

Q10 At baseline and yearly during the follow-up, the participants completed a 47-item questionnaire related to lifestyle variables, smoking status, medical history, and medication use. A validated Spanish version of the Minnesota Leisure-Time Physical Activity Questionnaire was administered in order to evaluate physical activity (26). To assess the degree of adherence to the MedDiet, a 14-item validated questionnaire was filled in for each participant (27). BMI was calculated as weight divided by height squared (kg/m^2). Participants' triacylglycerol and total, HDL, and LDL cholesterol concentrations were measured by using fasting plasma at baseline. Blood glucose and insulin concentrations were centrally assessed at baseline and at the end of the 1 y of Q11 follow-up. Insulin resistance was estimated by the HOMA-IR method with the use of the following equation (28): HOMA-IR = [fasting insulin (μIU/mL) $\times$ fasting glucose (mmol/L)]/22.5.

Statistical analysis

Baseline characteristics of cases and noncases are described as means $\pm$ SDs for quantitative variables and percentages or numbers for categorical variables. We applied a natural logarithmic transformation to approximate a normal distribution of metabolite ~~concentrations~~. Person-time of follow-up was calculated as the interval between the baseline visit and date of T2D event, death, or date of the last contact, whichever came first. We used Cox proportional hazard models, with Barlow weights (to account for the overrepresentation of cases), to estimate HRs and their 95% CIs for the risk of T2D. A crude model and 2 multivariable-adjusted Cox regression models were fitted as follows: *1*) multivariable model 1 adjusted for age (years), sex (male or female), BMI (kg/m^2), intervention group, and baseline fasting glucose (milligrams per deciliter) (adding a quadratic term to account for the departure from linearity) and *2*) multivariable model 2 additionally adjusted for smoking (never, current, or former), leisure-time physical activity (metabolic equivalent tasks in minutesper day), baseline dyslipidemia (yes or no), and hypertension (yes or no). We stratified the models according to recruitment center. Baseline metabolites were analyzed as both continuous variables (1-SD increment in their transformed levels) and by using quartiles (using cutoffs defined among noncases). To appraise the linear trend across quartiles, the median metabolite concentration within each quartile was included in the Cox regression models as a continuous variable. To account for multiple testing, we adjusted *P* values of the multivariable-adjusted associations between quartiles or 1-SD increment in metabolite ~~concentration~~ and T2D risk with the use of the Benjamini-Hochberg false discovery rate procedure (29). A false discovery rate *P* value < 0.05 was considered to be significant.

We also examined the associations of 1-y changes in metabolites with T2D risk. We used the same models as in the baseline value analyses but further adjusted for baseline metabolite ~~concentrations~~. With respect to metabolites, we first calculated the ratio between 1-y and baseline values and then normalized this ratio with the natural logarithmic transformation. To test the robustness of our results in relation to the association between TMAO and T2D risk, we conducted 2 sensitivity analyses: *1*) testing the association between baseline values and T2D risk after excluding early cases (<1 y) and *2*) testing the associations between the mean values at baseline and 1-y follow-up and subsequent T2D risk (T2D cases that occurred from baseline through the 1-y follow-up were excluded). To examine whether the association between baseline and 1-y changes in metabolites and incident T2D varied by intervention group, we stratified the analysis described above by intervention group (both merged MedDiet interventions compared with the control). We also added a multiplicative term (1 df) between intervention assignment (merged MedDiet compared with the control group) and metabolites (continuous) into the multivariable Cox models stratified on intervention assignment to test for interactions by means of likelihood ratio tests. In addition, we compared differences in 1-y changes in metabolites in the MedDiet group with changes in the control group (adjusted for the aforementioned covariates) with the use of ANCOVA. Finally, we applied multiple linear regression analyses to examine relations between TMAO ~~concentrations~~ and precursors (choline, betaine, and L-carnitine) at baseline and at the 1-y follow-up in the whole group, adjusting for age, sex, and Q12 HOMA-IR. Statistical analyses were performed with the use of Stata 13.1 (StataCorp.). A 2-sided *P* value < 0.05 was considered significant.

RESULTS

Baseline characteristics

The median follow-up of the study population was 3.8 y. The baseline characteristics of the 892 subjects (251 cases and 641 noncases) included in the present case-cohort study are shown in **Table 1**. The mean age of participants at baseline was 66.5 y, and the mean $\pm$ SD BMI was 30.1 $\pm$ 3.5. Compared with noncases, Q13 those participants who developed T2D were more likely to be men, current smokers, and to have a higher prevalence of hypertension in addition to a higher BMI and fasting glucose and triacylglycerol ~~concentrations~~ (Table 1).

Baseline metabolites and risk of T2D

Overall group

The associations between plasma metabolites with the risk of T2D in the overall group are presented in **Table 2**. In the group

TABLE 1
Baseline characteristics of the study population[1]

	Total	Cases	Noncases	*P*
n	892	251	641	
Age, y	66.5 ± 5.7	66.4 ± 5.7	66.5 ± 5.7	0.781
Sex, % female	61.2	55.0	63.6	0.017
BMI, kg/m^2	30.1 ± 3.5	30.8 ± 3.3	29.8 ± 3.6	<0.001
Physical activity, METs/d	240.7 ± 234.6	249.2 ± 233.5	237.4 ± 235.1	0.500
Intervention group, %				
MedDiet + EVOO	30.6	29.9	30.9	0.425
MedDiet + nuts	36.3	37.2	33.8	
Control group	33.1	36.3	31.8	
Hypertension, %	91.7	96.0	90.0	0.003
Dyslipidemia, %	84.3	79.7	86.1	0.018
Smoking, %				
Never	59.0	52.6	61.5	0.006
Former	22.4	22.3	22.5	
Current	18.6	25.1	16.0	
Score for adherence to MedDiet[2]	8.5 ± 2.0	8.4 ± 2.0	8.6 ± 1.9	0.186
Fasting blood glucose, mg/dL	103.3 ± 17.6	118.6 ± 18.0	97.8 ± 13.8	<0.001
Total cholesterol, mg/dL	222.2 ± 39.3	221.7 ± 42.3	222.4 ± 38.1	0.846
HDL cholesterol, mg/dL	54.7 ± 13.0	52.6 ± 12.7	55.7 ± 13.1	0.014
LDL cholesterol, mg/dL	139.7 ± 32.9	137.0 ± 31.6	141.0 ± 33.3	0.201
Triacylglycerol, mg/dL	140.1 ± 85.0	169.0 ± 121.0	128.6 ± 62.0	<0.001

[1]Values are means ± SDs unless otherwise indicated. Chi-square test was used for comparison of categorical variables and Student's *t* test was used for comparison of continuous variables. EVOO, extra-virgin olive oil; MedDiet, Mediterranean diet; MET, metabolic equivalent.
[2]This score is based on the 14-item dietary screener.

adjusted for age, sex, BMI, intervention group, glucose, smoking, leisure-time physical activity, dyslipidemia, and hypertension (multivariable model 2), the estimated HR for incident T2D reached significance only in the highest, compared with the lowest, quartile of TMAO concentrations (HR: 0.52; 95% CI: 0.29, 0.89). In both sensitivity analyses, TMAO concentrations in the highest quartile were significantly associated with lower T2D risk (a: HR: 0.52; 95% CI: 0.29, 0.93; b: HR: 0.44; 95% CI: 0.20, 0.96) compared with the lowest quartile. In the group adjusted for age, sex, BMI, intervention group, and glucose (multivariable model 1), the highest quartile of phosphocholine was associated with a lower risk of T2D (HR: 0.51; 95% CI: 0.27, 0.99), but these associations were no longer significant after further adjustment. With regard to betaine, the estimated HR for incident T2D in the highest compared with the lowest quartile was 0.41 (95% CI: 0.23, 0.74; $P = 0.003$). Concerning lyso-choline species, significant inverse associations with T2D incidence were observed for C16:0 LPC, C18:1 LPC, C18:0 LPC, C20:4 LPC, C22:6 LPC, and C18:1 LPC plasmalogen, either metabolites were modeled continuously (per 1 SD) or as quartiles (Table 2). Notably, per 1-SD increase in C18:1 LPC plasmalogen concentrations, a 54% lower risk (HR: 0.46; 95% CI: 0.35, 0.59) of T2D was observed ($P < 0.001$), and individuals in the highest quartile had an HR of 0.15 (95% CI: 0.06, 0.35; $P < 0.001$).

With regard to lyso-ethanolamine species, concentrations of C16:0 LPE (per 1-SD increase and highest quartile) were significantly associated with a reduced risk of T2D. Per 1-SD increase in L-carnitine, α-glycerophosphocholine, and betaine concentrations, a 19% (HR: 0.81; 95% CI: 0.67, 0.97; $P = 0.025$), 39% (HR: 0.61; 95% CI: 0.45, 0.83; $P = 0.002$), and 25% (HR: 0.75; 95% CI: 0.61, 0.91; $P = 0.004$) lower risk of T2D was found, respectively. These associations remained significant after accounting for multiple comparisons.

MedDiet and control group

In stratified analyses by intervention group, the estimated HR for incident T2D in the highest compared with the lowest quartile of α-glycerophosphocholine concentrations was 0.27 (95% CI: 0.12, 0.62; $P = 0.002$) in the MedDiet group (**Supplemental Table 1**) and 1.08 (95%: CI 0.14, 1.16; $P > 0.05$) in the control group (**Supplemental Table 2**). In the MedDiet group, the HR associated with a 1-SD increment in α-glycerophosphocholine concentration was 0.55 (95% CI: 0.37, 0.82; $P = 0.003$, P-interaction $= 0.041$) (**Figure 1**A, **Supplemental Table 3**), whereas in the control group no significant associations were observed (Figure 1B).

One-year changes in concentrations of metabolites and the risk of T2D

Associations between 1-y changes in metabolite concentrations across quartiles and the risk of T2D are shown in **Table 3**. In the highest quartile of increase in the concentrations of TMAO, a significant inverse association with T2D risk was found (HR: 0.49; 95% CI: 0.25, 0.98). Several lyso-choline species (C16:0 LPC, C18:1 LPC, and C18:0 LPC), including C18:1 LPC plasmalogen, C16:0 LPE, and α-glycerophosphocholine, were significantly associated with a decreased risk of T2D. After adjustment for multiple testing, only C18:1 LPC plasmalogen remained significant. We repeated the analyses with the use of a 1-SD increment in 1-y change in metabolite concentrations and,

TABLE 2
Associations of baseline individual metabolite ~~concentrations~~ with the risk of type 2 diabetes in the PREDIMED trial, 2003–2010, in the overall group.[1]

	Quartile of plasma metabolite ~~concentration~~				*P*				
Metabolite	1	2	3	4	*P*-trend	FDR-Adjusted p value (Q4 vs. Q1)	HR per 1-SD increment	Unadjusted	FDR-adjusted
Trimethylamine N-oxide									
Cases, *n*	73	65	68	44					
Crude model	Ref	0.98 (0.64, 1.53)	1.01 (0.66, 1.56)	0.60 (0.37, 0.98)	0.039		0.88 (0.75, 1.04)	0.162	
MV1	Ref	0.96 (0.60, 1.54)	0.95 (0.58, 1.56)	0.51 (0.29, 0.88)	0.012		0.86 (0.72, 1.04)	0.123	
MV2	Ref	0.98 (0.60, 1.59)	0.91 (0.55, 1.51)	0.52 (0.29, 0.89)	0.012	0.032	0.83 (0.69, 1.01)	0.059	0.078
Phosphocholine									
Cases, *n*	75	66	57	49					
Crude model	Ref	0.99 (0.64, 1.52)	0.77 (0.49, 1.21)	0.56 (0.32, 0.98)	0.029		0.90 (0.77, 1.06)	0.224	
MV1	Ref	1.11 (0.68, 1.80)	0.82 (0.48, 1.39)	0.51 (0.27, 0.99)	0.036		0.88 (0.74, 1.05)	0.157	
MV2	Ref	1.19 (0.71, 1.97)	0.89 (0.51, 1.56)	0.56 (0.29, 1.09)	0.079	0.124	0.90 (0.75, 1.08)	0.268	0.329
Choline									
Cases, *n*	52	67	63	65					
Crude model	Ref	1.20 (0.75, 1.90)	1.04 (0.63, 1.72)	1.34 (0.83, 2.18)	0.313		1.03 (0.86, 1.23)	0.729	
MV1	Ref	1.33 (0.80, 2.24)	1.07 (0.61, 1.88)	1.20 (0.70, 2.07)	0.698		0.94 (0.77, 1.14)	0.538	
MV2	Ref	1.42 (0.84, 2.42)	1.13 (0.63, 2.02)	1.23 (0.71, 2.13)	0.663	0.493	0.94 (0.77, 1.14)	0.523	0.557
C14:0 LPC									
Cases, *n*	72	65	55	56					
Crude model	Ref	1.05 (0.68, 1.64)	0.91 (0.57, 1.45)	0.91 (0.57, 1.44)	0.590		1.01 (0.85, 1.19)	0.910	
MV1	Ref	1.02 (0.61, 1.74)	0.89 (0.53, 1.49)	0.93 (0.55, 1.55)	0.669		1.03 (0.86, 1.24)	0.741	
MV2	Ref	1.05 (0.62, 1.78)	0.88 (0.52, 1.49)	0.88 (0.52, 1.50)	0.548	0.650	1.01 (0.84, 1.23)	0.879	0.879
C16:1 LPC									
Cases, *n*	78	61	58	50					
Crude model	Ref	0.89 (0.57, 1.38)	0.84 (0.54, 1.31)	0.72 (0.45, 1.16)	0.181		0.91 (0.77, 1.08)	0.317	
MV1	Ref	0.77 (0.47, 1.26)	0.93 (0.57, 1.52)	0.75 (0.45, 1.26)	0.387		0.91 (0.76, 1.11)	0.367	
MV2	Ref	0.77 (0.47, 1.28)	1.03 (0.63, 1.68)	0.75 (0.44, 1.27)	0.451	0.318	0.93 (0.77, 1.12)	0.461	0.526
C16:0 LPC									
Cases, *n*	102	62	51	36					
Crude model	Ref	0.60 (0.39, 0.92)	0.58 (0.37, 0.91)	0.41 (0.24, 0.69)	0.001		0.69 (0.56, 0.83)	<0.001	
MV1	Ref	0.53 (0.33, 0.85)	0.58 (0.36, 0.93)	0.41 (0.23, 0.74)	0.002		0.69 (0.56, 0.84)	<0.001	
MV2	Ref	0.50 (0.31, 0.82)	0.56 (0.35, 0.92)	0.42 (0.24, 0.75)	0.002	0.012	0.69 (0.56, 0.85)	<0.001	0.003
C18:1 LPC									
Cases, *n*	111	59	48	30					
Crude model	Ref	0.52 (0.34, 0.79)	0.44 (0.28, 0.68)	0.31 (0.18, 0.51)	<0.001		0.66 (0.56, 0.77)	<0.001	
MV1	Ref	0.51 (0.32, 0.82)	0.50 (0.32, 0.80)	0.34 (0.19, 0.60)	<0.001		0.67 (0.56, 0.80)	<0.001	
MV2	Ref	0.52 (0.32, 0.85)	0.54 (0.33, 0.86)	0.36 (0.20, 0.65)	<0.001	0.008	0.69 (0.58, 0.82)	<0.001	0.003
C18:0 LPC									
Cases, *n*	108	64	48	30					
Crude model	Ref	0.55 (0.36, 0.84)	0.42 (0.27, 0.66)	0.37 (0.21, 0.64)	<0.001		0.59 (0.48, 0.74)	<0.001	
MV1	Ref	0.59 (0.37, 0.96)	0.48 (0.29, 0.79)	0.34 (0.18, 0.68)	<0.001		0.63 (0.50, 0.79)	<0.001	
MV2	Ref	0.58 (0.36, 0.95)	0.47 (0.28, 0.79)	0.37 (0.19, 0.74)	0.001	0.013	0.65 (0.51, 0.82)	<0.001	0.003
C20:4 LPC									
Cases, *n*	103	56	47	43					
Crude model	Ref	0.52 (0.34, 0.80)	0.48 (0.31, 0.76)	0.45 (0.28, 0.73)	<0.001		0.67 (0.56, 0.80)	<0.001	
MV1	Ref	0.49 (0.30, 0.78)	0.54 (0.33, 0.87)	0.43 (0.25, 0.75)	0.001		0.66 (0.54, 0.80)	<0.001	
MV2	Ref	0.48 (0.29, 0.79)	0.57 (0.35, 0.93)	0.44 (0.25, 0.78)	0.003	0.013	0.67 (0.54, 0.81)	<0.001	0.003
C22:6 LPC									
Cases, *n*	93	72	52	33					
Crude model	Ref	1.03 (0.68, 1.55)	0.71 (0.47, 1.09)	0.39 (0.23, 0.66)	<0.001		0.76 (0.65, 0.89)	0.001	
MV1	Ref	1.12 (0.70, 1.79)	0.98 (0.63, 1.54)	0.40 (0.22, 0.74)	0.009		0.78 (0.66, 0.94)	0.007	
MV2	Ref	1.13 (0.71, 1.82)	1.07 (0.67, 1.70)	0.41 (0.22, 0.77)	0.022	0.014	0.80 (0.67, 0.96)	0.016	0.028
C18:1 LPC plasmalogen									
Cases, *n*	120	61	48	14					
Crude model	Ref	0.56 (0.37, 0.86)	0.38 (0.24, 0.59)	0.18 (0.09, 0.37)	<0.001		0.42 (0.33, 0.55)	<0.001	
MV1	Ref	0.68 (0.43, 1.09)	0.44 (0.27, 0.72)	0.14 (0.06, 0.32)	<0.001		0.45 (0.34, 0.58)	<0.001	
MV2	Ref	0.67 (0.42, 1.08)	0.45 (0.27, 0.74)	0.15 (0.06, 0.35)	<0.001	0.008	0.46 (0.35, 0.59)	<0.001	0.003
C16:0 LPE									
Cases, *n*	94	73	50	32					
Crude model	Ref	0.97 (0.64, 1.45)	0.59 (0.38, 0.92)	0.45 (0.26, 0.78)	0.001		0.73 (0.61, 0.87)	0.001	
MV1	Ref	0.86 (0.54, 1.36)	0.56 (0.35, 0.89)	0.49 (0.27, 0.88)	0.003		0.74 (0.61, 0.89)	0.002	
MV2	Ref	0.89 (0.55, 1.44)	0.55 (0.34, 0.88)	0.46 (0.25, 0.84)	0.002	0.026	0.73 (0.60, 0.89)	0.002	0.004

TABLE 2
Continued.

Metabolite	Quartile of plasma metabolite ~~concentration~~				*P*				
	1	2	3	4	*P*-trend	FDR-Adjusted p value (Q4 vs. Q1)	HR per 1-SD increment	Unadjusted	FDR-adjusted
C18:1 LPE									
Cases, *n*	73	66	61	49					
Crude model	Ref	0.89 (0.57, 1.38)	0.84 (0.54, 1.30)	0.60 (0.37, 0.95)	0.034		0.81 (0.69, 0.95)	0.011	
MV1	Ref	0.77 (0.47, 1.26)	0.79 (0.50, 1.26)	0.57 (0.34, 0.96)	0.045		0.82 (0.70, 0.99)	0.040	
MV2	Ref	0.79 (0.48, 1.32)	0.86 (0.53, 1.39)	0.59 (0.35, 1.00)	0.073	0.075	0.84 (0.69, 1.00)	0.058	0.078
L-Carnitine									
Cases, *n*	52	79	69	49					
Crude model	Ref	1.81 (1.12, 2.91)	1.44 (0.89, 2.34)	0.91 (0.54, 1.55)	0.379		0.90 (0.77, 1.05)	0.198	
MV1	Ref	1.76 (1.07, 2.90)	1.09 (0.64, 1.88)	0.75 (0.41, 1.36)	0.108		0.84 (0.70, 1.01)	0.071	
MV2	Ref	1.77 (1.06, 2.94)	1.07 (0.61, 1.88)	0.69 (0.37, 1.26)	0.066	0.280	0.81 (0.67, 0.97)	0.025	0.040
α-Glycerophosphocholine									
Cases, *n*	81	56	71	40					
Crude model	Ref	0.70 (0.44, 1.12)	0.81 (0.51, 1.28)	0.57 (0.32, 1.02)	0.091		0.70 (0.54, 0.92)	0.011	
MV1	Ref	0.58 (0.35, 0.98)	0.75 (0.46, 1.20)	0.47 (0.25, 0.90)	0.044		0.61 (0.45, 0.81)	0.001	
MV2	Ref	0.54 (0.31, 0.93)	0.75 (0.46, 1.23)	0.46 (0.24, 0.89)	0.051	0.035	0.61 (0.45, 0.83)	0.002	0.004
Betaine									
Cases, *n*	76	69	59	43					
Crude model	Ref	0.82 (0.53, 1.26)	0.82 (0.52, 1.29)	0.48 (0.29, 0.78)	0.005		0.83 (0.69, 0.98)	0.035	
MV1	Ref	0.90 (0.57, 1.43)	0.74 (0.45, 1.21)	0.41 (0.23, 0.73)	0.002		0.77 (0.64, 0.94)	0.009	
MV2	Ref	0.87 (0.54, 1.39)	0.73 (0.43, 1.24)	0.41 (0.23, 0.74)	0.003	0.012	0.75 (0.61, 0.91)	0.004	0.008

[1]Values are HRs (95% CIs) unless otherwise indicated. A natural logarithmic transformation was applied to the raw values of individual metabolites. Cox regression analysis was used. MV1 adjusted for age (years), sex (male or female), BMI (kg/m^2), intervention group (MedDiet + EVOO or MedDiet + nuts), and baseline fasting glucose (milligrams per deciliter) (centered on the sample mean and adding the quadratic term); MV2 additionally adjusted for smoking (never, current, or former), leisure-time physical activity (metabolic equivalent tasks in minutes per day), dyslipidemia, and hypertension. FDR-controlled adjustments were conducted by applying the method of Benjamini and Hochberg. EVOO, extra-virgin olive oil; FDR, false discovery rate; LPC, lyso-phosphatidylcholine; LPE, lyso-phosphatidylethanolamine; MedDiet, Mediterranean diet; MV, multivariable model; PREDIMED, Prevención con Dieta Mediterránea; Q, quartile; Ref, reference.

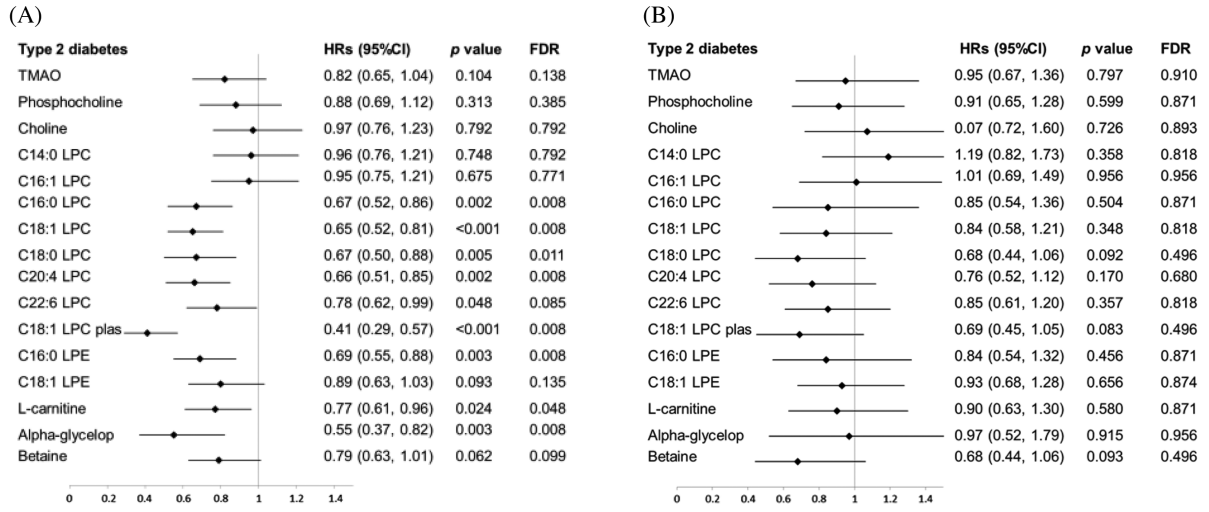

FIGURE 1 HRs (95% CIs) for type 2 diabetes according to baseline metabolites analyzed as continuous (per 1-SD increment) by both Mediterranean diet intervention groups (merged) (A) and the control group (B). All HRs were adjusted for age (y), sex (male or female), BMI (kg/m^2), baseline fasting glucose (mg/dL; mean + quadratic term of the centered mean), smoking (never, current, or former), leisure-time physical activity (metabolic equivalent tasks in min/d), dyslipidemia, and hypertension. A natural logarithmic transformation was applied to the raw value. FDR-controlled adjustments were conducted by applying the method of Benjamini and Hochberg. Alpha-glycelop, α-glycerophosphocholine; FDR, false discovery rate; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; plas, plasmalogen; TMAO, trimethylamine N-oxide.

after adjustment for multiple testing, we found that per 1-SD increase in ~~C16:0~~ LPC, ~~C18:1~~ LPC, ~~C18:0~~ LPC, and ~~C18:1~~ LPC plasmalogen concentrations, the risk of T2D decreased by 35% (HR: 0.65; 95% CI: 0.49, 0.86), 37% (HR: 0.63; 95% CI: 0.47, 0.85), 32% (HR: 0.68; 95% CI: 0.50, 0.92), and 42% (HR: 0.58; 95% CI: 0.42, 0.81), respectively. These significant inverse associations observed in the overall group persisted in the MedDiet group, whereas only C18:1 LPC was found to be significantly

TABLE 3
Associations of 1-y changes in individual metabolite ~~concentrations~~ with the risk of type 2 diabetes in the PREDIMED trial, 2003–2010, in the overall group[1]

	Quartile of plasma metabolite ~~concentration~~					
Metabolite	1	2	3	4	*P*-trend	FDR-adjusted *P* (Q4 vs. Q1)
Trimethylamine N-oxide						
Cases, *n*	44	44	45	48		
MV	Ref	0.58 (0.31, 1.10)	0.64 (0.32, 1.25)	0.49 (0.25, 0.98)	0.081	0.100
Phosphocholine						
Cases, *n*	44	44	45	48		
MV	Ref	0.75 (0.42, 1.36)	1.00 (0.59, 1.71)	0.74 (0.40, 1.37)	0.484	0.598
Choline						
Cases, *n*	49	44	50	37		
MV	Ref	1.11 (0.61, 2.02)	0.83 (0.46, 1.51)	1.04 (0.54, 2.00)	0.895	0.924
C14:0 LPC						
Cases, *n*	52	47	39	43		
MV	Ref	0.77 (0.42, 1.40)	0.79 (0.43, 1.47)	0.79 (0.38, 1.64)	0.587	0.769
C16:1 LPC						
Cases, *n*	57	35	43	45		
MV	Ref	0.47 (0.24, 0.91)	0.60 (0.33, 1.10)	0.86 (0.44, 1.68)	0.657	0.811
C16:0 LPC						
Cases, *n*	55	44	42	40		
MV	Ref	0.57 (0.31, 1.04)	0.38 (0.21, 1.69)	0.47 (0.24, 0.93)	0.014	0.100
C18:1 LPC						
Cases, *n*	44	53	36	44		
MV	Ref	0.76 (0.42, 1.38)	0.55 (0.29, 1.01)	0.44 (0.21, 0.90)	0.016	0.100
C18:0 LPC						
Cases, *n*	49	45	51	34		
MV	Ref	0.63 (0.36, 1.12)	0.47 (0.25, 0.90)	0.45 (0.22, 0.92)	0.018	0.100
C20:4 LPC						
Cases, *n*	48	41	42	49		
MV	Ref	0.74 (0.41, 1.35)	0.53 (0.27, 1.03)	0.54 (0.28, 1.04)	0.047	0.130
C22:6 LPC						
Cases, *n*	38	41	50	50		
MV	Ref	0.99 (0.55, 1.79)	0.55 (0.27, 1.10)	0.72 (0.36, 1.47)	0.334	0.598
C18:1 LPC plasmalogen						
Cases, *n*	52	57	40	32		
MV	Ref	0.76 (0.43, 1.33)	0.30 (0.15, 0.58)	0.33 (0.17, 0.66)	<0.001	0.032
C16:0 LPE						
Cases, *n*	44	51	45	34		
MV	Ref	0.87 (0.48, 1.58)	0.91 (0.51, 1.62)	0.49 (0.25, 0.98)	0.052	0.100
C18:1 LPE						
Cases, *n*	42	50	32	56		
MV	Ref	1.19 (0.66, 2.12)	0.69 (0.38, 1.27)	0.85 (0.43, 1.67)	0.408	0.811
L-Carnitine						
Cases, *n*	35	42	61	42		
MV	Ref	1.16 (0.58, 2.32)	2.05 (1.03, 4.11)	0.86 (0.38, 1.91)	0.725	0.811
α-Glycerophosphocholine						
Cases, *n*	49	47	51	33		
MV	Ref	0.40 (0.21, 0.78)	0.43 (0.22, 0.83)	0.45 (0.21, 0.98)	0.084	0.100
Betaine						
Cases, *n*	41	47	45	48		
MV	Ref	1.41 (0.77, 2.59)	0.90 (0.47, 1.73)	1.03 (0.54, 1.95)	0.732	0.924

[1] Values are HRs (95% CIs). We first calculated the ratio between 1-y and baseline ~~concentrations~~ of individual metabolites and then normalized this ratio with the natural logarithmic transformation. Cox regression analysis was used. The MV was adjusted for baseline ~~concentrations~~ of metabolites, age (years), sex (male or female), intervention group (MedDiet + EVOO or MedDiet + nuts), BMI (kg/m^2), baseline fasting glucose (milligrams per deciliter) (centered on the sample mean and adding the quadratic term), smoking (never, current, or former), leisure-time physical activity (metabolic equivalent tasks in minutes per day), dyslipidemia, and hypertension. FDR-controlled adjustments were conducted by applying the method of Benjamini and Hochberg. EVOO, extra-virgin olive oil; FDR, false discovery rate; LPC, lyso-phosphatidylcholine; LPE, lyso-phosphatidylethanolamine; MedDiet, Mediterranean diet; MV, multivariable model; PREDIMED, Prevención con Dieta Mediterránea; Q, quartile; Ref, reference.

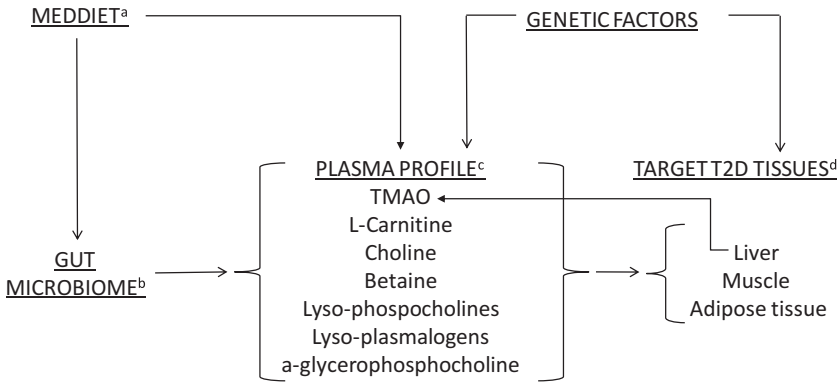

FIGURE 2 Open homeostasis pattern. [a]The role of the Mediterranean diet; [b]the role of the microbiome; [c]metabolites absorbed, produced and transformed, or methylated; [d]the role of T2D pathogenicity target tissues. MEDDIET, Mediterranean diet; T2D, type 2 diabetes; TMAO, trimethylamine-N-oxide.

associated with lower risk in the control group. However, *P*-interaction values between ~~C16:0~~ LPC, ~~C18:1~~ LPC, ~~C18:0~~ LPC, and the intervention group (MedDiet compared with the control group) ~~and~~ T2D were nonsignificant (**Supplemental Table 4**). There was a tendency toward higher T2D risk for those individuals in the control group with higher 1-y changes in betaine ~~concentrations~~, but the results were not significant (HR: 1.25; 95% CI: 0.81, 1.92; *P*-interaction = 0.012). There were no significant differences in mean 1-y changes in metabolite ~~concentrations~~ between the MedDiet and control groups (data not shown).

Predictors of TMAO concentrations from regression analyses

A multiple linear regression was used to assess potential relations between TMAO and choline, betaine, and L-carnitine, while adjusting for age, sex, and HOMA-IR (**Supplemental Tables 5** and **6**). Baseline and 1-y changes in L-carnitine ~~concentrations~~ were positively associated with TMAO ~~concentrations~~ at baseline and at 1 y, respectively.

DISCUSSION

With the use of a case-cohort design within the PREDIMED trial and aimed at identifying plasma metabolites potentially related to T2D in 892 individuals at high CVD risk, we observed that higher baseline ~~concentrations~~ of TMAO, L-carnitine, betaine, α-glycerophosphocholine, and several LPC and LPE species were associated with lower risk of T2D development, independently of recognized T2D risk factors (i.e., age, sex, BMI, blood glucose, smoking). At baseline, the participants in the highest quartile of TMAO plasma ~~concentrations~~ had a 48% lower risk of developing T2D compared with the lowest quartile. Notably, the association between TMAO and T2D persisted and remained significant after sensitivity analyses.

L-Carnitine affects insulin-mediated glucose uptake and oxidation in diabetics and healthy controls, improving insulin sensitivity and blood glucose ~~concentrations~~ in patients with T2D (11) and mitochondrial utilization of fatty acids (30). Betaine is associated with a lower risk of diabetes, presumably because it serves as a methyl donor in the methionine cycle and its administration decreases homocysteine ~~concentrations~~ (31–33). Recently, plasma betaine ~~concentrations~~ were reported to be reduced in insulin-resistant humans and were associated with insulin sensitivity (9). In addition, lower plasma ~~concentrations~~ of betaine were reported in patients with metabolic syndrome compared with a healthy population (34). The possible protective role of L-carnitine and betaine in T2D development was also clearly supported by our findings.

The gut microbiome is involved in gastrointestinal and immune function as well as the digestion of nutrients, and may thus affect the risk of obesity and T2D (35). L-Carnitine, choline, and betaine can be metabolized by the microbiome to TMA, which is absorbed by the gut and further oxidized to TMAO in the liver (**Figure 2**). Among these substrates, L-carnitine was identified in our study as the sole positive predictor of plasma TMAO ~~concentrations~~ (10, 36). L-Carnitine may be transformed to TMAO through ~~≥~~2 independent pathways catalyzed by L-carnitine dehydrogenase or carnitine oxygenase ~~or reductase~~ (10). TMAO is, on the other hand, considered to be a risk factor for CVD (36). However, circulating TMAO is influenced by the gut microbiome, kidney function, as well as a FMO3 genotype, factors that may confound the relation between TMAO and chronic disease (37). Whether TMAO is a causal factor for disease development and progression or simply a biomarker of metabolic adaptation is unclear and needs to be explored further.

With regard to the inverse associations between TMAO plasma ~~concentrations~~ and the risk of developing T2D, our findings are compatible with a recent prospective study (38) involving a consecutive sample of 37 obese subjects undergoing bariatric surgery, 17 of whom had diabetes. One year after surgery, TMAO plasma ~~concentrations~~ increased by ~~?~~2-fold compared with preoperative ~~concentrations~~, which was significantly correlated with the corresponding 1-y decrease in glycated hemoglobin after the bariatric surgery (r = –0.39, P = 0.025). In our study, Spearman's correlation analysis between TMAO and 1-y increase in HOMA-IR, a value used to quantify insulin resistance, showed a significant negative correlation (r = –0.13, P = 0.026).

On the other hand, a study conducted in 283 individuals that examined cardiometabolic risk factors and pathways associated with TMAO concentration reported that the presence of diabetes was associated with higher concentrations of plasma TMAO (7). Similarly, Shan et al. (39) measured plasma concentrations of TMAO in a case-control study in 2694 participants and noted that higher plasma TMAO was associated with increased odds

of T2D. In addition, in a larger study in 4007 patients undergoing coronary angiography, increased plasma concentrations of TMAO were related to increased concentrations of fasting glucose and diabetes (40). These studies (7, 39, 40) were not designed to investigate associations between TMAO and T2D prospectively, whereas our data showed that higher TMAO ~~concentrations~~ can be prospectively associated with decreased T2D risk. However, differences in study design and the populations studied can only partly explain the overall discrepancies. Because TMAO was reported to be highly variable in plasma and urine (log-normal reference change values ranging from 403% to 80% in plasma) of overweight people with T2D over a 2-y period (41), further research is needed to explain these variations. Overall, the exact mechanisms associating plasma TMAO ~~concentrations~~ and T2D remain unclear (10). On the other hand, the possibility that changes in TMAO just represent an epiphenomenon cannot be excluded.

TMAO and choline concentration changes were linked to risk profiles with altered concentrations of phospholipids and methylation markers, with total or specific LPC concentrations tending to be lower among diabetics (7, 42, 43). LPC may play an important role in glucose homeostasis by stimulating adipocyte glucose uptake, potentiating glucose-stimulated insulin secretion and lowering blood glucose concentrations (43, 44). In our study, Spearman's correlations between baseline metabolite ~~concentrations~~ and 1-y changes in HOMA-IR values indicated a significant negative correlation between ~~C18:1~~ LPC plasmalogen and HOMA-IR ($r = -0.14$, $P = 0.010$). Choline plasmalogens with 18:1 in the *sn*-2 position are strongly correlated with a wide range of risk factors for metabolic syndrome (45). Nonalcoholic fatty liver disease shares common characteristics with metabolic syndrome, such as insulin resistance (46), and has been associated with decreased plasma plasmalogens (47). In our study, higher ~~C18:1~~ LPC plasmalogen plasma ~~concentrations~~ may reflect a decrease in insulin resistance during follow-up and a potential improvement in liver function, resulting in increased secretion of plasmalogens into the circulation. Because the role of LPCs in diabetes pathophysiology is not completed understood, we cannot exclude the possibility that they do not offer any protection against the disease.

Another choline compound, α-glycerophosphocholine, which in our study was found to be associated with a lower risk of T2D, can produce phosphorylcholine and acetylcholine (Ach), a neurotransmitter responsible for initiating skeletal muscle contractions (48). Activation of muscarinic Ach receptors in skeletal muscle cells stimulates glucose uptake via a mechanism independent of the insulin-stimulated pathway (49). Because all muscle movements are related to contraction, and contraction is related to available Ach stores, maximizing Ach may optimize muscular performance and affect insulin sensitivity, ~~GLUT4~~ availability for glucose uptake, etc. Q27

We did not observe any significant difference in the association of most 1-y change metabolites with T2D risk between the MedDiet intervention and control groups. Furthermore, the intervention diets did not appear to significantly change the study metabolite ~~concentrations~~ during the intervention. We found a stronger inverse association only for α-glycerophosphocholine with T2D risk in the MedDiet intervention compared with the control group with a significant test of this interaction. This finding needs further investigation.

The results of the present study should be interpreted in the context of its limitations and strengths. First, participants were elderly Mediterranean individuals at high CVD risk and this may limit the generalizability of the findings to other age groups or populations. Second, even though we adjusted for several potential confounders, residual confounding may exist. With regard to strengths, the prospective evaluation of the association between metabolite ~~concentrations~~ and incident T2D, in the frame of a case-cohort design, minimizes biases that can affect case-control studies.

In conclusion, our study documented, for the first time to our knowledge, a strong inverse prospective association between plasma TMAO ~~concentrations~~ and relevant metabolite profile with incident T2D risk in an elderly population at high CVD risk. Because TMAO is a metabolite that has shown controversial associations with several chronic diseases, these results may be reported in the literature to avoid bias but must be interpreted cautiously and need to be replicated in the future in other populations. The potential mechanisms linking the aforementioned metabolites and diabetes risk must also be further investigated.

The authors' responsibilities were as follows—FBH and JS-S: designed the research; CP, MB, MR-C, MG-F, ET, DC, RE, ER, M Fitó, FA, M Fiol, JL, LS-M, EG-G, and JS-S: conducted the research; DC, RE, M Fitó, FA, M Fiol, JL, LS-M, EG-G, and JS-S: were the coordinators of subject recruitment at the outpatient clinics; CP and MB: analyzed the data; CP, MB, FBH, and JS-S: performed the statistical analysis and interpreted the data; CC: analyzed metabolomics data; CP: drafted the manuscript; FBH and JS-S: supervised the study; CP, MB, and JS-S: had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis; and all authors [illegible]ed the manuscript for important intellectual content and read and approved the final manuscript. The authors declared that they had no conflicts of interest related to this article. Q28

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