

# Gut microbe-generated metabolite trimethylamine-N-oxide as cardiovascular risk biomarker: a systematic review and dose-response meta-analysis

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## Aims

Gut microbiota-derived metabolite trimethylamine-N-oxide (TMAO) is emerging as a new potentially important cause of increased cardiovascular risk. The purpose of this meta-analysis was to systematically estimate and quantify the association between TMAO plasma levels, mortality, and major adverse cardio and cerebrovascular events (MACCE).

## Methods and results

MEDLINE, ISI Web of Science, and SCOPUS databases were searched for *ad hoc* studies published up to April 2017. Associations between TMAO plasma levels, all-cause mortality (primary outcome) and MACCE (secondary outcome) were systematically addressed. A total of 17 clinical studies were included in the analytic synthesis, enrolling 26 167 subjects. The mean follow-up in our study population was  $4.3 \pm 1.5$  years. High TMAO plasma levels were associated with increased incidence of all-cause mortality [14 studies for 16 cohorts enrolling 15 662 subjects, hazard ratio (HR): 1.91; 95% confidence interval (CI): 1.40–2.61,  $P < 0.0001$ ,  $I^2 = 94\%$ ] and MACCE (5 studies for 6 cohorts enrolling 13 944 subjects, HR: 1.67, 95% CI: 1.33–2.11,  $P < 0.00001$ ,  $I^2 = 46\%$ ). Dose-response meta-analysis revealed that the relative risk (RR) for all-cause mortality increased by 7.6% per each  $10 \mu\text{mol/L}$  increment of TMAO [summary RR: 1.07, 95% CI (1.04–1.11),  $P < 0.0001$ ; based on seven studies]. Association of TMAO and mortality persisted in all examined subgroups and across all subject populations.

## Conclusions

This is the first systematic review and meta-analysis demonstrating the positive dose-dependent association between TMAO plasma levels and increased cardiovascular risk and mortality.

## Keywords

Trimethylamine-N-oxide (TMAO) • Microbiota • Cardiovascular risk • Outcomes • Meta-analysis

## Introduction

Cardiovascular diseases (CVD) represent the leading cause of disability and death worldwide.<sup>1</sup> Despite the enormous progress made in patients' stratification according to the specific profile of

cardiovascular risk factors, understanding of all different components determining cardiovascular events and mortality is still not completely covered.

Dietary habitudes are widely recognized as modifiable risk factors with a strong impact on cardiovascular risk profile.<sup>2–4</sup> Recently, the

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interplay between dietary composition, gut microbiota and microbe-generated metabolites has been intensely investigated.<sup>5,6</sup> Trimethylamine N-oxide (TMAO) is a small, organic, gut microbiome-generated compound, whose blood concentration increases after ingesting dietary L-carnitine and phosphatidylcholine-rich foods such as red meat, eggs, and fish.<sup>7,8</sup> The interest for TMAO in cardiovascular research has recently arisen given the pre-clinical evidences revealing a mechanistic link between TMAO and CVD.<sup>8–10</sup> The adverse effects of TMAO on cardiovascular function have been associated with multiple pathways and mechanisms. TMAO promotes atherosclerosis, probably through an increased expression of macrophage scavenger receptors and formation of foam cells in the artery wall.<sup>9</sup> Accordingly, non-lethal inhibition of microbial production of TMAO inhibits atherosclerotic lesions formation in mice.<sup>11</sup> Of note, increased levels of TMAO have been associated with a significant reduction in 'reverse cholesterol transport' as well as defects in cholesterol metabolic pathways in general.<sup>8,12</sup> TMAO-dependent regulation of cholesterol transport and its relationship with atherosclerosis seems to be related also to the effects of TMAO on bile acid composition. It has been recently shown, in two different studies, that reduction in TMAO production by genetic inhibition of hepatic flavin mono-oxygenase 3 (FMO3), which is under control of the bile acid-activated nuclear farnesoid X receptor (FXR), inhibits atherosclerosis in different mouse models.<sup>13–15</sup> Finally, high levels of TMAO promote endothelial dysfunction, exacerbate platelet reactivity, and enhance thrombosis, affect lipid metabolism and inflammatory response, underlying the importance of this molecule in the cardiovascular system.<sup>10,16–18</sup>

A significant number of studies involving different population subsets have demonstrated various levels of association between plasma levels of TMAO and CVD or mortality.<sup>8,19–34</sup> However, the potential impact of TMAO as a novel biomarker of CVD and its prognostic role in disease progression has never been systematically addressed. Moreover, controversies regarding the potential causative role of TMAO in CVD have arisen. Fish represent one of the most important dietary sources of TMAO precursor phosphatidylcholine, and since both observational and randomized controlled trials<sup>35,36</sup> have clearly shown a protection from CVD after fish consumption, the possible maladaptive link between TMAO and CVD has been questioned. Along the same line, no univocal effect has been reported between another TMAO-generating compound, L-carnitine, and CVD. Two different recent meta-analyses analysing the association between L-carnitine supplementation and cardiovascular risk reported opposite results, feeding those controversies.<sup>37,38</sup> Given the need for synthetic quantitative data addressing the potential use of TMAO as novel biomarker in CVD, we conducted the first systematic review and dose-response meta-analysis of published studies to quantitatively assess the association between TMAO plasma levels and cardiovascular outcomes.

## Methods

### Literature search strategy

The study was designed according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) requirements<sup>39</sup> and the complete PRISMA checklist is provided in Supplementary material

online, *Figure S1*. MEDLINE, ISI Web of Science and SCOPUS databases were searched for studies published up to April 2017. Studies were identified using the major medical subject heading 'TMAO OR Trimethylamine-N-oxide' combined with 'Cardiovascular Disease OR Mortality OR Cardiovascular Mortality'. English was set as a language restriction. Two authors (AS and GGS) independently examined the title and abstract of citations. Full texts of potentially eligible studies were obtained and disagreements were resolved by discussion. To look for additional relevant studies, full texts and bibliography of all potential articles were also retrieved in detail. Abstracts, meeting proceedings, and personal communications were not used for the purpose of this study.

### Eligibility criteria

Studies were included if they reported estimates on the association between mortality and/or major adverse cardio and cerebrovascular events (MACCE) and TMAO plasma levels, reported as either categorical or continuous variable. Studies were excluded if any of the following criteria applied: (i) duplicate publication data; (ii) lack of data on TMAO plasma levels and their correlation with outcomes; (iii) the outcome of interest was not clearly reported or was impossible to extract or calculate from the published results; and (iv) number of included patients <100. Follow-up length was not set as a restriction.

### Data extraction

Two investigators (GGS and AS) independently screened articles for fulfilment of inclusion criteria and extracted, for each study included, information about baseline characteristics of the study sample, publication year, country of the study, duration of follow-up, as well as adjusted estimates for the outcomes of interest. Estimates and their 95% confidence intervals (CI) for TMAO plasma levels in relation to all-cause mortality or MACCE were extracted as they were presented in the original reports, including estimates per 1-unit, 10 µM, or per 1-SD increment, or for the highest vs. the lowest categories (e.g. tertiles, quartiles or quintiles) of TMAO, as reported in Supplementary material online, *Table S1*. Investigators compared selected trials and discrepancies were resolved by consensus. In studies reporting results from two different cohorts of subjects, the related estimates were analysed separately.

### Quality assessment

Internal study validity was assessed using a scale developed by the Methods Work Group for the US Preventive Service Task Force (USPSTF).<sup>40</sup> This scale allows the assessment of the internal validity of cohort studies included in meta-analysis on the basis of seven items: (i) initial comparability of groups; (ii) maintenance of comparability; (iii) follow-up duration and loss of data; (iv) adequateness of measurements; (v) clear definition of the interventions; (vi) relevance of the analysed outcomes; and (vii) analysis adjustments for confounders. Based on the above criteria, studies were rated as 'good', if they met all criteria; 'fair' if not all criteria were met but there were no fatal flaws; 'poor' if fatal flaws existed (group initially assembled were not close to be comparable or maintained through the study, invalid measurement instruments were used, key confounders given little or no attention; more than 20% loss to follow-up).

### End points and definitions

Two outcomes (end points) are reported in the present manuscript: (i) all-cause mortality (primary outcome) and (ii) MACCE (secondary outcome) which was defined as the incidence of death, myocardial infarction and stroke.

## Statistical analysis

### High vs. low trimethylamine-N-oxide plasma levels

In the primary studies, the exposure variable (TMAO concentration) was reported in different ways (either as a continuous or as a categorical trait) and the effect measures with their corresponding 95% CI were reported per different increments in TMAO values (e.g. per 1-unit, per 10  $\mu\text{mol/L}$ , or per 1-SD increment in the continuous trait; or per tertiles, quartiles or quintiles in TMAO levels; or comparing individuals above vs. below the median). In this meta-analysis, to harmonize the presentation of data, we provide effect measures for the top (High TMAO) vs. the bottom (Low TMAO) tertile of TMAO distribution. As described by Danesh et al.<sup>41</sup> and Schlesinger et al.,<sup>42</sup> if individual studies did not report effect measures per tertiles, we converted the results to a standard scale of effect, by giving an estimate per 2.18 SD units of TMAO, whereas the factor of 2.18 represents the difference in the means of the upper and lower tertile of the standard normal distribution. Hence, this scaling method assumes that the exposure variable (TMAO) is normally distributed and the association with all-cause mortality and/or MACCE is log linear. For primary studies reporting results by TMAO quartiles, quintiles or comparing individuals above vs. below the median, the estimate of the top vs. the bottom categorization was log-transformed, then multiplied by the factors 2.18/2.54, 2.18/2.80, or 2.18/1.59, respectively, and the resulting product was exponentiated to the base of *e* to derive hazard ratios (HRs) comparing the top vs. the bottom tertile.<sup>41</sup> In case of individual studies reporting estimates per 1-SD increment of TMAO levels, the log transformed value was multiplied per 2.18 and subsequently presented as effect measure of the top vs. bottom tertile. Finally, effect measures that were reported per 1-unit increment were multiplied by the study specific SD. Summary HRs and CIs from adjusted primary sources were abstracted (see Supplementary material online, Table S2). Estimates of effect were calculated with a random-effects model and expressed as HRs. Statistical significance was set at  $P \leq 0.05$  (2-tailed).

Subgroup analyses were performed according to the presence of chronic kidney disease (CKD), study location, internal study validity, and sample size. Interaction between subgroups was assessed as previously described.<sup>43</sup>

### Dose-response meta-analysis

Because most of the primary studies we examined reported different cut-offs of plasma TMAO, we performed a dose-response meta-analysis for our primary outcome by using the method proposed by Orsini et al.<sup>44</sup> and Greenland and Longnecker.<sup>45</sup> When the association between all-cause mortality and TMAO level was reported from categories of the biomarker, the method presented above required from each individual study the HR (with 95% CI), the median/mean level of exposure, and the number of cases and total participants or person years for at least three exposure categories. For the studies that did not present the median or mean doses of plasma TMAO, we chose the midpoint of each category. If the category was open-ended, the midpoint of this category was calculated by assuming the interval was the same as that of the adjacent interval. We used restricted cubic splines with three knots to explore a potential non-linear association between TMAO levels and all-cause mortality. The dose-response outcome was presented per 10  $\mu\text{mol/L}$  increment in plasma TMAO. To formally test for non-linearity, we used a likelihood ratio test.<sup>46</sup> Five studies were excluded from the dose-response meta-analysis because they did not report either the number of cases, the total number of patients for each category level of TMAO or the exposure level of TMAO.<sup>21,24,26,28,33</sup>

Meta-regression analysis was performed to assess potential important covariates (included in Table 1) that might exert substantial impact on between-study heterogeneity<sup>47</sup> (significance at  $P \leq 0.05$ ). If no significant covariates were found to be heterogeneous, the 'leave-one-out' sensitivity analysis<sup>48,49</sup> was

carried out to evaluate key studies (reporting same outcomes definition) with substantial impact on between-study heterogeneity. Fixed-effect model was used to confirm the results in case of significant heterogeneity.

Publication bias was assessed using funnel plots and Egger's test, consisting in a linear regression of the intervention effect estimates on their standard errors, weighting by  $1/(\text{variance of the intervention effect estimate})$ .

All data analyses were performed using STATA software (version 12.0), Prometa Software Version 2 and Reviewer Manager (RevMan, Version 5.2).<sup>50,51</sup>

## Results

### Literature search and characteristics of included studies

The initial search of literature identified 349 articles, of which 86 were retrieved for more detailed evaluation, and 17 clinical studies<sup>8,19–34</sup> were finally included in the meta-analysis, enrolling 26 167 subjects (Figure 1). Characteristics of the included studies are provided in Table 1. The mean follow-up in our study population was  $4.3 \pm 1.5$  years.

### Primary outcome: association between trimethylamine-N-oxide and all-cause mortality

Across all studies, higher TMAO plasma levels were associated with greater risk of all-cause mortality (14 studies for 16 cohorts enrolling 15 662 subjects, HR: 1.91; 95% CI: 1.40–2.61,  $P < 0.0001$ ,  $I^2 = 94\%$ ; comparing patients with 'high' vs. 'low' TMAO, Figure 2).

When the analysis for all-cause mortality was repeated stratifying the studies according to the presence or absence of CKD in the primary studies populations, a significant association between TMAO levels and all-cause mortality was found both in the CKD and non-CKD cohort (non-CKD: 10 studies for 11 cohorts enrolling 13 195 subjects, HR: 1.79, 95% CI: 1.23–2.60,  $P = 0.002$ ,  $I^2 = 94\%$ ; CKD: 5 studies enrolling 2467 subjects, HR: 2.27, 95% CI: 1.13–4.58,  $P = 0.02$ ,  $I^2 = 91\%$ ;  $P$  for interaction = ns; Figure 3A). Subsequently, we repeated the analysis stratifying the studies according to the geographical location where patients were enrolled. Statistical significance for the association between all-cause mortality and TMAO levels persisted also across different countries (USA: nine studies for 10 cohorts enrolling 10 906 subjects, HR: 1.79, 95% CI: 1.20–2.67,  $P = 0.004$ ,  $I^2 = 92\%$ ; Europe: five studies enrolling 4291 subjects, HR: 2.26, 95% CI: 1.07–4.79,  $P = 0.03$ ,  $I^2 = 97\%$ ; Other: one study enrolling 465 subjects, HR: 1.78, 95% CI: 1.15–2.76,  $P = 0.010$ ;  $P$  for interaction = ns; Figure 3B).

The dose-response meta-analysis revealed that the relative risk (RR) for all-cause mortality increased by 7.6% per each 10  $\mu\text{mol/L}$  increment of TMAO [summary RR: 1.07, 95% CI (1.04–1.11),  $P < 0.0001$ ; based on seven studies]. However, there was evidence for a non-linear association between TMAO plasma levels and all-cause mortality ( $P$  for non-linearity  $< 0.0001$ , based on seven studies; Figure 4).

### Secondary outcome: association between trimethylamine-N-oxide and major adverse cardio and cerebrovascular events

In patients with high TMAO plasma levels, the incidence of MACCE was significantly higher compared with patients with low TMAO

**Table 1** Baseline characteristics of the selected studies included in the meta-analysis

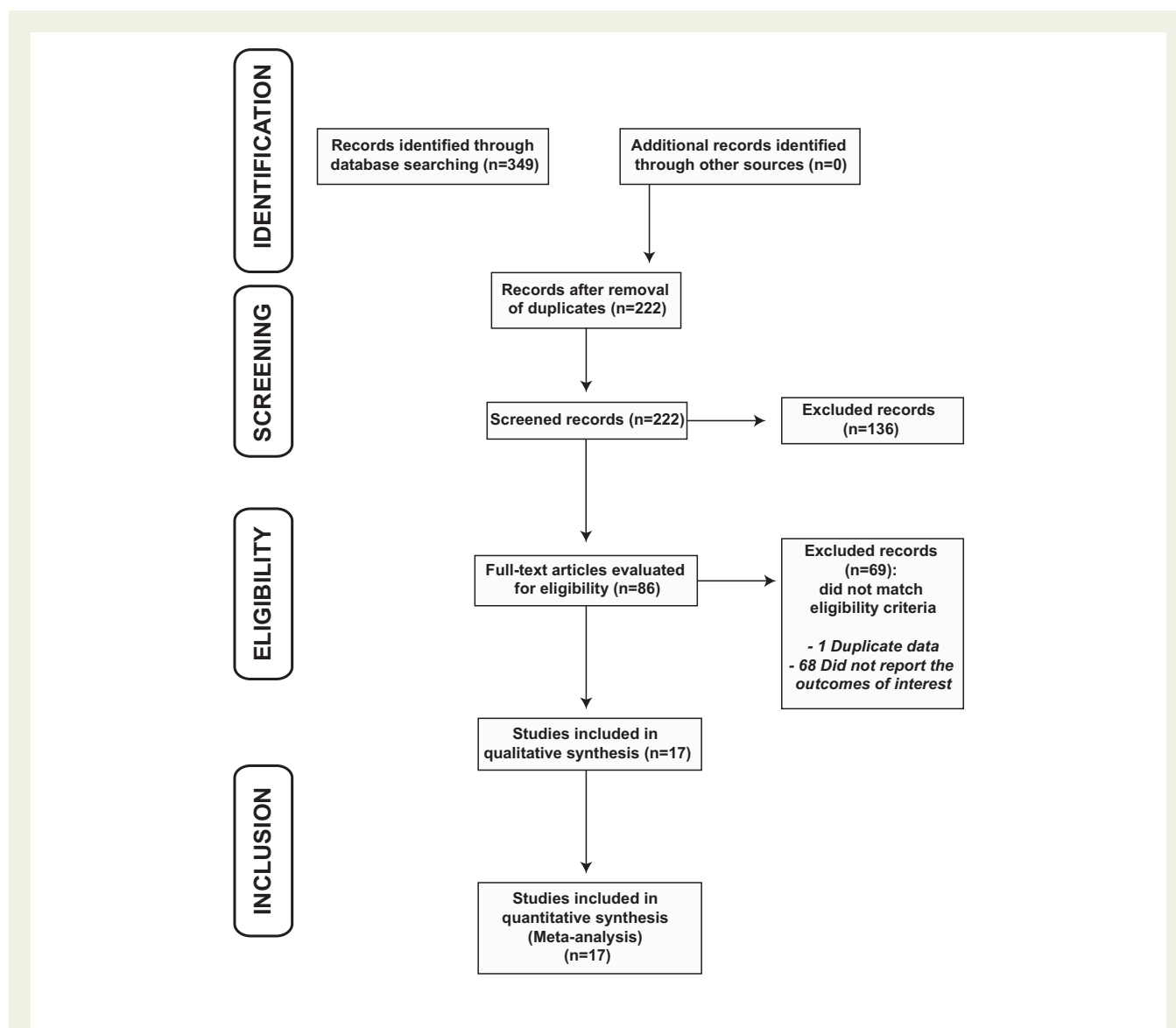
Authors (Ref. #)	Year	Location	Follow-up length (years)	n	Age (years)	Female (%)	BMI (kg/m <sup>2</sup> )	CAD (%)	CKD (%)	COPD (%)	Diabetes (%)	Dyslipidaemia (%)	EF (%)	Former/current smokers (%)	Hypertension (%)	TMAO levels (μmol/L)	hs-CRP (mg/L)	NT-proBNP (pmol/L)	MPO (pmol/L)
Kaysen <i>et al.</i> <sup>21</sup>	2015	USA	4	235	61.8 ± 14.2	44.7	29.7 ± 7.28	N/A	N/A	N/A	58.7	N/A	N/A	N/A	N/A	43 (27.5–66.6)	7.5 (3.95–13.0) <sup>b</sup>	N/A	N/A
Koeth <i>et al.</i> <sup>8</sup>	2013	USA	3	2595	62 (54–71)	30	29 (25–33)	78	N/A	N/A	28	85	N/A	69	72	N/A	2.3 (1.0–5.4)	N/A	113 (76–230)
Lever <i>et al.</i> <sup>22</sup>	2014	New Zealand	5	475	N/A	N/A	N/A	18.3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Li <i>et al.</i> (Cleveland Cohort) <sup>32</sup>	2017	USA	7	540	62.4 ± 13.9	42.5	N/A	48.9	N/A	N/A	27.2	50.0	N/A	61.1	65.6	4.28 (2.55–7.91)	N/A	N/A	N/A
Li <i>et al.</i> (Swiss Cohort) <sup>32</sup>	2017	Switzerland	7	1683	63.9 ± 12.4	22.2	N/A	36.8	N/A	N/A	17.5	62.6	N/A	67.8	59.3	2.87 (1.94–4.85)	N/A	N/A	N/A
Missalidis <i>et al.</i> <sup>23</sup>	2016	Sweden	5	259	58.5 ± 13	33.2	25.5 ± 4.5	N/A	69.1	N/A	18.5	N/A	N/A	13.9	N/A	53.4 (9.3–170.0) <sup>a</sup>	2.8 (0.6–16.8) <sup>a</sup>	N/A	N/A
Senthong <i>et al.</i> (JHAHA) <sup>34</sup>	2016	USA	5	2235	63 ± 11	29	N/A	95	N/A	N/A	35	71	N/A	70	76	3.8 (2.5–6.5)	2.6 (1.1–6.4)	N/A	1125 (74.5–239.6)
Senthong <i>et al.</i> (JHAHA) II <sup>31</sup>	2016	USA	5	821	66 ± 10	34	N/A	90	N/A	N/A	43	70	N/A	74	83	4.8 (2.9–8)	3.2 (1.3–8.3)	N/A	1186 (80.3–261.8)
Shafi <i>et al.</i> <sup>24</sup>	2016	USA	2.3	1232	57.7 ± 13.8	56.7	25.8 ± 5.4	79.2	100	N/A	45.0	N/A	N/A	N/A	32.2	88 (62–124)	1.0 ± 0.3	N/A	N/A
Stubbs <i>et al.</i> <sup>25</sup>	2016	USA	4	220	69.7 ± 10.3	57.3	30.6 ± 7	36.8	100	N/A	64.1	74.1	N/A	6.8	97.3	6.9 (4.8–10.9)	N/A	N/A	N/A
Suzuki <i>et al.</i> Clin Chem <sup>33</sup>	2017	UK	2	1079	67 (57–77)	38	N/A	33	N/A	N/A	23	89	N/A	42	52	3.7 (4.6–6.4)	N/A	812 (259–2199)	N/A
Suzuki <i>et al.</i> Heart <sup>26</sup>	2016	UK	1	972	78 (69–84)	39	N/A	40.4	N/A	9.2	33.8	24.4	N/A	9.6	58.3	5.6 (3.4–10.5)	N/A	2124 (969–3836)	N/A
Tang <i>et al.</i> Circ Res (CKD Cohort) <sup>27</sup>	2015	USA	5	521	70 ± 10	52	N/A	53	100	N/A	53	59	N/A	61	88	7.9 (5.2–12.4)	4.1 (1.8–9.6)	N/A	N/A
Tang <i>et al.</i> Circ Res (non-CKD Cohort) <sup>27</sup>	2015	USA	5	3166	62 ± 11	34	N/A	40	0	N/A	27	61	N/A	66	69	3.4 (2.3–5.3)	2.2 (0.9–5.0)	N/A	N/A
Tang <i>et al.</i> Clin Chem <sup>30</sup>	2017	USA	5	1216	64.4 ± 10.2	42	N/A	47	N/A	N/A	100	64	N/A	63	79	4.4 (2.8–7.7)	3.3 (1.3–8.3)	N/A	N/A
Tang <i>et al.</i> NEJM <sup>20</sup>	2013	USA	3	4007	63 ± 11	36	28.7 (25.6–32.5)	42	N/A	N/A	32.0	60	N/A	65	72	3.7 (2.4–6.2)	2.4 (1–5.9)	N/A	115.2 (76.4–245.7)
Tang <i>et al.</i> JACC <sup>28</sup>	2014	USA	5	720	66 ± 10	40	28.4 (25.1–33.1)	64	N/A	N/A	41	61	35 (25–50)	N/A	78	5 (3.0–8.5)	3.9 (1.6–9.0)	N/A	N/A
Troseth <i>et al.</i> <sup>29</sup>	2015	Norway	5.2	288	N/A	N/A	N/A	38	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Wang <i>et al.</i> <sup>19</sup>	2014	USA	3	3903	63 ± 11	36	N/A	61	N/A	N/A	31	N/A	N/A	65	72	3.7 (2.4–6.2)	2.4 (1.1–5.9)	N/A	N/A

All values are expressed as the mean ± SD, median (10th–90th percentile) or median (range interquartile), or number (%) as appropriate according to the primary studies. CAD refers to current/history of CAD or myocardial infarction as reported in the primary studies. Dyslipidaemia refers to % of patients treated with statins in the primary studies.

BMI, body mass index; CAD, coronary artery disease; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; hs-CRP, high-sensitivity C reactive protein; EF, ejection fraction; MPO, myeloperoxidase; NT-pro-BNP, N-terminal pro-brain natriuretic peptide; TMAO, trimethylamine N-oxide; N/A, not applicable (data not shown in the primary study or not available); SD, standard deviation.

<sup>a</sup>Values referred to CKD 3–5.

<sup>b</sup>Non-high-sensitivity CRP.



**Figure 1** Meta-analysis flow chart. Flow chart showing study search and selection.

levels (five studies for six cohorts enrolling 13 944 subjects, HR: 1.67, 95% CI: 1.33–2.11,  $P < 0.0001$ ,  $I^2 = 46\%$ , Supplementary material online, Figure S2).

## Study quality assessment and heterogeneity

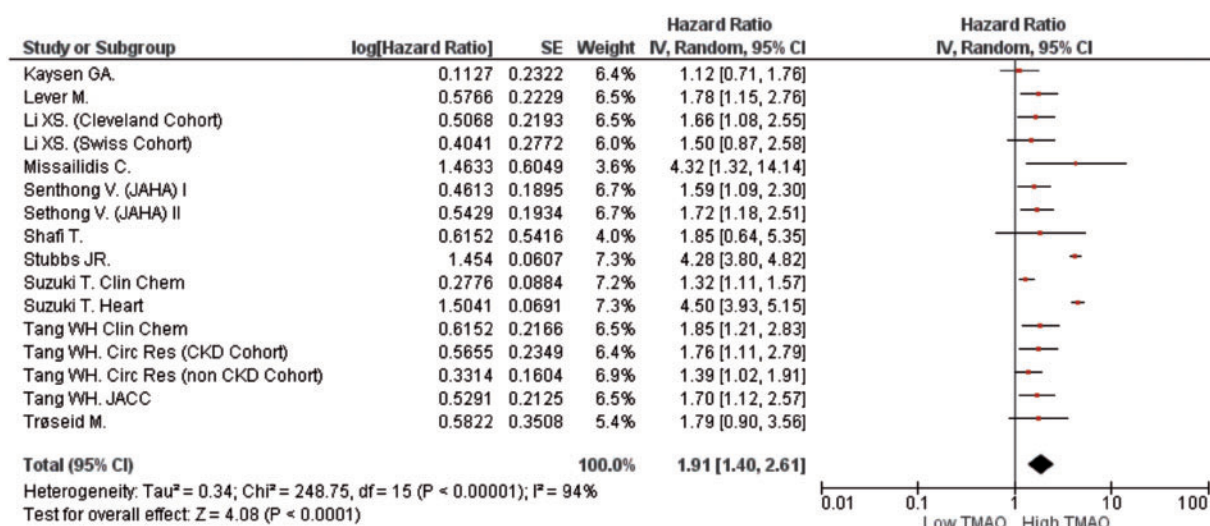
Heterogeneity assesses whether observed differences in results arise by chance alone. To assess the impact of study quality on heterogeneity, we applied the USPTF score to the primary studies included in the meta-analysis. All included studies fell into the categories 'good' or 'fair' (see Supplementary material online, Table S3), allowing us to identify two subgroups ('good' and 'fair') and to perform a subgroups analysis of our primary outcome. As shown in Supplementary material online, Figure S3, high TMAO plasma levels were associated to higher all-cause mortality in both subgroups of studies. Consistently,

the observed heterogeneity appeared to be driven essentially by the 'fair' study group. To further explain the residual heterogeneity, additional subgroup analyses were performed. We speculated that the population size of the studies included in the meta-analysis might have impacted on the heterogeneity. As shown in Supplementary material online, Figure S4, the analysis of all-cause mortality according to the population size revealed that the high heterogeneity was driven by the studies with lower numbers of subjects (i.e.  $<1000$  subjects). It was not possible to analyse the impact of internal study validity on MACCE incidence since all studies reporting this outcome were rated as good.

## Sensitivity analysis

The significantly higher incidence of all-cause mortality in the high TMAO levels group vs. the low TMAO levels group was confirmed





**Figure 2** Impact of trimethylamine-N-oxide (TMAO) plasma levels on all-cause mortality. Random effects hazard ratio (HR) and 95% confidence interval (CI) for all-cause mortality in overall population.

when meta-analyses were repeated removing one study at the time (see Supplementary material online, *Table S4*). For the same purpose, the analyses of all-cause mortality and MACCE were repeated using a fixed-effect model, which confirmed the significantly higher mortality in the high TMAO group vs. low TMAO group in all the subgroups analysed (see Supplementary material online, *Figures S5–S8*). Accordingly, using the fixed-effect model, the association between all-cause mortality and TMAO persisted also after adjustment for internal study validity and number of subjects included in the studies (see Supplementary material online, *Figures S9 and S10*).

## Meta-regression analysis and publication bias

To explore the potential impact of modifiers on the association between TMAO plasma levels and all-cause mortality outcome, we performed a meta-regression analysis of the baseline characteristics reported in more than 10 studies according to Cochrane guidelines.<sup>52</sup> Meta-regression analysis showed no relationship for all analysed effect modifiers and primary outcome of interest (all  $P$ -values  $> 0.05$ ), except for the covariate 'coronary artery disease' (CAD) with an increase of the association between high TMAO levels and mortality in studies with a higher % of CAD patients (*Table 2*, Supplementary material online, *Figure S11*).

Funnel plots and Egger's test did not show any publication bias for the analyses performed (see Supplementary material online, *Figures S12 and S13*).

## Discussion

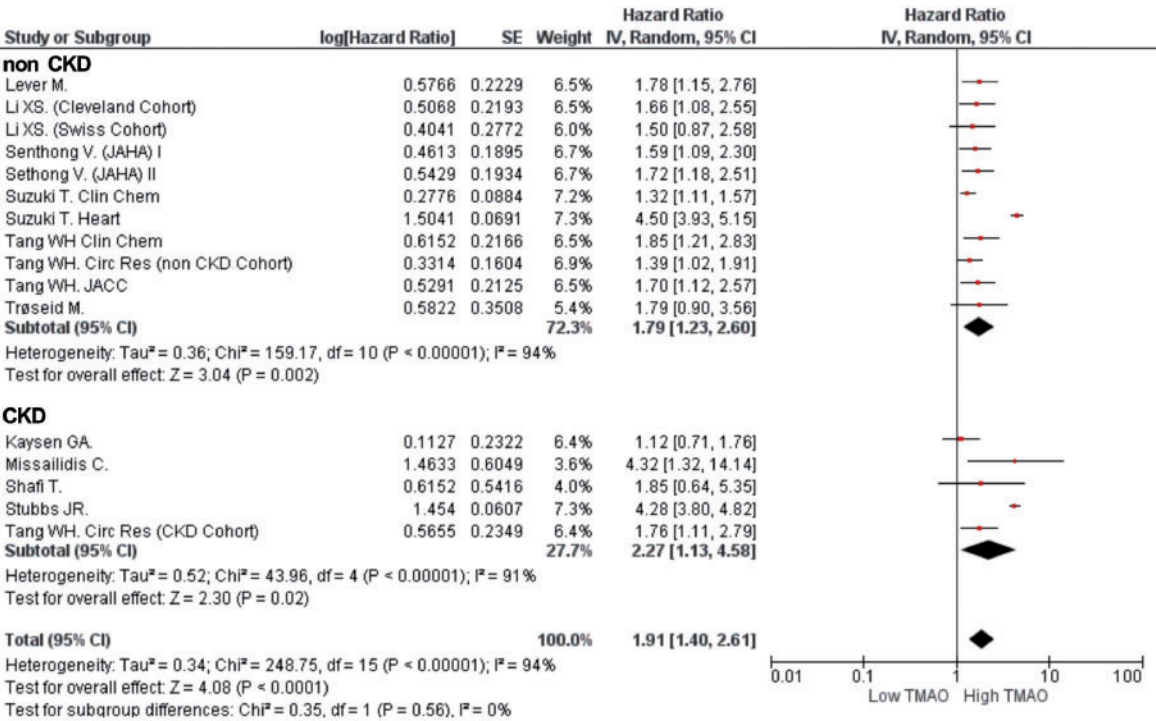
This is the first systematic review and meta-analysis evaluating the association between TMAO plasma levels and risk of mortality and

cardio/cerebrovascular events in a large population of subjects. The main results of our work were as follows. First, high-circulating concentrations of TMAO were associated with increased risk of all-cause mortality and MACCE. Second, there was a dose dependent, direct association between TMAO levels and all-cause mortality. Third, the association between TMAO plasma levels and all-cause mortality was robust in all examined subgroups and across all study populations. Fourth, its association with mortality was particularly strong in subject with CAD. Taken together, these data demonstrate a positive association between TMAO plasma levels and increased cardiovascular risk and mortality.

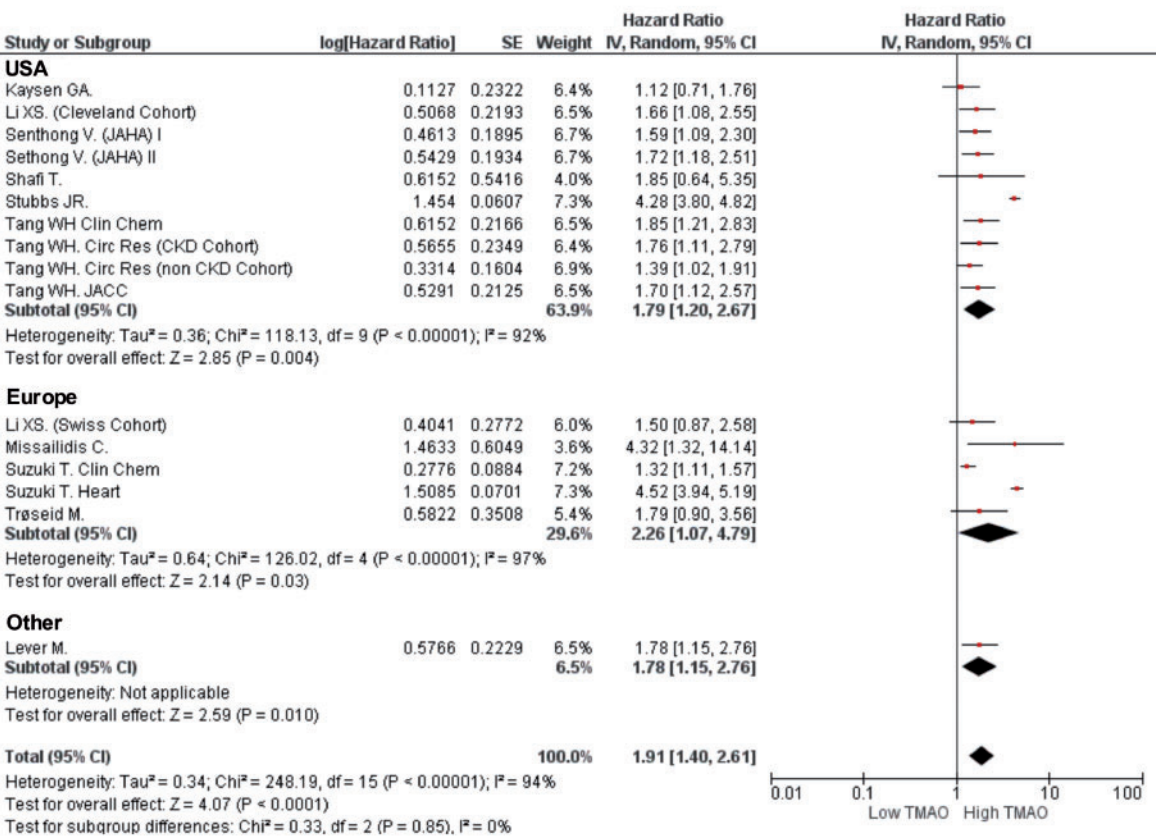
Surprisingly, although previous evidences have shown high-circulating TMAO levels in CKD,<sup>53</sup> our subgroup analysis did not show any significant difference in all-cause mortality in CKD cohort compared with non-CKD subjects. These results need to be considered carefully, and future investigations will be required to explore possible effects of kidney function on modifications of the structure and bioactivity of TMAO.

Another interesting evidence arisen from our study is that the geographic localization of populations included in the studies did not influence the primary outcome. Given the limited number of studies conducted outside 'Western countries', this observation should be taken with caution. It is possible to speculate that the different consumption of TMAO-producing foods might affect the inter-individual distribution of TMAO blood concentrations in the general population. The majority of studies on TMAO and cardiovascular risk were conducted in USA and Europe, and despite potential differences in dietary habitudes between these continents, they yielded similar results. Interestingly, all-cause mortality reported from other continents, based on results from a single study enrolling 475 patients in New Zealand, was also statistically associated with TMAO levels.<sup>22</sup> These results point out the importance to determine the relationship

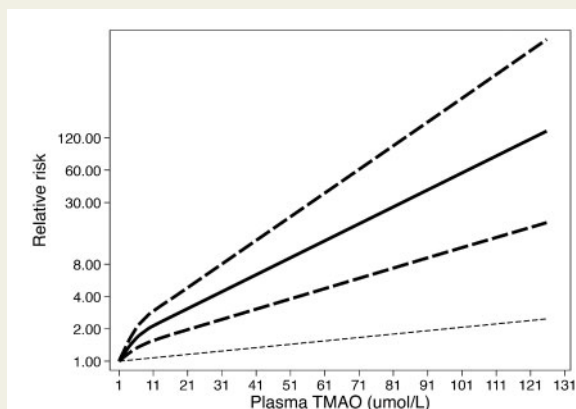
A



B



**Figure 3** Subgroup analyses of the impact of trimethylamine-N-oxide (TMAO) plasma levels on all-cause mortality. Random effects hazard ratio (HR) and 95% confidence interval (CI) for all-cause mortality in chronic kidney disease (CKD) and non-CKD subgroups (A) and according to geographical location of populations included in primary studies (B).



**Figure 4** Dose-response association between trimethylamine-N-oxide (TMAO) plasma levels and all-cause mortality risk Spline (solid line) and 95% confidence intervals (thick dashed lines) of pooled relative risk of all-cause mortality by 10  $\mu\text{mol/L}$  of plasma TMAO. Thin dashed line represents the linear association.

**Table 2** Meta-regression analysis for all-cause mortality

	All-cause mortality		
	Slope	P-value	Number of studies included in the analysis
Publication year	-0.08	0.486	16
Age	-0.06	0.078	14
Female gender	-0.01	0.264	14
BMI	N/A	N/A	5
CAD	0.01	0.007	12
CKD	N/A	N/A	5
COPD	N/A	N/A	1
Diabetes	0.00	0.831	14
Dyslipidaemia	N/A	N/A	9
EF	N/A	N/A	2
Smoke	0.00	0.065	11
Hypertension	0.00	0.864	12
TMAO levels	0.00	0.623	14
hs-CRP levels	N/A	N/A	9
NT-proBNP	N/A	N/A	3

BMI, body mass index; CAD, coronary artery disease; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; EF, ejection fraction; TMAO, trimethylamine N-oxide; hs-CRP, high-sensitivity C reactive protein; NT-pro-BNP, N-terminal pro-brain natriuretic peptide; MPO, myeloperoxidase. N/A, not applicable due to the presence of reported data in less than 10 studies.

between TMAO levels and cardiovascular risk in populations with significantly different dietary environments, including Asia and Middle East.

Meta-regression analysis showed that, except for the influence of CAD, the association of TMAO levels and mortality was not modified by the baseline characteristics of the subject populations included in

the primary studies. In particular, it seems that the presence of CAD further strengthens the proposed association. Based on this analysis, it is not possible to conclude if high TMAO plasma levels increased the risk of CAD or the presence of CAD, by itself, can somehow modify the circulating levels of TMAO. Therefore, this observation will need to be addressed more specifically, taking also into account the differences in microbiota composition and dietary profiles that could happen after any ischaemic insult.

High heterogeneity of TMAO ranges and populations in the included studies represented an important source of variability in interpreting the association between TMAO plasma levels and cardiovascular events. To investigate the source of heterogeneity in our primary analysis, we conducted a series of sensitivity and sub-group analyses. Leave-one-out sensitivity analysis, removing one study at a time from the primary analysis, did not change our main result, indicating that the overall increased risk of mortality in the high TMAO group was stable and significant (see Supplementary material online, Table S4). When the included studies were categorized on the basis of their quality, it turned out that in the subgroup of higher grade quality studies ('good'), the  $I^2$  for all-cause mortality was low (see Supplementary material online, Figure S3). When studies were categorized based on the number of patients included, the higher heterogeneity was found in those with <1000 patients (see Supplementary material online, Figure S4). Finally, conducting all-cause mortality and MACCE analyses with a fixed-effect model (see Supplementary material online, Figures S5–S10) we confirmed our previous random-effect model results. Collectively, these results allow us to conclude that pooling good quality studies with fairly high numbers of subjects confirm the significant association between high TMAO plasma levels and mortality.

Since our meta-analysis was based on results from clinical studies including patients with CVD, the demonstrated association of TMAO plasma levels and analysed outcomes could still be subject to residual confounding or reverse causation bias ('reverse causality'), and therefore does not allow inference of a cause-effect association. Although recent mechanistic pre-clinical studies have identified TMAO as a promising molecule involved in disease progression and associated with cardiovascular events,<sup>3,9,13,20</sup> it is conceivable that the presence of CVD might *per se* affect gut-microbiota composition and lead, in turn, to increased production of TMAO, feeding the vicious circle of 'chicken or egg' dilemma. Therefore, to ultimately address the role of TMAO in primary prevention and potentially identify this molecule as a novel biomarker of cardiovascular risk, further *ad hoc* prospective studies will be needed to clarify the association of TMAO and cardiovascular events in general population.

## Study limitations

This study presents the known limitations of meta-analyses related to the inherent differences in characteristics and definitions of the included studies. Therefore, the results should be interpreted with caution and considered as hypothesis generating. Since the distribution of TMAO levels in the general population is currently unknown and 'standardized normal values' are not currently available, the possibilities to perform a systematic association between specific values of plasmatic TMAO and outcomes were limited. However, we were able to assess the risk of all-cause mortality associated with specific, quantitative values of TMAO as reported in the dose-response



analysis. Moreover, variability in TMAO range concentrations across the studies due to the presence of several different subject populations, contributed to the high heterogeneity observed in this meta-analysis, resolved, in part, by the number of several sensitivity and subgroup analyses we performed.

## Conclusions

Data presented in this study showed a significant positive dose-dependent association between plasma TMAO levels, cardiovascular events, and mortality. Additional meta-analyses based on individual participant data will be required to characterize the shape of these dose-response associations, to characterize the nature of this association in populations with different dietary environments, and to explore the usefulness of TMAO plasma levels in prediction of risk of CVD. Future prospective studies will also be needed to determine whether the modulation of TMAO levels or its precursors might represent a novel potential therapeutic approach to change CVD prognosis.

## Supplementary material

Supplementary material is available at *European Heart Journal* online.

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