Full title: Choline Metabolism and Risk of Atrial Fibrillation and Heart Failure in the PREDIMED Study

Running title: Choline metabolites and cardiac outcomes

Christopher Papandreou,[a,b,c,d] Mònica Bulló,[a,b,c,d] Pablo Hernández-Alonso,[a,b,c,d] Miguel Ruiz-Canela,[c,e,f] Jun Li,[g] Marta Guasch-Ferré,[a,b,g,r] Estefanía Toledo,[c,e,f] Clary Clish,[h] Dolores Corella,[c,i] Ramon Estruch,[c,j] Emilio Ros,[c,k] Montserrat Fitó,[c,l] Angel Alonso-Gómez,[c,m] Miquel Fiol,[c,n] José M. Santos-Lozano,[c,o] Lluís Serra-Majem,[c,p] Liming Liang, [q] Miguel A. Martínez-González,[c,e,f,g] Frank B. Hu,[g,q,r] Jordi Salas-Salvadó,[a,b,c,d]

[a]Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, Unitat de Nutrició, Reus, Spain

[b]Institut d'Investigació Sanitària Pere Virgili (IISPV), Reus, Spain

[c]Centro de Investigación Biomédica en Red Fisiopatologia de la Obesidad y la Nutrición (CIBEROBN), Institut de Salud Carlos III, Madrid, Spain

[d]University Hospital of Sant Joan de Reus, Nutrition Unit, Reus, Spain

[e]University of Navarra, Department of Preventive Medicine and Public Health, Pamplona, Spain

[f]Navarra Institute for Health Research (IdiSNA), Pamplona, Navarra, Spain.

[g]Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, MA, USA

[h]Broad Institute of MIT and Harvard University, Cambridge, MA, USA

[i]Department of Preventive Medicine, University of Valencia, Valencia, Spain

[j]Department of Internal Medicine, Department of Endocrinology and Nutrition Institut d'Investigacions Biomèdiques August Pi Sunyer (IDIBAPS), Hospital Clinic, University of Barcelona, Barcelona, Spain

[k]Lipid Clinic, Department of Endocrinology and Nutrition Institut d'Investigacions Biomèdiques August Pi Sunyer (IDIBAPS), Hospital Clinic, University of Barcelona, Barcelona, Spain

[l]Cardiovascular and Nutrition Research Group, Institut de Recerca Hospital del Mar, Barcelona, Spain

[m]Bioaraba Health Research Institute; Osakidetza Basque Health Service, Araba University Hospital; University of the Basque Country UPV/EHU; Vitoria-Gasteiz, Spain

[n]Institute of Health Sciences IUNICS, University of Balearic Islands and Hospital Son Espases, Palma de Mallorca, Spain

[o]Department of Family Medicine, Distrito Sanitario Atención Primaria Sevilla, San Pablo Health Center, Sevilla, Spain.

[p]Research Institute of Biomedical and Health Sciences IUIBS, University of Las Palmas de Gran Canaria, Las Palmas, Spain

[q]Departments of Epidemiology and Statistics, Harvard T.H. Chan School of Public Health, Boston, MA, USA

[r]Channing Division for Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, MA, USA

Address for correspondence: Christopher Papandreou, PhD and Jordi Salas-Salvadó, MD, PhD, Human Nutrition Unit, Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili, C/Sant Llorenç 21, 43201 Reus, Spain (Phone: +34 977759313, FAX: +34 977759322, Email: papchris10@gmail.com, Email: jordi.salas@urv.cat).

Keywords: Trimethylamine-N-oxide; choline metabolism; atrial fibrillation; heart failure; PREDIMED

List of abbreviations: AF, atrial fibrillation; ECGs, electrocardiograms, HF, heart failure; PREDIMED, Prevención con Dieta Mediterránea; TMAO, trimethylamine-N-oxide.

ABSTRACT

BACKGROUND: Few studies have examined the associations of trimethylamine-N-oxide (TMAO) and its precursors (choline, betaine, dimethylglycine and L-carnitine) with the risk of atrial fibrillation (AF) and heart failure (HF). This study sought to investigate the association of TMAO and its precursors with risk of AF and HF.

METHODS: Prospective associations of these metabolites with incident AF and HF were examined among participants at high cardiovascular risk in the PREDIMED study (PREvención con DIeta MEDiterránea) after follow-up for ≈10 years. Two nested case-control studies were conducted, including 509 AF incident cases matched to 618 controls and 326 HF incident cases matched to 426 controls. Plasma levels of TMAO and its precursors were semi-quantitatively profiled with liquid chromatography tandem mass spectrometry. Odds ratios were estimated with multivariable conditional logistic regression models.

RESULTS: After adjustment for classical risk factors and accounting for multiple testing, participantsin the highest quartile vs. the lowest quartile of baseline choline and betaine levels had a higher risk of AF [OR (95% CI): 1.85 (1.30-2.63) and 1.57 (1.09-2.24), respectively]. The corresponding OR for AF for extreme quartiles of dimethylglycine was 1.39 (0.99-1.96). One SD increase in ln-transformed dimethylglycine was positively associated with AF risk (OR, 1.17; 1.03-1.33). The corresponding ORs for HF for extreme quartiles of choline, betaine and dimethylglycine were 2.51 (1.57-4.03), 1.65 (1.00-2.71) and 1.65 (1.04-2.61), respectively. TMAO and L-carnitine levels were not associated with AF or HF.

CONCLUSION: Our findings support the role of the choline metabolic pathway in the pathogenesis of AF and HF.

Introduction

Atrial fibrillation (AF) and heart failure (HF) represent important emerging cardiovascular epidemics contributing to the increasing cardiovascular disease (CVD) burden and growing health care costs globally (1). The prevalence of AF and HF has risen (1) partially due to the aging of the Western populations and also to increases in shared risk factors such as obesity, type 2 diabetes (T2D), hypertension and dyslipidemia (2). However, these conventional risk factors do not fully explain the variability in AF and HF occurrence, therefore it is important to identify novel risk markers (3). By examining these two outcomes with the same putative markers, a better understanding of their common underlying mechanisms can be acquired (4) in order to develop more effective prevention and treatment strategies. In recent years, there has been growing interest in metabolic impairment as a contributor to AF and HF development and progression. Metabolomics, through a systematic evaluation of small-molecule metabolites in biological samples, may help to identify novel metabolic pathways involved in the development of CVD (5).

Production of trimethylamine-N-oxide (TMAO) via the gut microbiota has recently been proposed as a key pathophysiological mechanism linking intake of animal-derived foods and the development of CVD, coronary heart disease and stroke (6). However, few studies have examined the association of this metabolite and its related metabolic pathways with AF and HF. No previous study has assessed the role of TMAO and its precursors in relation to both outcomes jointly. Previous studies suggested that higher circulating TMAO concentrations were associated with increased risk of AF (7) and HF (8, 9). Recently, higher plasma concentrations of TMAO precursors choline and betaine were reported to be associated with increased risk of AF (10). Furthermore, two previous studies found that elevated plasma betaine

concentrations were independently associated with HF risk (11, 12). Dimethylglycine, the immediate product of betaine, has also been related to increased risk of HF (11), but the relationship with AF has not been examined. In addition, the role of L-carnitine, another precursor of TMAO, in AF and HF remains unknown.

Early identification of metabolites reflecting pathophysiological processes would help to identify individuals who are at risk for the development of AF or HF and could be targeted for preventive measures. It is unclear whether the potential role of TMAO and its precursors can help to elucidate aspects of metabolic dysfunction contributing to AF and HF risk and improve their early detection and diagnosis.Therefore, the aim of the present two prospective case-control studies, nested within the PREDIMED trial, conducted in a Mediterranean population, was to investigate the association of plasma TMAO, choline, betaine, dimethylglycine and L-carnitine with risk of AF and HF.

Materials and Methods

Study design and participants

The current study used two case-control studies nested within the PREDIMED study (www.predimed.es, ISRCTN35739639), a multicenter, single-blind, controlled trial, conducted in Spanish primary health care centers. The detailed methods and design of this study have been described elsewhere (13). The study had a period from October 1, 2003 to December 1, 2010 (median follow-up of 4.8 years) where information about CVD-related outcomes was collected and analyzed (14) and an extended follow-up till December 2017. The primary endpoint of the PREDIMED study was a major cardiovascular event (myocardial infarction, stroke or death from cardiovascular causes. Here we analyzed the AF and HF events (secondary endpoints

of the PREDIMED study) identified during the period 2003- 2017. Five hundred nine incident AF events and 326 incident HF events were ascertained after excluding prevalent cases, and incident cases without available samples (**Supplementary Fig. 1**). Incidence density sampling with replacement was used as the control sampling method (15). The controls were randomly selected among participants at risk at the time of the incidence case occurrence, and selected controls could be selected again as a control for another index case and they could become later a case (15). Controls were matched by age at recruitment (± 5 years), sex and recruitment center. In one of the PREDIMED centers, no cases and controls were selected between 2015 and 2017 because of lack of event information during this extended follow-up period. Following the strategy described above, 2 or 3 controls were selected for overlapping cases (between AF and HF cases) with different event dates to match time at risk for each pair. The number of controls was 618 for AF and 426 for HF cases. There were 108 overlapping cases of AF and HF. The protocol of the PREDIMED trial was approved by the Research Ethics Committees of all participating centers.

Metabolomic profiling

Fasting plasma EDTA samples were collected from subjects and stored at −80 °C. Samples for each participant were randomly ordered and shipped on dry ice to the Broad Institute (Boston, Massachusetts, USA) for metabolomics analyses. Liquid chromatography-tandem mass spectrometrywas used to profile TMAO, choline, betaine, dimethylglycine and L-carnitine (16). 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. Metabolite identities were confirmed using authentic reference standards. Raw data were processed via TraceFinder software (Thermo Fisher Scientific).Internal standard peak areas were

monitored for quality control and to ensure system performance throughout analyses. Pooled plasma reference samples were also inserted every 20 samples as an additional quality control.

Ascertainment of AF and HF Cases

During the first study period 2003 to 2010, information on AF and HF was collected from contacts with participants and primary health care physicians, annual follow-up visits and yearly ad-hoc reviews of medical charts. During the extended follow-up period up to 2017 information on AF and HF was collected by reviewing the medical charts of the participants. Study physicians who were blinded to the intervention groups collected this information. If a clinical diagnosis of AF or HF was made, all relevant documentation, including clinical records of hospital discharge, outpatient clinics and family physicians' records were obtained. The medical charts were labelled only with the study identification number and were sent anonymously to the Clinical End-Point Adjudication Committee. The End-Point Adjudication Committee, chaired by a cardiologist, adjudicated the events according to pre-specified criteria. Two cardiologists independently evaluated the documentation and if they did not agree on the classification of the event, a third cardiologist (the committee's chair) intervened. The diagnostic criteria and procedures have been reported in detail elsewhere (17, 18).

a) AF was initially identified from an annual review of all medical records of each participant and yearly electrocardiograms (ECGs) performed during follow-up examinations. If AF was mentioned anywhere in the medical record or AF was present in the ECG, all relevant documentation was submitted to the Clinical End-point Committee following the procedure explained above

b) HF was defined according to the 2005 (time of study design) guidelines of the European Society of Cardiology on the diagnosis and treatment of acute and chronic HF (19). Based on these guidelines, an event was classified as HF if patients had symptoms and/ or signs of HF (frequent breathlessness or fatigue at rest or during exertion, or ankle swelling) attributable to objective evidence of cardiac dysfunction at rest (preferably by echocardiography). The clinical picture may have a sudden onset or develop progressively.

Covariate Assessment

Information about lifestyle variables, smoking status, medical history and medication use was collected through a questionnaire at baseline. A validated semiquantitative 137-item food frequency questionnaire was used to assess food intake (20). Physical activity was assessed using a validated Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire (21). Participants were considered to have T2D, dyslipidemia, or hypertension if they had previously been diagnosed and/or they were being treated with antidiabetic, cholesterol-lowering, or antihypertensive agents, respectively. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2).

Statistical analyses

Baseline characteristics of AF and HF cases and matched controls are described as means (SDs) for quantitative variables and percentages for categorical variables. Baseline characteristics were compared between cases and controls using Student's t-test for continuous variables and χ^2 tests for categorical variables. We applied a natural logarithmic transformation to approximate a normal distribution of metabolite levels. A correlation (Spearman) matrix of the metabolites under study was visualized

through a heat map (R statistical package version 3.1.1; R Development Core Team, 2011; http://cran.r-project.org).

To investigate the association of the metabolites of interest with AF or HF, we conducted conditional logistic regressions separately using the two different case-control studies, where the outcome was the case/control status of either outcome. A crude model and 2 multivariable-adjusted conditional logistic regression models were fitted as follows: 1) multivariable model 1 adjusted for potential confounders including smoking(never, current, or former), family history of premature coronary heart disease (CHD) (yes or no), physical activity(metabolic equivalent tasks in minutes per day), alcohol intake (g/day), BMI (kg/m^2), intervention group assignments (MedDiet + EVOO, MedDiet + nuts or control interventions), hypertension (yes or no), dyslipidemia (yes or no),and T2D (yes or no) and 2) multivariable model 2 additionally adjusted for medication use (lipid-modifying, antihypertensive, and antidiabetic medications). Metabolites were analyzed as both continuous variables [1-standard deviation (SD) (1-SD) increment in their ln-transformed levels calculated among controls and then applied to all sample] and by using quartiles (with cut-offs defined among controls). To appraise the linear trend across quartiles, the median metabolite concentration within each quartile was included in the conditional logistic regression models as a continuous variable. To account for multiple testing, we adjusted P for trend and P values of the multivariable-adjusted associations between quartiles or 1-SD increments in metabolite level and AF or HF risk with the use of the Benjamini-Hochberg false discovery rate (FDR) procedure (22). A FDR-adjusted P-value < 0.05 was considered to be statistically significant after adjustment for 5 tests corresponding to the 5 metabolites included in the present analyses. We further examined the shape of the association between

metabolites and AF or HF risk nonparametrically by fitting cubic splines to a conditional logistic regression model. Tests for nonlinearity used the likelihood ratio test, comparing the model with only the linear term to the model with the linear and the cubic spline terms.

We additionally conducted stratified analyses by age group (<65 yrs vs. ≥65 yrs), sex (male, female), T2D (yes, no) and obesity status (<30.0, ≥30.0 kg/m^2). Potential effect modification was examined by adding a multiplicative term (1 df) between stratifying variables and metabolites (continuous) into a multivariable unconditional logistic regression to test for interactions by using the likelihood ratio tests. Since this prospective study was conducted in the framework of dietary interventions, possible interactions of each metabolite with the intervention groups (MedDiet+EVOO and MedDiet+nuts vs. control group) was evaluated using the likelihood ratio test.

To test the robustness of the associations of the metabolites with the risk of AF and HF, we conducted 2 sensitivity analyses: 1) using unconditional logistic regressions to examine whether the associations of metabolites with AF and HF were modified by matching factors such as age, sex, and center. For these analyses, we used the aforementioned multivariable model 2 additionally adjusted for age, sex and center, and 2) further adjusting the multivariable model 2 for dietary sources of TMAO and its precursors (23) such as meat, fish, eggs, bread, cereals, dairy and legumes.

Finally, we applied multivariable linear regression analyses to examine cross-sectional relations of TMAO and its precursors with lipids [total cholesterol (chol), low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol

(HDL-C), triacylglycerols (TAG)], blood glucose, BMI and leukocyte count. Models were adjusted for age, sex, center, smoking, physical activity, alcohol intake, BMI, dyslipidemia, hypertension, and T2D. Lipid-modifying medication or anti-diabetic medication was also included as confounder for lipids or blood glucose, respectively. Cholesterol, LDL-C, HDL-C, TAG and glucose levels were natural logarithmically transformed to normalize their distributions, while leukocyte counts were log transformed. Statistical analyses were performed using Stata 14.1 (Stata Corp.).

Results

The follow-up of the study population was ≈10 years. The baseline characteristics of the 1879 subjects (509 AF cases and 618 controls, 326 HF cases and 426 controls) included in the present study are shown in Table 1. Compared with the controls, participants who developed AF and HF were more likely to have a higher BMI and higher prevalence of hypertension (**Table 1**). Participants with incident AF were also more likely to use antihypertensive medication, whereas those participants with incident HF were more likely to have a higher prevalence of T2D and use oral antidiabetic agents. Heatmaps of Spearman correlation coefficients of the plasma metabolites analyzed are shown in Supplementary Fig. 2. Betaine was moderately correlated with choline (r = 0.45) and dimethylglycine (r = 0.42) in the AF case-control study. Moderate correlations were also observed between betaine and choline (r = 0.46) in the HF case-control study. TMAO was weakly correlated with its precursors.

Plasma metabolites and risk of AF

The associations between plasma metabolites with AF risk are presented in **Table 2**. In the fully adjusted model, the estimated OR for incident AF reached significance

only in the highest, compared with the lowest, quartile of plasma levels of choline, and betaine [1.85 (95% CI, 1.30–2.63, P for $trend_{AdjFDR}$ =0.005) and 1.57 (95% CI, 1.09–2.24, P for $trend_{AdjFDR}$ =0.022), respectively]. One SD increment in levels of choline betaine and dimethylglycine was significantly associated with higher risk of AF incidence (OR=1.23, 95% CI, 1.08–1.40, OR=1.19, 95% CI, 1.05–1.35, and OR=1.17, 95% CI, 1.03–1.33, respectively). After adjustment for multiple testing, these associations remained significant. Cubic spline curves (**Supplementary Fig. 3**) showed that these metabolites were nonlinearly associated with AF risk (P value for nonlinearity with AF risk was 0.001, 0.020, and 0.028 for choline, betaine and dimethylglycine, respectively). No significant associations of TMAO and L-carnitine with AF were observed.

Plasma metabolites and risk of HF

Associations between plasma metabolite levels and the HF risk are shown in **Table 3**. Comparing the highest quartile of choline, betaine and dimethylglycine levels, significant positive associations with HF risk were found for choline [OR=2.51, (95% CI, 1.57–4.03, P for $trend_{AdjFDR}$ =0.005), for betaine, OR=1.65 (95% CI, 1.00–2.71, P for $trend_{AdjFDR}$ =0.045), and for dimethylglycine, OR=1.65 (95% CI, 1.04–2.61, P for $trend_{AdjFDR}$ =0.040), respectively]. After adjustment for multiple testing, the risk of HF significantly increased per 1-SD increase in choline and dimethylglycine levels (OR=1.42, 95% CI, 1.20–1.67 and OR=1.25, 95% CI, 1.07–1.46, respectively). Restricted cubic spline analysis (**Supplementary Fig. 4**) suggested nonlinear associations of choline and dimethylglycine with HF risk (P value for nonlinearity with HF risk of choline was <0.001, and 0.018 for dimethylgycine). Betaine was linearly associated with HF. Similarly than for AF, no significant associations of TMAO and L-carnitine with HF were observed.

Analyses adjusting for matching factors also yielded consistent results for both clinical outcomes (**Supplementary Tables 1 and 2**).After further adjustment for food group intake the associations between choline, betaine, dimethylglycine and AF and HF remained significant (**Supplementary Tables 3 and 4**). Furthermore, adjusting for incident CVD cases did not substantially affect these associations (data not shown). Excluding 45 participants with incident AF diagnosed before the development of HF did not noticeably change the results (data not shown).

Using TMAO, L-carnitine, choline, betaine, and dimethylglycine as the primary exposures, we observed no significant effect modification by age, sex, T2D, or obesity status. Similarly, the interactions between the MedDiet+EVOO or MedDiet+nuts interventions and the metabolites were not significant.

Metabolites in relation to lipids, glucose, BMI and leukocyte count

In the AF case-control study, choline was directly associated with BMI (β=0.43) and leukocyte count (β=0.007), whereas betaine was inversely associated with LDL-C cholesterol (β=-0.04), TAG (β=-0.10) and blood glucose (β=-0.03) (**Supplementary Table 7**). Dimethylglycine was positively associated with leukocyte count (β=0.006) (**Supplementary Table 7**). Similarly, in the HF case-control study, choline was positively associated with BMI (β=0.38) but not leukocyte count (**Supplementary Table 8**). Betaine was inversely associated with TAG (β=-0.06), glucose (β=-0.03) and BMI (β=-0.45) (**Supplementary Table 8**).

Discussion

Using two prospective case-control studies nested within the PREDIMED study cohort, we observed that baseline plasma choline, betaine, and dimethylglycine, but

not TMAO and L-carnitine levels were independently associated with increased risk of both incident AF and HF after 10 years of follow-up. Most of these associations were nonlinear, such that the associations with AF and HF were more pronounced among individuals with higher levels of the metabolites, while the magnitude of these associations was stronger for choline. These associations underscore the complexity of the pathophysiology concerning these two cardiac outcomes (24) that are only partially explained by perturbations in the choline metabolic pathway (25).

Circulating choline levels have been associated with an unfavourable cardiovascular risk profile (26) and CVD incidence (11). In fact, our group has recently reported positive associations between plasma choline and risk of major cardiovascular events (myocardial infarction, stroke, and CVD death) in the PREDIMED cohort (27). However, few studies have analysed the associations between circulating levels of this metabolite and AF. In a recent cross-sectional study of 49 AF patients, plasma choline concentrations were higher than in their non-AF counterparts (28). Results from 3 prospective cohort studies are also consistent with our findings with respect to the association between plasma choline and AF incidence (9). A previous study also reported elevated plasma choline levels in HF patients (8). In our cross-sectional analysis, we also observed an association between this metabolite and specific cardiovascular risk factors such as BMI and leukocytes that support our hypothesis.

Regarding betaine, the downstream metabolite of choline oxidation, it was previously found to be associated with both AF (10) and HF (11, 12), which is in agreement with our findings. Interestingly, in our study betaine was not associated with any unfavourable cardiometabolic risk profile. Our findings concerning the positive relationship between dimethylglycine, the catabolic product of betaine, and

AF are novel. Although unexplored in relation to AF, higher concentrations of this metabolite were previously found to be associated with HF incidence in patients with coronary artery disease (11).

On the other hand, we did not observe any significant association of TMAO and L-carnitine with AF or HF. In a similar way, our previous analyses did not support significant associations between TMAO and overall CVD incidence (28). While data evaluating the association between TMAO and AF are sparse, one study including two Norwegian cohorts has shown that plasma levels of TMAO are associated with increased risk of incident AF (7). Elevated plasma levels of TMAO have also been reported in HF patients compared to control individuals (8, 9). A positive association of high plasma TMAO and L-carnitine levels with CVD risk has been previously reported among individuals undergoing cardiac evaluation (29) However, it was recently suggested that L-carnitine might have favourable effects in HF patients (30).

Mitochondrial dysfunction may play a significant role in the mechanisms leading to AF and HF (31). The associations of choline, betaine and dimethylglycine with AF and HF involve the presence of metabolic interactions affecting both processes. This notion is further supported by the fact that: 1) the correlations between food such as meat (source of choline, dimethylglycine), fish (source of choline), eggs (source of choline), bread/cereals (source of choline, betaine) (23) and the metabolites under investigation were weak or absent (**Supplementary Tables 5 and 6)**; 2) the associations between these metabolites and the two cardiac outcomes were independent of food intake (**Supplementary Tables 3 and 4**); 3) there was not significant interaction between the dietary interventions and these metabolites, suggesting that the aforementioned associations are more likely related to metabolic pathway disruptions instead of dietary intakes. Choline can be metabolized to betaine

and to further downstream metabolites such as dimethylglycine by mitochondrial pathways in the liver and kidney (25). A disruption of mitochondrial choline oxidation to betaine as part of mitochondrial dysfunction may precede the development of AF and HF. Experimental data in animal models suggest a causal link between increased mitochondrial oxidative stress and AF (32), and HF (33). It is worth mentioning that in our study higher choline levels were associated with an increased number of circulating leukocytes. Oxidative stress and inflammation may impair betaine-homocysteine methyltransferase (BHMT) activity, resulting in accumulation of circulating choline and betaine (34). A growing body of evidence links oxidative stress and inflammation to adverse atrial structural and electrical remodelling that leads to AF development and maintenance (35). Oxidative stress and inflammation are also involved in cardiac muscle dysfunction and in the onset of HF (36).

Dimethylglycine, which is formed from betaine during the remethylation of homocysteine to methionine by BHMT (25), was also associated with leukocytes in our study. Dimethylglycine is a feedback inhibitor of BHMT and is normally excreted in urine or metabolized to sarcosine (25). Dysregulation in dimethylglycine metabolism may lead to increased levels in plasma, which would inhibit BHMT activity possibly causing elevation in homocysteine concentrations (37). Elevated homocysteine has been associated with an increased risk of AF (38) and HF (39).

Our study has several limitations. First, participants were older Mediterranean individuals at high cardiovascular risk, which limits the generalizability of our findings to other populations. Second, although we adjusted for several potential confounders, residual confounding cannot be ruled out especially considering the complexity of metabolomic profiling. Third, we used a single measure of metabolites at baseline. A single measurement relies on the assumption that metabolite levels vary

little over a medium-term period. Using the same LC-MS platform as in the present study, Townsend et al. (40) demonstrated that these metabolites can be accurately measured in conditions mimicking those of prospective epidemiologic studies. Fourth, even though the AF diagnosis was relied on both ECG and clinical records, underreporting of incident AF cases cannot be discarded.

In conclusion, the results of two nested case-control studies using prospectively identified cases suggest that among older Mediterranean adults at high cardiovascular risk, higher baseline levels of choline, betaine and dimethylglycine are associated with an increased risk of both AF and HF. These findings underscore the potential role of the choline metabolic pathway in the pathogenesis of AF and HF that are likely to share common pathophysiologic pathways. Further studies are needed to investigate potential mechanisms linking these metabolites to the genesis of AF and HF.

Author contributions: FH, JS-S and MM-G designed research; CP, MB, PH-A, MR-C, JL, MG-F, ET, CC, DC, RE, ER, MF, FA, MFiol, JL, LS-M, LL, MM-G, FH and JS-S conducted research; DC, RE, ER, MF, FA, MFiol, LS-M, MM-G and JS-S were the coordinators of subject recruitment at the outpatient clinics; CP and JS-S analyzed the data; CP, MB, FBH and JS-S interpreted statistical analysis and data; CC acquired and processed metabolomics data; CP drafted the paper; FH, JS-S and MM-G supervised the study and MB and JS-S took the responsibility for the integrity of the data and the accuracy of the data analysis. All authors revised the manuscript for important intellectual content, read and approved the final manuscript.

Disclosures: ER has received grants for research through his institution from the California Walnut Commission (CWC), as well as fees for lectures and participation in the CWC's Health Research Advisory Group, and support for travel and accommodation. He is also a non-paid member of the Scientific Advisory Council of the CWC. He has also received honoraria for presentations and support for travel and accommodation from Danone. JS-S is a non-paid member of the Scientific Committee of the International Nut and Dried Fruit Foundation. He has received grants/research support from the American Pistachio Growers and International Nut and Dried Fruit Foundation through his Institution. He has received honoraria from Nuts for Life, Danone and Eroski. He reports personal fees from Danone. He is a member of the executive committee of Instituto Danone Spain. None of the other co-authors have potential conflicts of interest to disclose relevant to the subject matter or materials included in this work.

Funding: This study was funded by the National Institutes of Health (R01HL118264), the Spanish Ministry of Health (Instituto de Salud Carlos III) and the Ministerio de Economía y Competitividad-Fondo Europeo de Desarrollo Regional

(Projects CNIC-06/2007, RTIC G03/140, CIBER 06/03, PI06-1326, PI07-0954, PI11/02505, SAF2016-80532, SAF2009-12304 and AGL2010-22319-C03-03) and by the Generalitat Valenciana (PROMETEO 17/2017, ACOMP2010-181, AP111/10, AP-042/11, ACOM2011/145, ACOMP/2012/190, ACOMP/2013/159 and ACOMP/213/165). Dr. CP is recipient of the Instituto de Salud Carlos III Miguel Servet fellowship (grant CP 19/00189). Dr. PH-A is supported by a postdoctoral fellowship (Juan de la Cierva-Formación, FJCI-2017-32205). Dr. MG-F was supported by American Diabetes Association grant #1-18-PMF-029. Professor JS-S, senior author of this work is partially supported by ICREA under the ICREA Academia programme.

References

1. Braunwald E. Shattuck lecture--cardiovascular medicine at the turn of the millennium: triumphs, concerns, and opportunities. N Engl J Med 1997;337:1360-9.
2. Lee Park K, Anter E. Atrial Fibrillation and Heart Failure: A Review of the Intersection of Two Cardiac Epidemics. J Atr Fibrillation 2013;6:751.
3. Schnabel RB, Rienstra M, Sullivan LM, Sun JX, Moser CB, Levy D, et al. Risk assessment for incident heart failure in individuals with atrial fibrillation. Eur J Heart Fail 2013;15:843-9.
4. Khan MA, Satchithananda DK, Mamas MA. The importance of interactions between atrial fibrillation and heart failure. Clin Med (Lond) 2016;16:272-26.
5. Barish R, Lynce F, Unger K, Barac A. Management of Cardiovascular Disease in Women With Breast Cancer. Circulation 2019;139:1110-20.

6. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 2011;472:57-63.
7. Svingen GFT, Zuo H, Ueland PM, Seifert R, Løland KH, Pedersen ER, et al. Increased plasma trimethylamine-N-oxide is associated with incident atrial fibrillation. Int J Cardiol 2018;267:100-6.
8. Trøseid M, Ueland T, Hov JR, Svardal A, Gregersen I, Dahl CP, et al. Microbiota-dependent metabolite trimethylamine-N-oxide is associated with disease severity and survival of patients with chronic heart failure. J Intern Med 2015;277:717-26.
9. Tang WH, Wang Z, Fan Y, Levison B, Hazen JE, Donahue LM, et al. 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.
10. Zuo H, Svingen GFT, Tell GS, Ueland PM, Vollset SE, Pedersen ER, et al. Plasma Concentrations and Dietary Intakes of Choline and Betaine in Association With Atrial Fibrillation Risk: Results From 3 Prospective Cohorts With Different Health Profiles. J Am Heart Assoc 2018;7:e008190.
11. Lever M, George PM, Elmslie JL, Atkinson W, Slow S, Molyneux SL, et al. Betaine and secondary events in an acute coronary syndrome cohort. PLoS One 2012;7:e37883.
12. Lever M, George PM, Slow S, Bellamy D, Young JM, Ho M, et al. Betaine and Trimethylamine-N-Oxide as Predictors of Cardiovascular Outcomes Show Different Patterns in Diabetes Mellitus: An Observational Study. PLoS One 2014;9:e114969.

13. Martínez-González MÁ, Corella D, Salas-Salvadó J, Ros E, Covas MI, Fiol M, et al. Cohort profile: design and methods ofthe PREDIMED study. Int J Epidemiol 2012;41:377-85.
14. Estruch R, Ros E, Salas-Salvadó J, Covas MI, Corella D, Arós F, et al. Primary Prevention of Cardiovascular Disease with a Mediterranean Diet Supplemented with Extra-Virgin Olive Oil or Nuts. N Engl J Med 2018;378:e34.
15. Wang M, Shugart YY, Cole SR, Platz EA. A Simulation Study of Control Sampling Methods for Nested Case-Control Studies of Genetic and Molecular Biomarkers and Prostate Cancer Progression. Cancer Epidemiol Biomarkers Prev 2009;18:706-711.
16. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, et al. Metabolite profiles and the risk of developing diabetes. Nat Med 2011;17:448-53.
17. Papadaki A, Martínez-González MÁ, Alonso-Gómez A, Alonso-Gómez A, Rekondo J, Salas-Salvadó J, et al. Mediterranean diet and risk of heart failure: results from the PREDIMED randomized controlled trial Eur J Heart Fail 2017;19:1179-85.
18. Martínez-González MÁ, Toledo E, Arós F, Fiol M, Corella D, Salas-Salvadó J, et al. Extra virgin olive oil consumption reduces risk of atrial fibrillation: the PREDIMED (Prevención con Dieta Mediterránea) trial. Circulation 2014;130:18-26.
19. Swedberg K, Cleland J, Dargie H, Drexler H, Follath F, Komajda M, et al; Task Force for the Diagnosis and Treatment of Chronic Heart Failure of the European Society of Cardiology. Guidelines for the diagnosis and treatment of

chronic heart failure: executive summary (update 2005): The Task Force for the Diagnosis and Treatment of Chronic Heart Failure of the European Society of Cardiology. Eur Heart J 2005;26:1115-40.

20. Fernández-Ballart JD, Piñol JL, Zazpe I, Corella D, Carrasco P, Toledo E, et al. Relative validity of a semi-quantitative food-frequency questionnaire in an elderly Mediterranean population of Spain. Br J Nutr 2010;103:1808-16.
21. Elosua R, Marrugat J, Molina L, Pons S, Pujol E. Validation of the Minnesota Leisure Time Physical Activity Questionnaire in Spanish men. The MARATHOM Investigators. Am J Epidemiol 1994;139:1197-209.
22. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J R Stat Soc Ser B Methodol 1995;57:289-300.
23. Papandreou C, Moré M, Bellamine A. Trimethylamine N-Oxide in Relation to Cardiometabolic Health-Cause or Effect? Nutrients 2020;12(5):1330.
24. Lee Park K, Anter E. Atrial Fibrillation and Heart Failure: A Review of the Intersection of Two Cardiac Epidemics. J Atr Fibrillation 2013;6(1):751.
25. Holm PI, Ueland PM, Kvalheim G, Lien EA. Determination of choline, betaine, and dimethylglycine in plasma by a high-throughput method based on normal-phase chromatography-tandem mass spectrometry. Clin Chem 2003;49(2):286-94.
26. Konstantinova SV, Tell GS, Vollset SE, Nygård O, Bleie Ø, Ueland PM. Divergent associations of plasma choline and betaine with components of metabolic syndrome in middle age and elderly men and women. J Nutr 2008; 138:914-20.

27. Guasch-Ferré M, Hu FB, Ruiz-Canela M, Bulló M, Toledo E, Wang DD, 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.
28. Lai S, Hua X, Gao R, Zeng L, Song J, Liu J, et al. Combinational Biomarkers for Atrial Fibrillation Derived from Atrial Appendage and Plasma Metabolomics Analysis. Sci Rep 2018;8:16930.
29. Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat Med 2013;19:576-85.
30. Wang ZY, Liu YY, Liu GH, Lu HB, Mao CY. l-Carnitine and heart disease. Life Sci 2018;194:88-97.
31. Ozcan C, Li Z, Kim G, Jeevanandam V, Uriel N. Molecular Mechanism of the Association Between Atrial Fibrillation and Heart Failure Includes Energy Metabolic Dysregulation Due to Mitochondrial Dysfunction. J Card Fail 2019;25:911-20.
32. Xie W, Santulli G, Reiken SR, Yuan Q, Osborne BW, Chen BX, et al. Mitochondrial oxidative stress promotes atrial fibrillation. Sci Rep 2015;5:11427.
33. Ide T, Tsutsui H, Kinugawa S, Suematsu N, Hayashidani S, Ichikawa K, et al. Direct evidence for increased hydroxyl radicals originating from superoxide in the failing myocardium. Circ Res 2000;86:152-7.
34. Martínez-Vega R, Garrido F, Partearroyo T, Cediel R, Zeisel SH, Martínez-Álvarez C, et al. Folic acid deficiency induces premature hearing loss through

mechanisms involving cochlear oxidative stress and impairment of homocysteine metabolism. FASEB J 2015;29:418-32.

35. Karam BS, Chavez-Moreno A, Koh W, Akar JG, Akar FG. Oxidative stress and inflammation as central mediators of atrial fibrillation in obesity and diabetes. Cardiovasc Diabetol 2017;16:120.
36. Mann DL. Inflammatory mediators and the failing heart: past, present, and the foreseeable future. Circ Res 2002;91:988-98.
37. McGregor DO, Dellow WJ, Lever M, George PM, Robson RA, Chambers ST. Dimethylglycine accumulates in uremia and predicts elevated plasma homocysteine concentrations. Kidney Int 2001;59:2267-72.
38. Kubota Y, Alonso A, Heckbert SR, Norby FL, Folsom AR. Homocysteine and Incident Atrial Fibrillation: The Atherosclerosis Risk in Communities Study and the Multi-Ethnic Study of Atherosclerosis. Heart Lung Circ 2019;28:615-22.
39. Vasan RS, Beiser A, D'Agostino RB, Levy D, Selhub J, Jacques PF, et al. Plasma homocysteine and risk for congestive heart failure in adults without prior myocardial infarction. JAMA 2003;289:1251-7.
40. Townsend MK, Clish CB, Kraft P, Wu C, Souza AL, Deik AA, et al. Reproducibility of metabolomic profiles among men and women in 2 large cohort studies. Clin Chem 2013;59:1657-67.

Table 1. Baseline characteristics of the study population.

	AF Cases	Controls	HF Cases	Controls
n	509	618	326	426
Age (years)	68.2 (6.1)	68.5 (6.1)	70.3 (5.8)	70.4 (5.9)
Sex (% Women)	49.7	49.2	58.3	54.2
Body mass index, kg/m^2	30.7 (3.8)	29.8 (3.8)*	31.1 (3.8)	29.4 (3.6)*
Physical activity, MET-min/week	226 (209)	232 (217)	217 (196)	215 (217)
Intervention group, %				
MedDiet+EVOO	31.4	36.4	30.9	37.6
MedDiet+Nuts	31.4	28.6	32.5	26.5
Control group	37.0	34.9	36.5	35.9
Alcohol intake (g/day)	8.9 (13.3)	9.7 (15.0)	7.9 (14.6)	8.1 (12.1)
Family history of premature CHD, %	19.1	20.1	19.3	19.2
Type 2 diabetes, %	47.9	49.8	59.5	52.1*
Hypertension, %	88.4	82.8*	87.4	82.2*
Dyslipidemia, %	65.2	68.4	64.1	69.0
Antihypertensive medication, (%)	79.5	72.5*	76.4	75.1
Oral antidiabetic agents, (%)	30.8	31.4	40.5	32.6*
Insulin medication, (%)	7.3	7.4	10.4	7.9
Lipid-lowering medication, (%)	36.4	35.4	37.1	38.5
Smoking, %				
Never	58.7	57.9	59.8	61.3
Former	26.9	28.8	25.8	27.5
Current	14.3	13.3	14.4	11.3

Data are presented as mean (SD)or percentage.The x^2 test was used for comparison of categorical variables and Student's t-testwas used for comparison of continuous variables. AF, atrial fibrillation; HF, heart failure; CHD, coronary heart disease; MedDiet, Mediterranean diet; EVOO, extra-virgin olive oil; MET, metabolic equivalent.

*P value<0.05

There were 108 overlapping cases of AF and HF.

Table 2. Associations of baseline individual metabolites levels with the risk of incident atrial fibrillation in a nested case-control study of the PREDIMED Trial[1].

		Quartiles of plasma metabolite levels					
Metabolite	Q1	Q2	Q3	Q4	Ptrend[2]	OR per 1 SD increment	P value[2]
Trimethylamine N-oxide							
Cases	122	133	134	120			
Crudemodel	Ref.	1.09 (0.79-1.51)	1.16 (0.83-1.61)	1.06 (0.76-1.48)	0.966	1.05 (0.94-1.18)	0.377
MV1	Ref.	1.06 (0.76-1.48)	1.07 (0.76-1.51)	1.02 (0.72-1.44)	0.933	1.04 (0.92-1.17)	0.548
MV2	Ref.	1.06 (0.76-1.48)	1.05 (0.74-1.46)	1.02 (0.72-1.44)	0.955	1.04 (0.92-1.17)	0.552
L-carnitine							
Cases	114	105	139	151			
Crudemodel	Ref.	0.95 (0.67-1.33)	1.23 (0.88-1.70)	1.39 (1.00-1.94)	0.028	1.14 (1.01-1.28)	0.041
MV1	Ref.	0.90 (0.64-1.28)	1.18 (0.83-1.66)	1.32 (0.93-1.87)	0.068	1.10 (0.97-1.25)	0.155
MV2	Ref.	0.90 (0.63-1.28)	1.17 (0.83-1.65)	1.30 (0.92-1.86)	0.078	1.10 (0.97-1.25)	0.172
Choline							
Cases	100	115	115	179			
Crudemodel	Ref.	1.13 (0.79-1.62)	1.21 (0.85-1.71)	1.96 (1.39-2.75)	0.005	1.26 (1.11-1.43)	0.005
MV1	Ref.	1.12 (0.77-1.62)	1.17 (0.82-1.67)	1.86 (1.31-2.64)	0.005	1.24 (1.09-1.41)	0.005
MV2	Ref.	1.12 (0.77-1.62)	1.16 (0.81-1.66)	1.85 (1.30-2.63)	0.005	1.23 (1.08-1.40)	0.005
Betaine							
Cases	113	120	126	150			
Crudemodel	Ref.	1.06 (0.75-1.51)	1.15 (0.82-1.61)	1.43 (1.02-1.99)	0.035	1.15 (1.02-1.30)	0.031
MV1	Ref.	1.11 (0.77-1.61)	1.29 (0.90-1.83)	1.58 (1.11-2.26)	0.017	1.19 (1.05-1.35)	0.015
MV2	Ref.	1.12 (0.78-1.63)	1.31 (0.92-1.88)	1.57 (1.09-2.24)	0.022	1.19 (1.05-1.35)	0.017
Dimethylglycine							
Cases	115	106	122	166			
Crudemodel	Ref.	0.96 (0.68-1.35)	1.13 (0.80-1.59)	1.50 (1.09-2.07)	0.010	1.21 (1.07-1.36)	0.005
MV1	Ref.	0.95 (0.67-1.36)	1.12 (0.78-1.60)	1.42 (1.01-1.99)	0.026	1.18 (1.04-1.33)	0.015
MV2	Ref.	0.95 (0.67-1.36)	1.11 (0.78-1.60)	1.39 (0.99-1.96)	0.038	1.17 (1.03-1.33)	0.023

[1]Values are OR (95% CI). A natural logarithmic transformation was applied to the raw values of individual metabolites. Conditional logistic regression analysis was used. MV1 adjusted for smoking, family history of premature coronary heart disease, physical activity, alcohol intake, BMI (kg/m^2), intervention group (MedDiet + EVOO or MedDiet + nuts), dyslipidemia, hypertension and type 2 diabetes; MV2 additionally adjusted for medication use (lipid-modifying, antihypertensive, and antidiabetic medication). Abbreviations: Ref, reference.

[2]Adjusted with the Benjamini-Hochberg False Discovery Rate method.

Case and control subjects were matched on age, sex and recruitment center.

Table 3. Associations of baseline individual metabolites levels with the risk of incident heart failure in a nested case-control study of the PREDIMED Trial[1].

		Quartiles of plasma metabolite levels					
Metabolite	Q1	Q2	Q3	Q4	Ptrend[2]	OR per 1 SD increment	P value[2]
Trimethylamine N-oxide							
Cases	87	70	97	72			
Crudemodel	Ref.	0.84 (0.56-1.27)	1.16 (0.78-1.73)	0.87 (0.57-1.33)	0.825	0.97 (0.83-1.12)	0.814
MV1	Ref.	0.72 (0.46-1.14)	0.97 (0.63-1.19)	0.75 (0.48-1.19)	0.419	0.93 (0.79-1.09)	0.455
MV2	Ref.	0.71 (0.44-1.12)	0.99 (0.64-1.56)	0.72 (0.45-1.15)	0.324	0.91 (0.77-1.08)	0.350
L-carnitine							
Cases	82	92	76	76			
Crudemodel	Ref.	1.11 (0.75-1.65)	0.90 (0.60-1.36)	0.95 (0.63-1.43)	0.825	0.94 (0.81-1.08)	0.650
MV1	Ref.	1.26 (0.82-1.92)	1.10 (0.69-1.75)	1.26 (0.79-2.02)	0.419	1.04 (0.88-1.22)	0.626
MV2	Ref.	1.26 (0.82-1.93)	1.20 (0.75-1.93)	1.34 (0.83-2.16)	0.324	1.07 (0.91-1.27)	0.387
Choline							
Cases	60	81	59	126			
Crudemodel	Ref.	1.40 (0.91-2.14)	1.10 (0.70-1.73)	2.47 (1.61-3.78)	0.005	1.41 (1.20-1.66)	0.005
MV1	Ref.	1.28 (0.80-2.03)	1.04 (0.64-1.69)	2.52 (1.58-4.04)	0.005	1.43 (1.20-1.69)	0.005
MV2	Ref.	1.24 (0.77-1.98)	1.04 (0.64-1.71)	2.51 (1.57-4.03)	0.005	1.44 (1.21-1.71)	0.005
Betaine							
Cases	81	78	90	77			
Crudemodel	Ref.	1.00 (0.66-1.51)	1.13 (0.74-1.72)	1.02 (0.66-1.58)	0.839	1.02 (0.87-1.19)	0.814
MV1	Ref.	1.07 (0.68-1.68)	1.58 (0.98-2.52)	1.59 (0.97-2.60)	0.083	1.18 (0.99-1.40)	0.116
MV2	Ref.	1.07 (0.68-1.69)	1.59 (0.98-2.58)	1.65 (1.00-2.71)	0.045	1.19 (0.99-1.42)	0.096
Dimethylglycine							
Cases	84	68	66	108			
Crudemodel	Ref.	0.87 (0.59-1.30)	0.92 (0.60-1.39)	1.42 (0.94-2.12)	0.100	1.21 (1.04-1.41)	0.037
MV1	Ref.	0.89 (0.58-1.38)	0.93 (0.59-1.46)	1.45 (0.93-2.27)	0.083	1.23 (1.04-1.45)	0.042
MV2	Ref.	0.97 (0.62-1.50)	0.97 (0.61-1.53)	1.65 (1.04-2.61)	0.040	1.27 (1.07-1.51)	0.015

[1]Values are OR (95% CI). A natural logarithmic transformation was applied to the raw values of individual metabolites. Conditional logistic regression analysis was used. MV1 adjusted for smoking, family history of premature coronary heart disease, physical activity, alcohol intake, BMI (kg/m^2), intervention group (MedDiet + EVOO or MedDiet + nuts), dyslipidemia, hypertension and type 2 diabetes; MV2 additionally adjusted for medication use (lipid-modifying, antihypertensive, and antidiabetic medication). Abbreviations: Ref, reference.

[2]Adjusted with the Benjamini-Hochberg False Discovery Rate method.

Case and control subjects were matched on age, sex and recruitment center.

Supplementary Table 1. Associations of baseline individual metabolites levels with the risk of atrial fibrillation after further adjustment of the multivariable model for the matching factors in a nested case-control study of the PREDIMED Trial[1].

		Quartiles of plasma metabolite levels					
Metabolite	Q1	Q2	Q3	Q4	P trend[2]	OR per 1 SD increment	P value[2]
Trimethylamine N-oxide							
Cases	122	133	134	120			
MV	Ref.	1.06 (0.76-1.48)	1.04 (0.75-1.45)	0.96 (0.69-1.35)	0.658	1.02 (0.91-1.15)	0.730
L-carnitine							
Cases	114	105	139	151			
MV	Ref.	0.89 (0.63-1.26)	1.15 (0.82-1.61)	1.24 (0.88-1.75)	0.122	1.08 (0.96-1.22)	0.227
Choline							
Cases	100	115	115	179			
MV	Ref.	1.21 (0.85-1.71)	1.17 (0.82-1.66)	1.80 (1.29-2.51)	0.005	1.20 (1.07-1.35)	0.010
Betaine							
Cases	113	120	126	150			
MV	Ref.	1.08 (0.77-1.51)	1.20 (0.85-1.68)	1.43 (1.02-2.01)	0.050	1.15 (1.02-1.29)	0.045
Dimethylglycine							
Cases	115	106	122	166			
MV	Ref.	0.94 (0.67-1.33)	1.02 (0.72-1.43)	1.39 (1.00-1.95)	0.047	1.15 (1.03-1.29)	0.040

[1]Values are OR (95% CI). A natural logarithmic transformation was applied to the raw values of individual metabolites. Unconditional logistic regression analysis was used. MV adjusted for age, sex, smoking, family history of premature coronary heart disease, physical activity, alcohol intake, BMI (kg/m^2), intervention group (MedDiet + EVOO or MedDiet + nuts), dyslipidemia, hypertension, type 2 diabetes, medication use (lipid-modifying, antihypertensive, and antidiabetic medication) and center. Abbreviations: Ref, reference.

[2]Adjusted with the Benjamini-Hochberg False Discovery Rate method.

Supplementary Table 2. Associations of baseline individual metabolites levels with the risk of heart failure after further adjustment of the multivariable model for the matching factors in a nested case-control study of the PREDIMED Trial[1].

		Quartiles of plasma metabolite levels					
Metabolite	Q1	Q2	Q3	Q4	P trend[2]	OR per 1 SD increment	P value[2]
Trimethylamine N-oxide							
Cases	87	70	97	72			
MV	Ref.	0.74 (0.48-1.13)	0.97 (0.65-1.46)	0.78 (0.51-1.20)	0.489	0.94 (0.81-1.09)	0.510
L-carnitine							
Cases	82	92	76	76			
MV	Ref.	1.20 (0.79-1.81)	1.09 (0.71-1.69)	1.21 (0.78-1.88)	0.489	1.00 (0.87-1.16)	0.940
Choline							
Cases	60	81	59	126			
MV	Ref.	1.26 (0.81-1.96)	1.03 (0.65-1.63)	2.18 (1.42-3.36)	0.005	1.33 (1.15-1.54)	0.005
Betaine							
Cases	81	78	90	77			
MV	Ref.	1.02 (0.66-1.56)	1.47 (0.96-2.26)	1.44 (0.91-2.26)	0.096	1.15 (0.98-1.36)	0.136
Dimethylglycine							
Cases	84	68	66	108			
MV	Ref.	0.87 (0.56-1.34)	0.91 (0.59-1.42)	1.61 (1.06-2.46)	0.015	1.23 (1.07-1.43)	0.010

[1]Values are OR (95% CI). A natural logarithmic transformation was applied to the raw values of individual metabolites. Unconditional logistic regression analysis was used. MV adjusted for age, sex, smoking, family history of premature coronary heart disease, physical activity, alcohol intake, BMI (kg/m^2), intervention group (MedDiet + EVOO or MedDiet + nuts), dyslipidemia, hypertension, type 2 diabetes, medication use (lipid-modifying, antihypertensive, and antidiabetic medication) and center. Abbreviations: Ref, reference.

[2]Adjusted with the Benjamini-Hochberg False Discovery Rate method.

Supplementary Table 3. Associations of baseline individual metabolites levels with the risk of atrial fibrillation after further adjustment of the multivariable model for dietary sources of TMAO and its precursors in a nested case-control study of the PREDIMED Trial[1].

		Quartiles of plasma metabolite levels					
Metabolite	Q1	Q2	Q3	Q4	P trend[2]	OR per 1 SD increment	P value[2]
Trimethylamine N-oxide							
Cases	122	133	134	120			
MV	Ref.	1.07 (0.75-1.53)	1.08 (0.75-1.56)	1.06 (0.74-1.53)	0.881	1.06 (0.93-1.20)	0.366
L-carnitine							
Cases	114	105	139	151			
MV	Ref.	0.86 (0.59-1.24)	1.23 (0.86-1.76)	1.32 (0.92-1.91)	0.061	1.10 (0.96-1.26)	0.191
Choline							
Cases	100	115	115	179			
MV	Ref.	1.19 (0.81-1.76)	1.16 (0.79-1.68)	1.89 (1.30-2.73)	0.005	1.23 (1.07-1.40)	0.015
Betaine							
Cases	113	120	126	150			
MV	Ref.	1.18 (0.80-1.72)	1.36 (0.94-1.96)	1.60 (1.00-2.33)	0.025	1.19 (1.04-1.35)	0.030
Dimethylglycine							
Cases	115	106	122	166			
MV	Ref.	0.97 (0.67-1.41)	1.17 (0.80-1.72)	1.41 (0.98-2.02)	0.045	1.16 (1.02-1.32)	0.045

[1]Values are OR (95% CI). A natural logarithmic transformation was applied to the raw values of individual metabolites. Conditional logistic regression analysis was used. MV adjusted for smoking, family history of premature coronary heart disease, physical activity, alcohol intake, BMI (kg/m^2), intervention group (MedDiet + EVOO or MedDiet + nuts), dyslipidemia, hypertension, type 2 diabetes, medication use (lipid-modifying, antihypertensive, and antidiabetic medication), center and food groups (meat, fish, eggs, bread, cereals, dairy, legumes). Abbreviations: Ref, reference.

[2]Adjusted with the Benjamini-Hochberg False Discovery Rate method.

Supplementary Table 4. Associations of baseline individual metabolites levels with the risk of heart failure after further adjustment of the multivariable model for dietary sources of TMAO and its precursors in a nested case-control study of the PREDIMED Trial[1].

		Quartiles of plasma metabolite levels					
Metabolite	Q1	Q2	Q3	Q4	P trend[2]	OR per 1 SD increment	P value[2]
Trimethylamine N-oxide							
Cases	87	70	97	72			
MV	Ref.	0.78 (0.47-1.29)	1.02 (0.63-1.67)	0.78 (0.47-1.30)	0.451	0.93 (0.78-1.11)	0.426
L-carnitine							
Cases	82	92	76	76			
MV	Ref.	1.26 (0.80-1.99)	1.22 (0.74-2.02)	1.41 (0.86-2.33)	0.245	1.09 (0.92-1.29)	0.382
Choline							
Cases	60	81	59	126			
MV	Ref.	1.28 (0.78-2.11)	1.09 (0.65-1.83)	2.76 (1.67-4.56)	0.005	1.50 (1.25-1.80)	0.002
Betaine							
Cases	81	78	90	77			
MV	Ref.	1.16 (0.71-1.89)	1.68 (1.00-2.81)	1.68 (1.00-2.87)	0.056	1.19 (0.99-1.45)	0.115
Dimethylglycine							
Cases	84	68	66	108			
MV	Ref.	1.06 (0.66-1.69)	1.00 (0.62-1.62)	1.82 (1.12-2.97)	0.025	1.35 (1.12-1.62)	0.002

[1]Values are OR (95% CI). A natural logarithmic transformation was applied to the raw values of individual metabolites. Conditional logistic regression analysis was used. MV adjusted for smoking, family history of premature coronary heart disease, physical activity, alcohol intake, BMI (kg/m^2), intervention group (MedDiet + EVOO or MedDiet + nuts), dyslipidemia, hypertension, type 2 diabetes, medication use (lipid-modifying, antihypertensive, and antidiabetic medication), center and food groups (meat, fish, eggs, bread, cereals, dairy, legumes). Abbreviations: Ref, reference.

[2]Adjusted with the Benjamini-Hochberg False Discovery Rate method.

Supplementary Table 5. Multiple linear regression coefficients for TMAO, L-carnitine, choline, betaine, dimethylglycine (per 1 SD increment) in relation to lipids, glucose, body mass index and leukocyte count using the AF case-control study.

Parameters	TMAO	L-carnitine	Choline	Betaine	Dimethylglycine
LnChol, mg/dl[1]	-0.008 ± 0.008	-0.01 ± 0.008	0.01 ± 0.008	-0.02 ± 0.008	-0.005 ± 0.008
LnHDL-C, mg/dl[1]	0.01 ± 0.01	-0.03 ± 0.01*	0.02 ± 0.01	1.02 ± 0.01	-0.002 ± 0.009
LnLDL-C, mg/dl[1]	-0.01 ± 0.01	-0.03 ± 0.01	-0.03 ± 0.01	-0.04 ± 0.01*	-0.02 ± 0.01
LnTAG, mg/dl[1]	-0.01 ± 0.01	0.008 ± 0.02	0.02 ± 0.01	-0.10 ± 0.01*	-0.008 ± 0.01
LnGlucose, mg/dl[2]	0.0004 ± 0.01	-0.04 ± 0.01*	0.008 ± 0.01	-0.03 ± 0.01*	-0.008 ± 0.01
BMI, kg/m^2 [3]	0.05 ± 0.10	0.27 ± 0.11*	0.43 ± 0.10*	-0.13 ± 0.11	0.03 ± 0.10
LogLeukocyte count (10^9/L)[4]	-0.002 ± 0.002	-0.004 ± 0.002	0.007 ± 0.002*	0.002 ± 0.003	0.006 ± 0.002*

[1]Multiple linear regression analysis for lipids was adjusted for age, sex, center, smoking, physical activity, alcohol intake, BMI (kg/m^2), dyslipidemia, hypertension, type 2 diabetes and use of lipid-modifying medication.

[2]Multiple linear regression analysis for glucose was adjusted for age, sex, center, smoking, physical activity, alcohol intake, BMI (kg/m^2), dyslipidemia, hypertension and anti-diabetic medication.

[3]Multiple linear regression analysis for BMI was adjusted for age, sex, center, smoking, physical activity, alcohol intake, dyslipidemia, hypertension and type 2 diabetes.

[4]Multiple linear regression analysis for leukocyte count was adjusted for age, sex, center, smoking, physical activity, alcohol intake, BMI (kg/m^2), dyslipidemia, hypertension and type 2 diabetes.

Values are expressed as β ± SE.

*P value <0.05.

Abbreviations: AF, atrial fibrillation; TMAO, Trimethylamine N-oxide; Chol, total cholesterol; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; TAG, triacylglycerols; BMI, body mass index.

Supplementary Table 6. Multiple linear regression coefficients for TMAO, L-carnitine, choline, betaine, dimethylglycine (per 1 SD increment) in relation to lipids, glucose, body mass index and leukocyte count using the HF case-control study.

Parameters	TMAO	L-carnitine	Choline	Betaine	Dimethylglycine
LnChol, mg/dl[1]	-0.005 ± 0.01	0.006 ± 0.009	0.02 ± 0.009	-0.005 ± 0.01	-0.006 ± 0.009
LnHDL-C, mg/dl[1]	0.003 ± 0.01	-0.01 ± 0.01	0.01 ± 0.01	0.002 ± 0.01	-0.01 ± 0.01
LnLDL-C, mg/dl[1]	-0.009 ± 0.02	-0.01 ± 0.01	-0.001 ± 0.02	0.02 ± 0.02	0.04 ± 0.01
LnTAG, mg/dl[1]	-0.008 ± 0.02	0.04 ± 0.02*	0.03 ± 0.02	-0.06 ± 0.02*	-0.01 ± 0.01
LnGlucose, mg/dl[2]	-0.02 ± 0.01	-0.03 ± 0.01*	-0.003 ± 0.01	-0.03 ± 0.01*	-0.13 ± 0.01
BMI, kg/m^2 [2 3]	-0.01 ± 0.13	-0.25 ± 0.12*	0.38 ± 0.13*	-0.45 ± 0.14*	-0.13 ± 0.12
LogLeukocyte count (10^9/L)[4]	0.003 ± 0.003	-0.0001 ± 0.002	0.003 ± 0.002	0.005 ± 0.003*	0.009 ± 0.01

[1]Multiple linear regression analysis for lipids was adjusted for age, sex, center, smoking, physical activity, alcohol intake, BMI (kg/m^2), dyslipidemia, hypertension, type 2 diabetes and use of lipid-modifying medication.

[2]Multiple linear regression analysis for glucose was adjusted for age, sex, center, smoking, physical activity, alcohol intake, BMI (kg/m^2), dyslipidemia, hypertension and anti-diabetic medication.

[3]Multiple linear regression analysis for BMI was adjusted for age, sex, center, smoking, physical activity, alcohol intake, dyslipidemia, hypertension and type 2 diabetes.

[4]Multiple linear regression analysis for leukocyte count was adjusted for age, sex, center, smoking, physical activity, alcohol intake, BMI (kg/m^2), dyslipidemia, hypertension and type 2 diabetes.

Values are expressed as β ± SE.

*P value <0.05.

Abbreviations: HF, heart failure; TMAO, Trimethylamine N-oxide; Chol, total cholesterol; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; TAG, triacylglycerols; BMI, body mass index.

Supplementary Table 7. Spearman's correlation analysis between metabolites and food groups using the AF case-control study.

Food group	TMAO	L-carnitine	Choline	Betaine	Dimethylglycine
Meat	0.02	0.02	-0.05	-0.06	-0.03
Fish	0.20*	0.06*	0.07*	0.05	0.03
Eggs	-0.06*	-0.04	0.10*	0.009	0.04
Bread (white)	-0.04	0.09*	0.03	0.07*	0.10*
Cereals	-0.03	0.03	0.06*	0.07*	0.08*
Dairy	-0.01	-0.04	-0.09*	-0.06	0.01
Legumes	-0.05	0.04	0.06	0.08*	0.02

*P value <0.05.

Abbreviations: TMAO, Trimethylamine N-oxide.

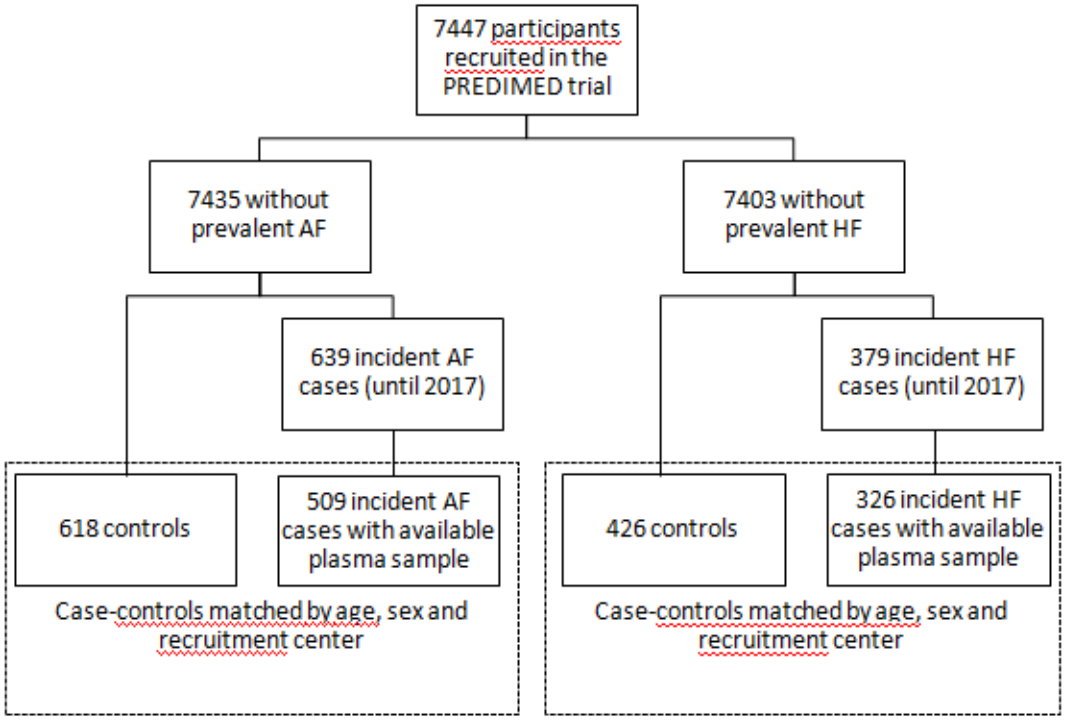
Supplementary Table 8. Spearman's correlation analysis between metabolites and food groups using the HF case-control study.

Food group	TMAO	L-carnitine	Choline	Betaine	Dimethylglycine
Meat	-0.007	-0.01	-0.03	-0.04	-0.05
Fish	0.23*	0.07	0.05	0.05	0.04
Eggs	-0.001	-0.07*	-0.01	0.006	0.05
Bread (white)	0.04	0.02	0.02	0.12*	0.05
Cereals	0.03	0.04	-0.007	0.08*	0.09*
Dairy	0.03	-0.009	-0.10*	-0.11*	-0.05
Legumes	-0.08	0.03	0.08	0.08*	0.05

*P value <0.05.

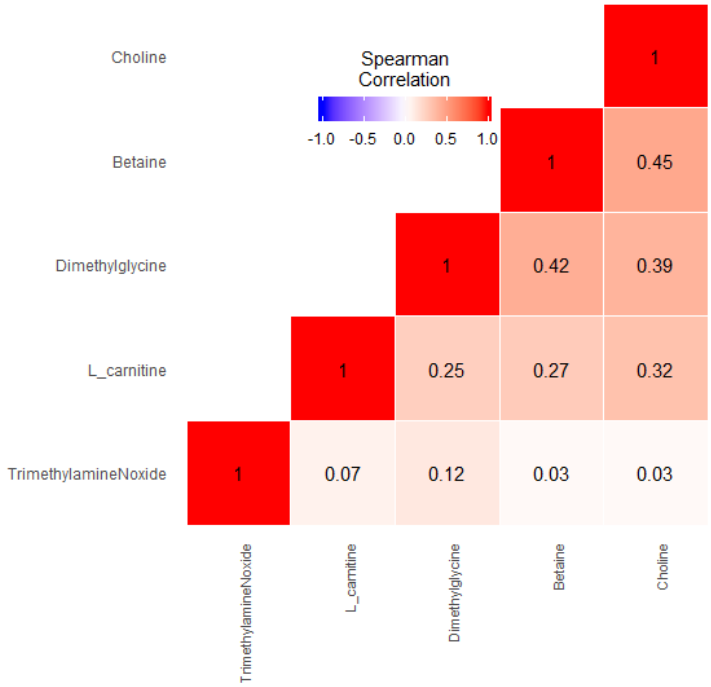
Abbreviations: TMAO, Trimethylamine N-oxide.

Supplemental Fig. 1. Flow-chart of study participants.

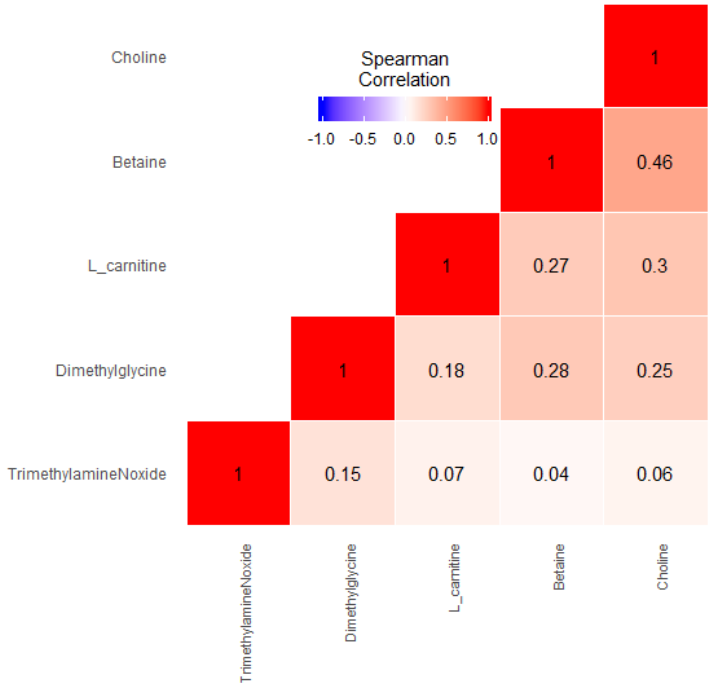


Supplementary Fig. 2. Correlation heatmap of the metabolites under study. (A) Spearman correlation coefficients of the metabolites using the AF case-control study. (B) Spearman correlation coefficients of the metabolites using the HF case-control study.

A

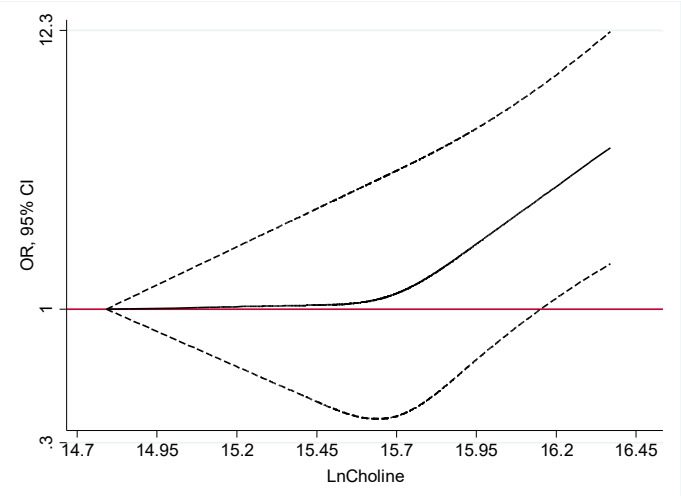


B

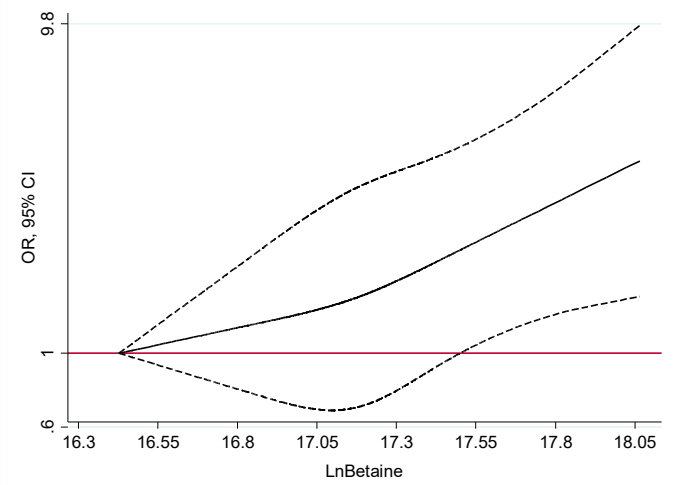


Supplementary Fig. 3. Spline of levels of (A) choline, (B) betaine, (C) dimethylglycine and incident atrial fibrillation in a nested case-control study (509 cases, 618 controls) of the PREDIMED study. Dotted lines are 95% confidence intervals of the spline; horizon line is the reference.

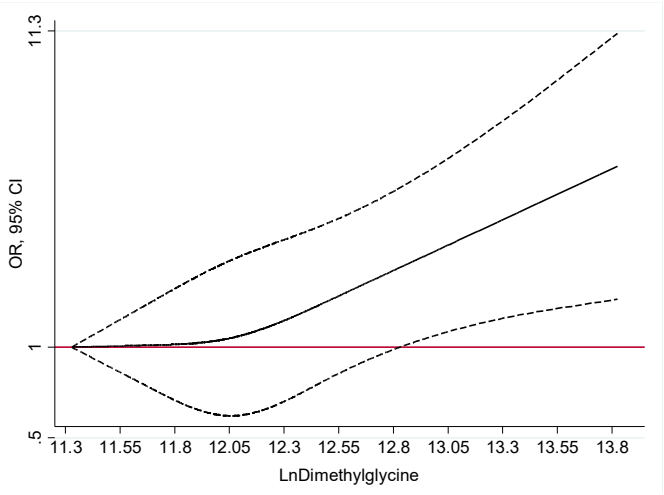
A



B

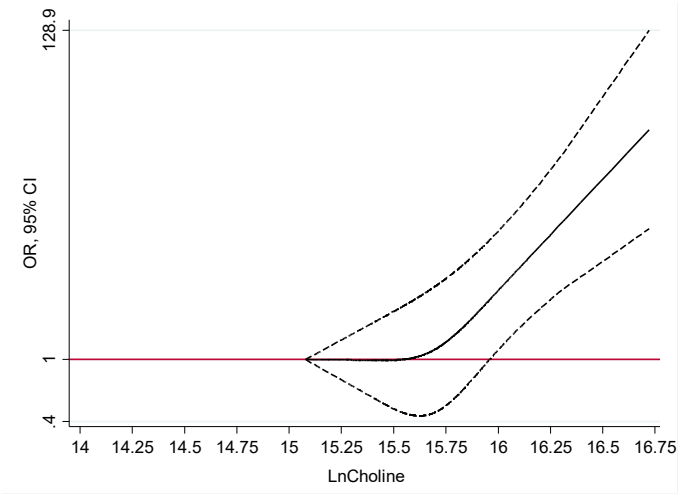


C

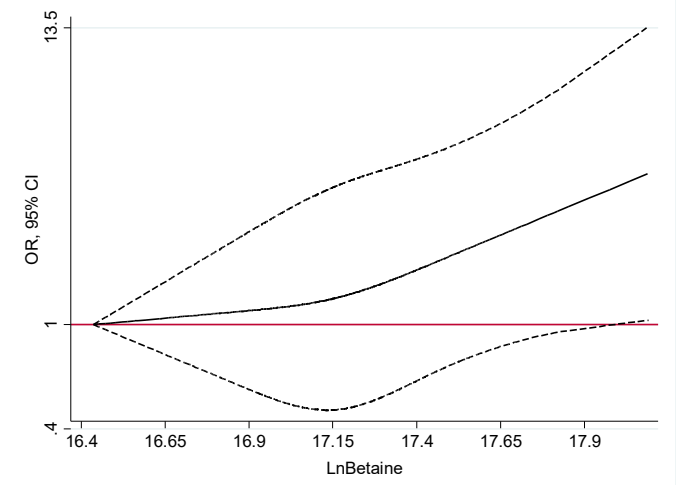

11.3
1
.5
OR, 95% CI
11.3
11.55
11.8
12.05
12.3
12.55
12.8
13.05
13.3
13.55
13.8
LnDimethylglycine

Supplementary Fig. 4. Spline of levels of (A) choline, (B) betaine, (C) dimethylglycine and incident heart failure in a nested case-control study (326 cases, 426 controls) of the PREDIMED study. Dotted lines are 95% confidence intervals of the spline; horizon line is the reference.

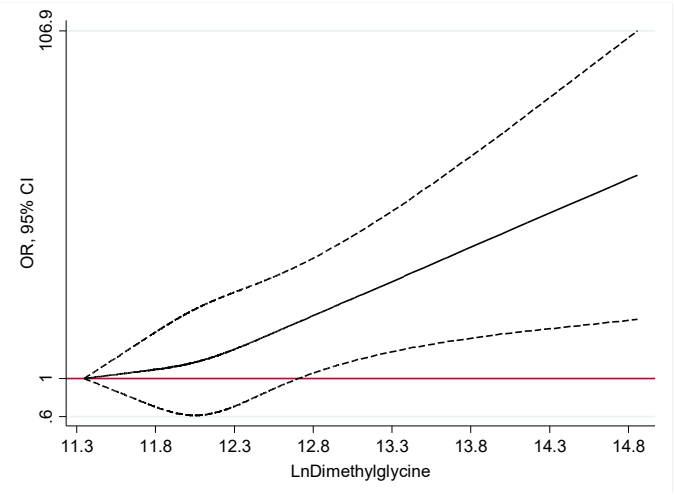
A



B



C


106.9
1
.6
OR, 95% CI
11.3
11.8
12.3
12.8
13.3
13.8
14.3
14.8
LnDimethylglycine