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Geographical location affects the levels and association of trimethylamine N-oxide with heart failure mortality: a post-hoc analysis of BIOSTAT-CHF

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Elevated circulating levels of the gut microbiota-derived metabolite, trimethylamine N-oxide (TMAO), are associated with adverse outcomes in heart failure (HF) (1-4). Geographical differences of TMAO levels or on their association with HF outcomes have not been reported. However, we have noticed differences in the reported relationship between TMAO levels and outcomes according to reported geographical region. Independent reports from a British acute HF cohort (1) and from a Norwegian chronic HF cohort (2) showed attenuation of association of TMAO levels with outcomes after adjustment for main confounders, namely renal function. In contrast, in a German chronic HF population, TMAO levels were reported to be associated with mortality and had a better predictive value than N-terminal pro-B-type natriuretic peptide (NT-proBNP) even after adjustment for glomerular filtration rate (eGFR) (3). We therefore wondered whether geographical location might account for this apparent difference (5) and investigated this hypothesis using the BIOSTAT-CHF cohort (4, 6, 7), a multinational study done across 11 countries in Europe in which we recently reported the measurements of plasma TMAO levels and their association with outcomes (4).

A diet-based categorization by country of enrolment was performed using the European Nutrition and Health Report classification (8) into the Northern/Western group (NW: France, Netherlands, Norway, Sweden, and United Kingdom), Central/Eastern group (CE: Germany, Poland, Serbia, and Slovenia), and Southern group (S: Greece and Italy) (Figure 1). The primary study endpoint was 2-year all-cause mortality. The association between TMAO and mortality was assessed by Cox proportional hazards analysis within each geographical group, adjusted for the modified BIOSTAT full risk model (Figure 1) (6) and gender, and for further specific TMAO confounders: eGFR, and body mass index (BMI). The effects of geographical location on addition of TMAO or NT-proBNP to the BIOSTAT risk model for mortality and interaction with TMAO were investigated. Further, genetic effects on TMAO by the enzyme critical for conversion of TMAO from its precursor TMA, the flavin-containing

monooxygenase isoform 3 (FMO3) gene (i.e. four common gene polymorphisms of the A allele of rs2266782 and rs1736557, G allele of rs2266780, and T allele of rs909530) (9), were investigated using linear regression under an additive mode of inheritance further adjusting for the first 10 genetic principle components. Genotyping was carried out on the Affymetrix Axiom UK Biobank array and called using Affymetrix Power Tools 1.16.1. A p-value of <0.05 was considered statistically significant.

Of 2234 patients in BIOSTAT-CHF, 952 (43%) were classified as NW, 714 (32%) as CE, and 568 (25%) as S. Geographical differences in demographics, comorbidities, and mortality were in line with previous reports on geographical characteristics of patients with HF (i.e. CE patients showing a younger age, a higher percentage of ischemic disease, and different outcomes) (5). CE patients were younger, had higher eGFR, and higher percentage of ischemic aetiology compared to NW but were similar to S patients (Figure 1 and Table 1). When adjusted for age, eGFR, and BMI, TMAO levels in CE remained significantly lowest amongst regions (median [IQR]: 6.2 µM [4.8-7.8], 7.2 µM [5.5-8.8], and 6.5 µM [5.0-8.2] respectively for CE, NW and S, p for trend <0.001). Results were essentially similar when there was additional adjustment for protein intake (Maroni formula) (median [IQR]: 6.2 µM [4.8-6.8], 7.2 µM [5.4-8.9], and 6.5 µM [5.0-8.5] respectively for CE, NW and S, p for trend <0.001). Approximately 24% of patients (n=531) reached the primary endpoint during a median follow-up of 21.5 [15.7-24.3] months. Mortality rate varied amongst regions [NW 26.7% (n=254), CE 22.0% (n=157), and S 21.1% (n=120), *p for trend*=0.019]. In a Cox model adjusted for confounders including BIOSTAT risk model factors, the CE group alone showed significant association of higher TMAO levels with mortality (HR: 2.56 (1.44-4.57); *p*=0.001) (Figure 1). When interaction between geographical differences with TMAO or NT-proBNP levels and outcome was investigated, a statistically significant interaction for TMAO alone was observed but not for NT-proBNP (*p interaction*= 0.033 and 0.586 respectively). Further, NT-proBNP levels were

significantly associated with mortality after adjustment for confounders in all groups. However, TMAO levels significantly improved risk prediction when added to the basic model (BIOSTAT risk model and eGFR, BMI and gender) in CE patients alone, as shown by changes in C-statistic (0.723 to 0.748, p=0.031), NRI (21.3 [0.1-42.6], p=0.049) and IDI (2.0 [0.7-3.4], p=0.003) (Supplementary table 1). In contrast, for NT-proBNP there was a statistically significant gain in C-statistic, NRI, and IDI in all groups (Supplementary table 1). Associations of four previously identified genetic variants in the FMO3 gene with TMAO levels were not observed (Supplementary table 2).

This is the first report on effects of regional differences on association between TMAO levels and mortality risk in HF. There are two main findings of the present analysis. First, TMAO levels of HF patients differed by region, even after adjustment for confounders. Second, there was a different association with outcome by region (i.e. mortality risk of patients with elevated TMAO levels was higher in CE patients than in NW and S patients). In addition to these main findings, in CE patients, TMAO levels were predictive of mortality on C-statistic analysis as well as NT-proBNP levels. Finally, known FMO3 gene variants were not associated with TMAO levels. Collectively, our findings demonstrate the different associations of TMAO with HF outcomes in a European population suggesting that geographical differences apart from measured demographic and associated comorbidities might represent at least one possible explanation for this (1-3). The BIOSTAT-CHF cohort has two features that made it an ideal cohort to investigate regional discrepancies. First, patients from different European regions (NW, CE and S) were well represented in our cohort. Second, with more than 99% of patients being Caucasian, the role of ethnicity and the genetic pool was mitigated as possible confounders of underlying geographical differences (5). However, there were several limitations in the present study. We did not have any information regarding the actual dietary

records, gut microbiota composition or intestinal permeability to confirm the impact of diet on TMAO levels.

In conclusion, geographical differences affect the levels and association of TMAO with heart failure mortality, regardless of main confounders. There may still be other under-investigated factors that affect associations of TMAO and HF adverse outcomes (e.g. changes in gut flora with age, and association with dietary history and/or physical activity).

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Conflict of Interest

All of the other authors have no conflicts to report.

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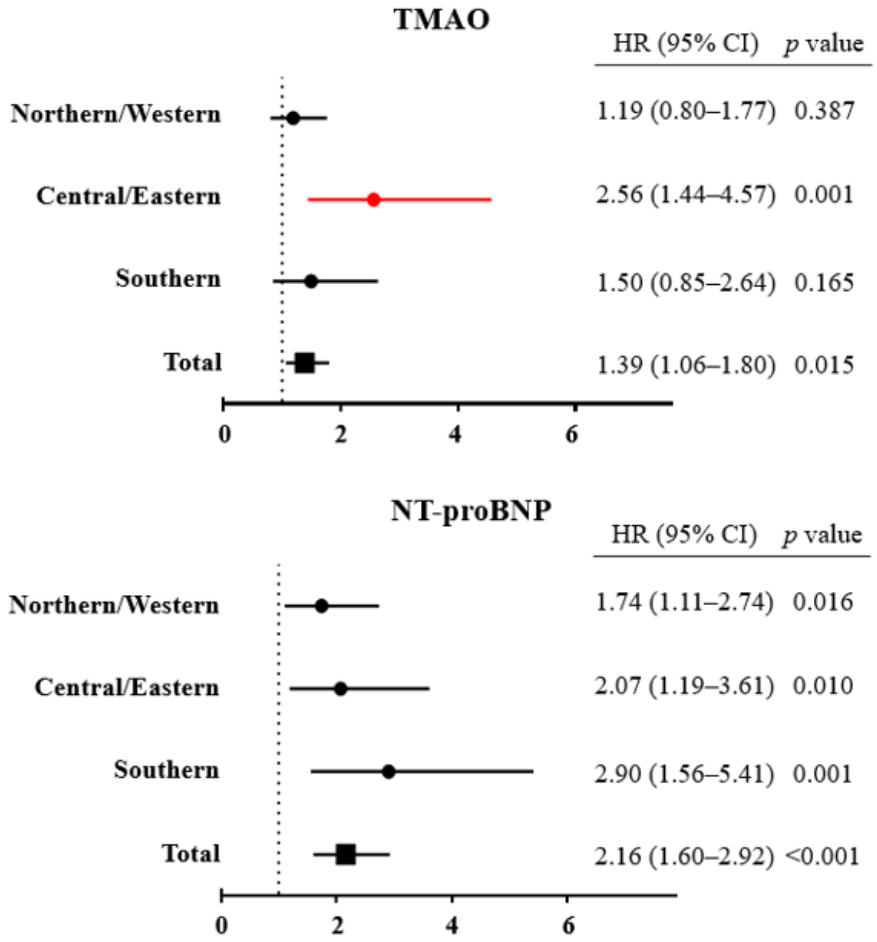
Table 1. Patient characteristics regarding geographical groups.

	Northern/Western (n=952)	Central/Eastern (n=714)	Southern (n=568)	*p for trend*
Demographics				
Age	74 [64–80] †	67 [59–75]	68 [59–76] †	<0.001
Male	627 (66%)*	548 (77%)	479 (84%)	<0.001
Body mass index (kg/m2)	26.9 [23.7–30.9] *	27.5 [24.7–31.1]	27.5 [24.4–29.8]	0.005
Current smoker	122 (13%)	106 (15%)	84 (15%)	0.407
Ischemic aetiology	477 (51%)*	432 (62%)	305 (56%)	<0.001
Hypertension	516 (54%)*	540 (76%)	345 (61%)*	<0.001
Diabetes mellitus	273 (29%)	246 (35%)	211 (37%)	0.001
Atrial fibrillation	470 (49%) †	293 (41%)	243 (43%)	0.002
COPD	180 (19%) †	101 (14%)	106 (19%)	0.025
Previous HF hospitalisation	260 (27%)*	229 (32%)	214 (38%)	<0.001
NYHA class (%) I/II/III/IV	2/34/51/12†	1/39/53/7	5/33/45/17†	<0.001
LV ejection fraction (%)	30 [25–40] †	30 [25–36]	30 [25–35]	<0.001
Clinical signs				
Pulmonary congestion	513 (57%)†	325 (46%)	311 (56%) †	<0.001
Peripheral oedema	523 (69%) †	358 (55%)	222 (50%)*	<0.001
Systolic blood pressure (mmHg)	120 [110–139] *	125 [110–140]	120 [110–130] *	<0.001
Diastolic blood pressure (mmHg)	71 [63–83] *	78 [70–85]	70 [69–80] *	<0.001
Heart rate (beat/min)	79 [68–95] †	75 [66–84]	75 [66–86] †	<0.001
Medication				
Beta-blocker	755 (79%)*	629 (88%)	479 (84%)	0.017
ACE inhibitor or ARB	673 (71%)*	569 (80%)	396 (70%)*	<0.001
MRA	379 (40%)*	475 (67%)	339 (60%)	<0.001
Diuretics	950 (100%)	714 (100%)	568 (100%)	0.260
Laboratory				
Haemoglobin (g/dL)	13.0 [11.7-14.4] *	13.7 [12.3-14.7]	13.2 [11.8-14.4] *	<0.001
Urea (mmol/L)	9.0 [6.7-13.1]	9.5 [6.9-15.0]	18.2 [12.5-26.1]	<0.001
Sodium (mmol/L)	139 [137-141] *	140 [138-142]	139 [137-142] *	<0.001
Outcomes (2 years)				
Mortality	254 (27%) †	157 (22%)	120 (21%)	0.019

Data are expressed as median [interquartile range] for continuous variables or n (%) for categorical values. *P* values are quoted for Kruskal-Wallis tests for continuous variables and Chi-square tests for categorical variables. ACE=angiotensin-converting enzyme; ARB=angiotensin receptor blocker; COPD=chronic obstructive pulmonary disease; HF=heart failure; LV=left ventricular; MRA=mineralocorticoid receptor antagonist; NT-proBNP=N-terminal pro-B-type natriuretic peptide; NYHA=New York Heart Association;

†Significantly higher compared to CE; *significantly lower compared to CE pairwise analyses.



	Northern/Western (n=952)	Central/Eastern (n=714)	Southern (n=568)	*p for trend*
TMAO (µmol/L)	**7.0 [4.1–13.6]**	**4.6 [2.9–7.5]**	**6.2 [3.6–11.5]**	< 0.001
Protein intake (g/day)	52.1 [45.5–60.5]	56.9 [48.1–65.3]	52.7 [46.1–60.4]	< 0.001
eGFR (ml/min/1.73m^2)	59 [44-78]	66 [51-83]	63 [49-79]	< 0.001
NT-proBNP (pg/mL)	3567 [1813-6775]	2025 [833-5008]	2128 [975-4721]	< 0.001
Mortality at 2 years	254 (27%)	157 (22%)	120 (21%)	0.019

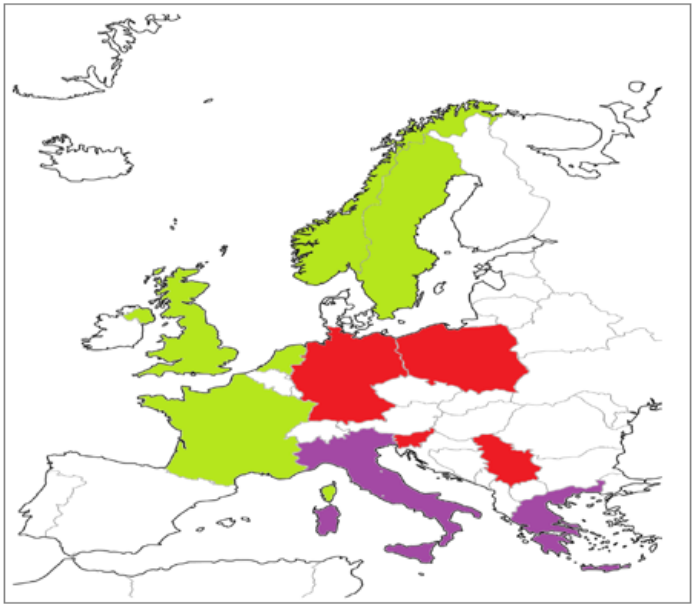


Figure 1. Association between TMAO/NT-proBNP and all-cause mortality at 2 years according to geographical groups.

Forest plot of the hazard ratio (HR) of 2-year mortality risk per log TMAO/NT-proBNP increase. Whiskers represent 95% confidence intervals (CI).HRs adjusted for modified BIOSTAT-CHF full risk model factors (age, log urea, haemoglobin, use of beta-blocker at baseline, log NT-proBNP/ log TMAO, ischemic aetiology, chronic obstructive pulmonary disease, diastolic blood pressure, and sodium), glomerular filtration rate, body mass index, and gender.

Supplementary table 1. Added value performance for TMAO or BNP over the BIOSTAT risk model according to geographical groups.

	C-statistic		p value	NRI [% (95% CI)]	p value	IDI [% (95% CI)]	p value
	Basic model	adding					
TMAO							
North and West	0.716	0.717	0.879	7.3 (-9.0 - 23.6)	0.381	0.1 (-0.1 - 0.2)	0.574
Centre and East	0.723	0.748	0.031	21.3 (0.1 - 42.6)	0.049	2.0 (0.7 - 3.4)	0.003
South	0.733	0.736	0.663	12.1 (-11.6 - 35.8)	0.316	0.4 (-0.4 - 1.3)	0.310
NT-proBNP							
North and West	0.716	0.742	0.008	35.6 (19.5 - 51.7)	<0.001	2.7 (1.4 - 4.0)	<0.001
Centre and East	0.723	0.754	0.026	50.9 (30.4 - 71.4)	<0.001	4.2 (2.2 - 6.2)	<0.001
South	0.733	0.778	0.024	53.6 (30.9 - 76.3)	<0.001	6.7 (3.9 - 9.6)	<0.001

IDI= integrated discrimination improvement; NRI= net reclassification improvement; NT-proBNP=N-terminal pro-B-type natriuretic peptide; TMAO=trimethylamine-N-oxide. Basic model: modified BIOSTAT risk model included age, log urea, haemoglobin, use of beta-blocker at baseline, ischemic aetiology, chronic obstructive pulmonary disease, diastolic blood pressure, and sodium, and eGFR, BMI, and gender.

Supplementary table 2. Association between genetic variants of FMO3 and TMAO levels.

SNP	Position (b37)	EA	EAF	Beta	95% CI	*P* value
rs2266782	171076966	A	0.399	-0.019	-0.091, 0.053	0.604
rs1736557	171080080	A	0.072	-0.043	-0.105, 0.020	0.183
rs909530	171083174	T	0.220	-0.008	-0.108, 0.092	0.878
rs2266780	171083242	G	0.162	-0.009	-0.062, 0.045	0.756

SNP= Single nucleotide polymorphism; EA= Effect allele; EAF= Effect allele frequency. Results are adjusted for gender, age, geographical location, BMI, eGFR, and the first 10 genetic principle components.