Supplementary material: Plasma Trimethylamine N-oxide and risk of cardiovascular events in patients with type 2 diabetes

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Plasma Trimethylamine N-oxide and risk of cardiovascular events in patients with type 2 diabetes

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Quantification of plasma TMAO and related metabolites – TMAO and its precursors (betaine, choline and carnitine) were analyzed in plasma samples by liquid chromatography-tandem mass spectrometry (LC-MS/MS). All solvents were LC-MS grade and purchased from Biosolve (Valkenswaard, Netherlands). Standard compounds were obtained from Sigma Aldrich (Saint-Quentin Fallavier, France). A pool of reference standard solutions was prepared and serially diluted in acetonitrile to obtain 7 standard solutions ranging 0.05-100 µmol/L. Ten microliters (10 µL) of exogenous internal standards at 25 µmol/L in acetonitrile ($^{2}H_9$-choline, $^{2}H_9$-carnitine, $^{13}C_2$-betaine, and $^{2}H_9$-TMAO) were added to 20 µL of standard solutions and plasma samples. All samples were then treated with 75 µL of *ter*-butyl-bromoacetate at 50 mmol/L in acetonitrile and 10 µL of 70% ammonium hydroxide solution before being mixed and incubated in the dark at room temperature for 30 min. Then, 50 µL of acetonitrile containing 1% formic acid were added and samples were centrifuged 10 min at 10,000 × g (20 °C). Supernatants were then transferred to vials for LC-MS/MS analyses that were performed on a Xevo® TQD mass spectrometer with an electrospray interface and an Acquity H-Class® UPLC™ device (Waters Corporation, Milford, MA, USA). Samples (5 µL) were injected onto a HILIC-BEH column (1.7 µm; 2.1 × 100 mm, Waters Corporation) held at 35 °C, and compounds were separated with a linear gradient of mobile phase B (98% acetonitrile, 0.1% formic acid) in mobile phase A (10 mmol/L ammonium acetate, 0.1% formic acid) at a flow rate of 400 µL/min. Mobile phase A was kept constant for 1 min at 1%, linearly increased from 1% to 45% for 6.5 min, kept constant for 1 min, returned to the initial condition over 1 min, and kept constant for 1.5 min before the next injection. Targeted compounds were then detected by the mass spectrometer with the electrospray interface operating in the positive ion mode (capillary voltage, 1.5 kV; desolvatation gas (N_2) flow and temperature, 650 L/h and 350 °C; source temperature, 150 °C). The multiple reaction monitoring mode was applied for MS/MS detection as detailed in supplementary Table 1. Chromatographic peak area ratios between unlabeled compounds and their respective internal standards constituted the detector responses. Standard solutions were used to plot calibration curves for quantification. Linearity was expressed by mean r^2, which was greater than 0.997 for all compounds (linear regression, 1/x weighting, origin excluded). The intra- and inter-assay imprecisions of the analytical method were assessed throughout experiments in spiked samples with known

concentrations (16 experiments, 5 replicates per experiment for 4 spiked concentrations), and were below 9.7% for all compounds. At completion of the study, a representative set of samples (10% of the cohort) was arbitrarily reanalyzed and the new concentrations did not vary by more than 5.6% in comparison with the first analysis. Recoveries were assessed with internal standards and exceeded 96%.

Supplemental Table S1 – Multiple Reaction Monitoring (MRM) parameters used for LC-MS/MS analysis.

Compounds	**MRM (m/z)**	**Cone voltage (V)**	**Collision energy (eV)**
TMAO	75.9 → 58.9	20	11
$^{2}H_{9}$-TMAO	85.0 → 68.0	20	11
Betaine	118.1 → 58.1	40	22
$^{13}C_{2}$-betaine	120.1 → 58.1	40	22
Choline	104.1 → 60.1	40	15
$^{2}H_{9}$-choline	113.2 → 69.1	40	15
Carnitine	162.1 → 103.0	25	14
$^{2}H_{9}$-carnitine	171.1 → 69.1	25	14

Supplemental Table S2 – Recovery rates of TMAO and its derivates in samples, after 1, 2 and 3 freeze/thaw cycles in 10 independent samples.

Compounds	**Freeze/thaw cycle 1**	**Freeze/thaw cycle 2**	**Freeze/thaw cycle 3**	**Recovery rate cycle 2 vs 1**	**Recovery rate cycle 3 vs 1**
TMAO	7.13 (3.23- 37.03)	7.32 (3.38- 35.55)	7.98 (3.46- 45.61)	102.1 ± 3.19	110.3 ± 5.78
Betaine	42.09 (20.57- 58.97)	43.39 (19.06- 58.95)	42.35 (21.24- 57.04)	100.3 ± 5.29	101.1 ± 5.28
Choline	1.52 (0.79- 2.59)	1.56 (0.85- 2.69)	1.63 (0.98- 2.82)	103.8 ± 5.84	111.8 ± 8.64
Carnitine	44.97 (23.3- 69.91)	44.26 (26.11- 71.50)	44.02 (25.00- 71.52)	101.0 ± 5.02	102.2 ± 5.60

Data are expressed as median (min-max) and recovery rate was calculated as:

Concentration at freeze/thaw cycle (2 or 3) × 100 ÷ concentration at freeze/thaw cycle 1.

Supplemental Table S3 – Multivariable Cox model analysis for risk of MACE.

MACE	TMAO			Betaine			Choline		
	HR	**95% CI**	***p* value**	**HR**	**95% CI**	***p* value**	**HR**	**95% CI**	***p* value**
Model 1									
Sex (reference men)	0.84	0.68-1.03	0.1007	0.86	0.70-1.06	0.1542	0.88	0.72-1.09	0.2450
Age (yr)	1.06	1.05-1.08	<0.0001	1.07	1.05-1.08	<0.0001	1.06	1.05-1.07	<0.0001
Personal history of MI (reference no)	1.86	1.47-2.35	<0.0001	1.80	1.42-2.28	<0.0001	1.70	1.34-2.15	<0.0001
Biomarker (reference: Q1-Q3)			<0.0001			0.0903			<0.0001
Q4	1.86	1.52-2.28		1.21	0.97-1.52		1.72	1.39-2.12	
Model 2									
Sex (reference men)	0.86	0.70-1.07	0.1758	0.87	0.70-1.08	0.1998	0.87	0.70-1.08	0.1943
Age (yr)	1.05	1.04-1.07	<0.0001	1.05	1.04-1.07	<0.0001	1.05	1.04-1.07	<0.0001
Personal history of MI (reference no)	1.70	1.34-2.16	<0.0001	1.65	1.30-2.09	<0.0001	1.65	1.30-2.10	<0.0001
eGFR	0.92	0.87-0.97	0.0025	0.90	0.86-0.95	<0.0001	0.91	0.86-0.96	0.0005
uACR (log)	1.55	1.36-1.76	<0.0001	1.55	1.37-1.77	<0.0001	1.55	1.36-1.76	<0.0001
Biomarker (reference: Q1-Q3)			0.019			0.265			0.239
Q4	1.32	1.05-1.66		1.14	0.91-1.43		1.15	0.91-1.46	
Model 3									
Sex (reference men)	0.86	0.70-1.07	0.1684	0.85	0.68-1.05	0.1283	0.86	0.69-1.07	0.1648
Age (yr)	1.04	1.03-1.06	<0.0001	1.04	1.03-1.06	<0.0001	1.04	1.03-1.06	<0.0001
Personal history of MI (reference no)	1.35	1.05-1.72	0.0172	1.33	1.04-1.70	0.0243	1.31	1.03-1.68	0.0286
eGFR	1.00	0.95-1.06	0.9325	0.98	0.93-1.03	0.4676	0.99	0.93-1.05	0.6615
uACR (log mg/mmol)	1.34	1.17-1.53	<0.0001	1.35	0.18-1.54	<0.0001	1.34	1.17-1.53	<0.0001
NT-proBNP (log pg/mL)	2.36	1.91-2.91	<0.0001	2.37	1.92-2.92	<0.0001	2.35	1.91-2.89	<0.0001
Biomarker (reference: Q1-Q3)			0.0282			0.8973			0.4493
Q4	1.31	1.03-1.66		0.99	0.78-1.24		1.10	0.86-1.40	

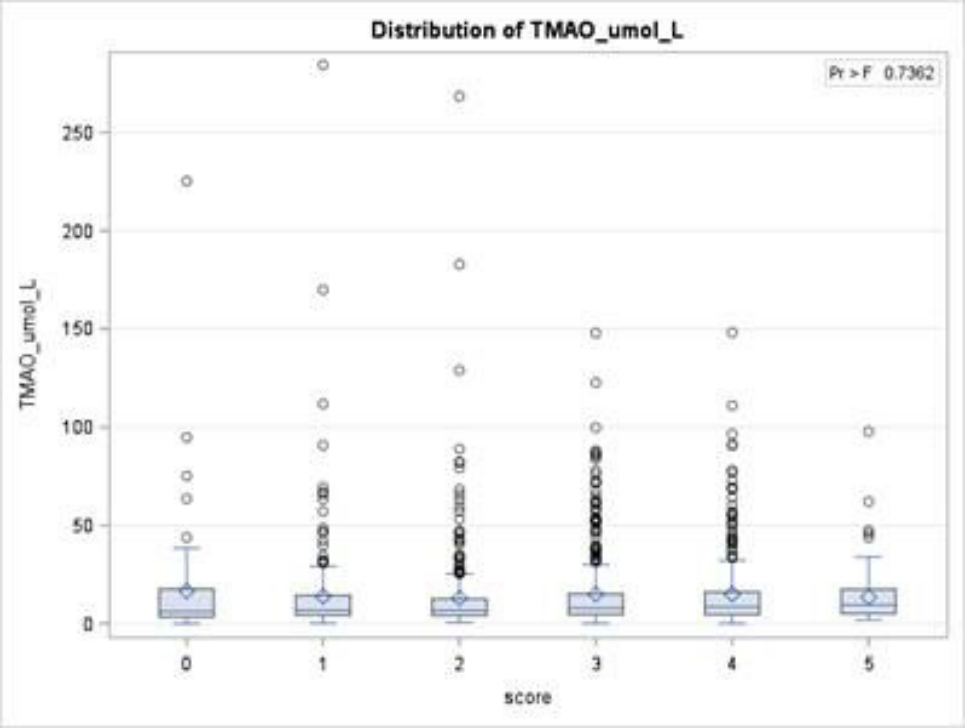
HR, hazard ratio; CI, confidence interval; TMAO, trimethylamine N-oxide; MI, myocardial infarction; eGFR, estimated glomerular filtration rate; uACR, urine albumin-to-creatinine ratio; NT-proBNP, N-terminal pro-brain natriuretic peptide; Q, quartile.

Supplemental Table S4 – Multivariable Cox model analysis for risk of all-cause mortality

Mortality	TMAO			Betaine			Choline		
	HR	95% CI	p value	HR	95% CI	p value	HR	95% CI	p value
Model 1									
Sex (reference men)	0.65	0.54-0.77	<0.0001	0.68	0.57-0.81	<0.0001	0.70	0.59-0.84	<0.0001
Age (yr)	1.08	1.07-1.09	<0.0001	1.08	1.07-1.09	<0.0001	1.08	1.07-1.09	<0.0001
Biomarker (reference: Q1-Q3)			<0.0001			0.0024			<0.0001
Q4	1.79	1.50-2.13		1.34	1.11-1.62		1.64	1.37-1.97	
Model 2									
Sex (reference men)	0.66	0.55-0.79	<0.0001	0.70	0.58-0.83	<0.0001	0.72	0.60-0.86	0.0003
Age (yr)	1.08	1.07-1.09	<0.0001	1.08	1.07-1.09	<0.0001	1.07	1.06-1.08	<0.0001
Sinus rhythm (reference yes)	1.82	1.37-2.44	<0.0001	1.89	1.41-2.50	<0.0001	1.82	1.35-2.44	<0.0001
SBP (mm Hg)	1.01	1.00-1.01	0.0005	1.01	1.00-1.01	0.0003	1.01	1.00-1.01	0.0004
Biomarker (reference: Q1-Q3)			<0.0001			0.0053			<0.0001
Q4	1.75	1.17-2.09		1.31	1.08-1.58		1.59	1.33-1.91	
Model 3									
Sex (reference men)	0.64	0.54-0.77	<0.0001	0.67	0.56-0.80	<0.0001	0.65	0.54-0.78	<0.0001
Age (yr)	1.07	1.06-1.08	<0.0001	1.07	1.06-1.08	<0.0001	1.07	1.06-1.08	<0.0001
Sinus rhythm (reference yes)	1.54	1.15-2.08	0.0036	1.54	1.15-2.08	0.0040	1.56	1.15-2.08	<0.0033
SBP (mm Hg)	1.01	1.00-1.01	0.0164	1.01	1.00-1.01	0.0154	1.01	1.00-1.01	0.0152
Angptl2 (log ng/mL)	1.77	1.07-2.94	0.0275	1.81	1.09-2.99	00212	1.80	1.08-2.98	0.0234
sTNFR1 (log pg/mL)	5.46	3.41-8.75	<0.0001	6.05	3.86-9.50	<0.0001	5.73	3.59-9.12	<0.0001
Biomarker (reference: Q1-Q3)			0.1514			0.0094			0.3621
Q4	1.16	0.95-1.42		1.18	1.06-1.55		1.10	0.90-1.34	

HR, hazard ratio; CI, confidence interval; TMAO, trimethylamine N-oxide; SBP, systolic blood pressure; Angptl2, angiopoietin-2; sTNFR1, soluble tumor necrosis factor receptor 1; Q, quartile.

Supplementary Figure - Relationship between the number of markers of insulin resistance and TMAO concentrations.



"HOMA-IR proxy Score" was not associated with TMAO in our dataset (p=0.7362).

The following clinical characteristics were taken into account, following a modified IDF definition of the metabolic syndrome to identify the number of markers of insulin resistance:

1/ Obesity (of note waist circumference was unfortunately not available in our study)

- Up to 24.9 Kg/m2: 0 point
- 25- 29.9 Kg/m2: 1 point
- Above 30: 2 points

2/ Triglycerides

- Fasting TG concentration > 149 mg/dl and/or fibrate treatment: 1 point (otherwise 0 point)

3/ HDL-C

- Fasting HDL-C concentration < 40 mg/dl in males and 50 mg/dl in females: 1 point (otherwise 0 point)

4/ Blood pressure (BP treatment was not considered, as BP medications were not specific for hypertension but could be also used for CVD/renal risk management)

- SBP/DBP equal to or higher than 135/85 mm Hg: 1 point (otherwise 0 point)