Electronic Supplementary Material

Trimethylamine *N*-oxide and prognosis in acute heart failure

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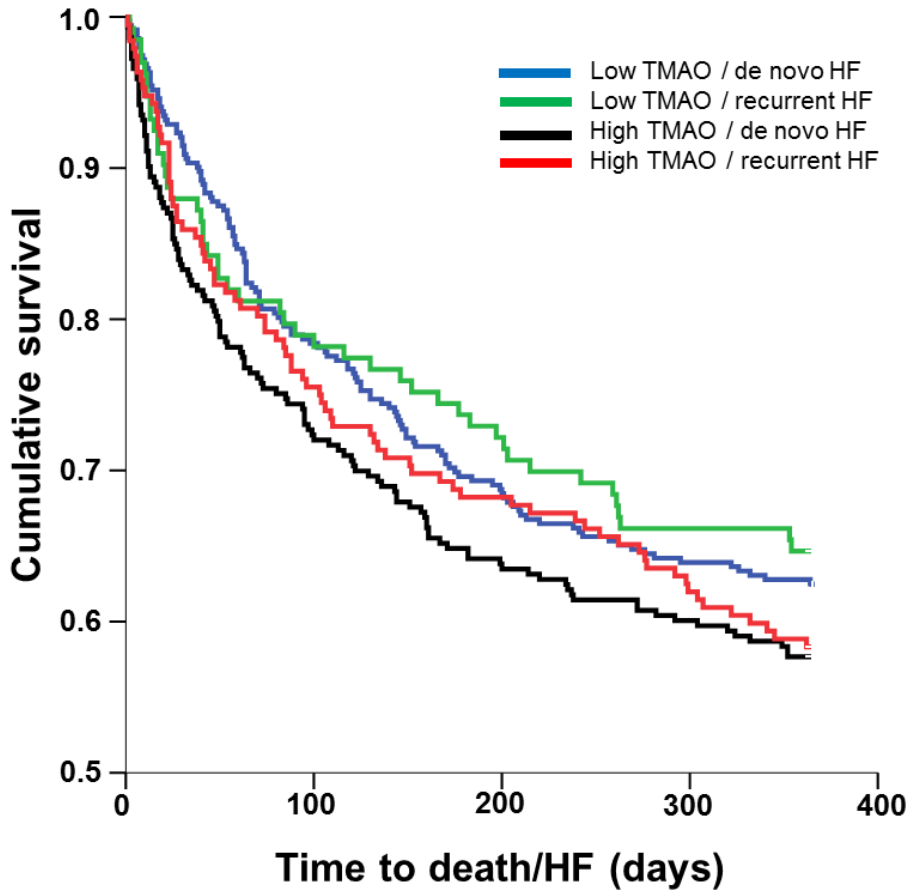
Sample preparation and analysis

Samples were prepared for analysis as reported previously [Heaney et al. 2015] using stable-isotope dilution by mixing 20 µL of plasma with 80 µL of 10 µmol/L deuterated TMAO (D9-TMAO) in methanol. Protein precipitation was achieved by a 1 min vortex period followed by centrifugation at 16900 x g for 20 min. After centrifugation, the supernatant was removed and transferred to a vial for analysis.

Extracted samples were analysed by liquid chromatography-time of flight mass spectrometry with multiple reaction monitoring, following a method described previously [Heaney et al. 2015]. Briefly, an Acquity UPLC with a 100 mm Acquity UPLC BEH HILIC analytical column was coupled to a Synapt G2-S high resolution mass spectrometer (Waters Corp., Milford, MA, USA). Mobile buffer A was 0.025% ammonium hydroxide, 0.045% formic acid (pH 8.1) and buffer B pure acetonitrile. A concentration gradient was applied starting with 95% B and reducing linearly to 4% at 0.8 min and returning to 95% B by 1.9 min and being held until a total analysis time of 2.5 min. TMAO and D9-TMAO were monitored using precursor ions of m/z 76.1 and 85.1 and their product ions of m/z 58.066/59.073 and 66.116/68.130, respectively. Peak areas were calculated on the primary product ion and response ratios of TMAO : D9-TMAO were calculated. Results were converted to and reported in µmol/L using a previously defined calibration.

Control reagents for TMAO were purchased from Sigma-Aldrich (Gillingham, UK) and D9-TMAO from Cambridge Isotopes (Cambridge, MA, USA). Water, acetonitrile, methanol, formic acid (all Optima™ LC/MS grade) and 25% extra pure ammonium hydroxide in H2O (Acros Organics) were purchased from Fisher Scientific (Loughborough, UK).

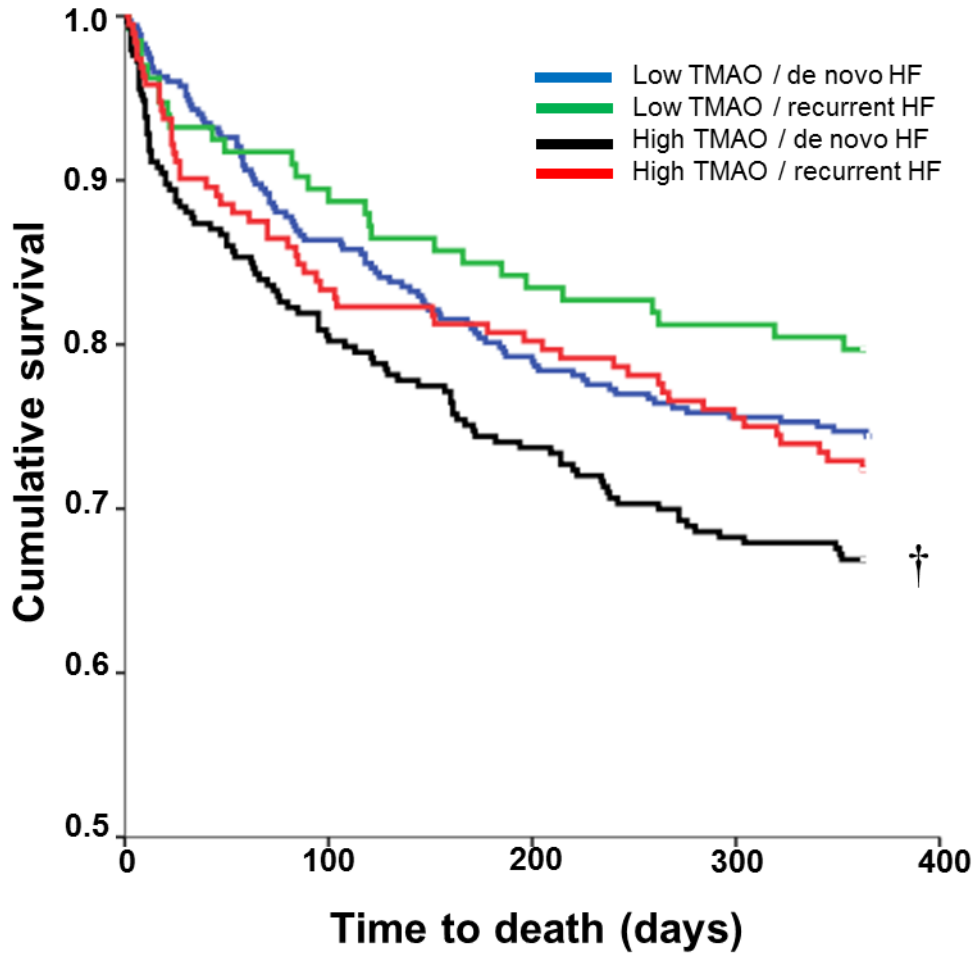
Figure S1. Kaplan-Meier survival curves for outcome of death or rehospitalisation due to heart failure at one year (Death/HF) with patients stratified by above or below median trimethylamine *N*-oxide (TMAO) and recurrent or de novo heart failure (HF). Statistical *p* values for each curve comparison by log rank test are detailed below.



Unadjusted p Values	**Low TMAO de novo HF**	**Low TMAO recurrent HF**	**High TMAO de novo HF**	**High TMAO recurrent HF**
Low TMAO de novo HF				
Low TMAO recurrent HF	0.682			
High TMAO de novo HF	0.144	0.149		
High TMAO recurrent HF	0.368	0.292	0.720	

Note: A Bonferroni adjusted α value of 0.0083 is deemed as significant

Figure S2. Kaplan-Meier survival curves for outcome of all-cause mortality at one year with patients stratified by above or below median trimethylamine *N*-oxide (TMAO) and recurrent or de novo heart failure (HF). Statistical *p* values for each curve comparison by log rank test are detailed below.



Unadjusted p Values	**Low TMAO de novo HF**	**Low TMAO recurrent HF**	**High TMAO de novo HF**	**High TMAO recurrent HF**
Low TMAO de novo HF				
Low TMAO recurrent HF	0.220			
High TMAO de novo HF	0.032	0.008†		
High TMAO recurrent HF	0.632	0.141	0.178	

Note: A Bonferroni adjusted α value of 0.0083 is deemed as significant

Figure S3. 3D column chart to show the percentage contribution of total in-hospital deaths stratified by above and/or below median values for trimethylamine N-oxide (TMAO) and N-terminal pro B-type natriuretic peptide (NT-proBNP)

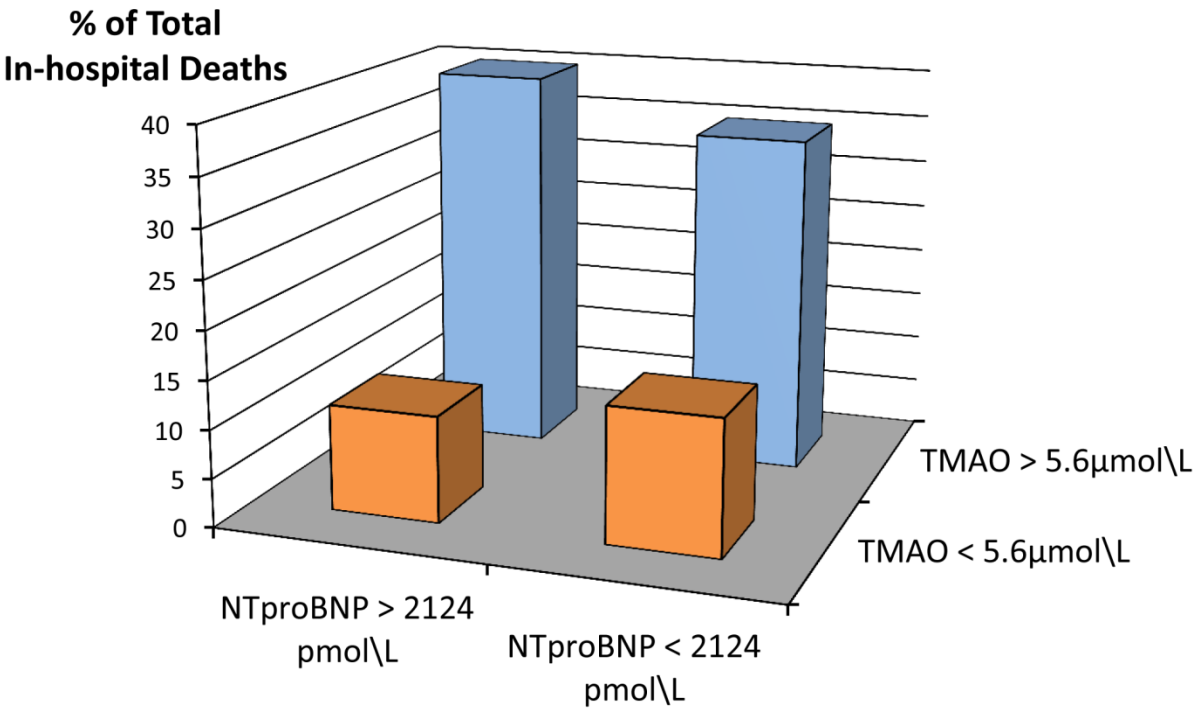


Table S1. Cox hazard rations (HR) with 95 % confidence intervals (lower and upper CI) for cardiac risk factors (including cardiac troponin, *n* = 503) in multivariate analysis for death or heart failure rehospitalisation at one year, without inclusion of renal indices.

Variable	HR	Lower CI	Upper CI	*p* Value
Age	1.02	1.00	1.04	0.016
Sex	1.01	0.75	1.37	0.934
PH heart failure	1.35	0.99	1.84	0.061
PH IHD	1.04	0.77	1.40	0.808
PH hypertension	0.77	0.57	1.04	0.084
PH diabetes	1.36	0.99	1.87	0.059
NYHA class	1.58	1.21	2.07	0.001
Current smoker	1.07	0.66	1.75	0.777
Oedema	0.83	0.62	1.12	0.233
Atrial Fibrillation	0.84	0.63	1.13	0.252
Systolic BP	1.00	0.99	1.00	0.104
Heart rate	0.99	0.99	1.00	0.057
Heamoglobin	1.00	0.99	1.01	0.663
Respiratory rate	1.04	1.02	1.06	0.001
Sodium	0.98	0.95	1.01	0.135
NT-proBNP	1.14	0.92	1.42	0.239
TMAO	1.27	1.10	1.46	0.001
Cardiac troponin	0.97	0.91	1.03	0.304

BP = blood pressure; IHD = ischemic heart disease; NT-proBNP = N-terminal pro B-type natriuretic peptide; NYHA = New York Heart Association; PH = past history; TMAO = trimethylamine N-oxide

Table S2. Cox hazard rations (HR) with 95 % confidence intervals (lower and upper CI) for cardiac risk factors (including cardiac troponin, *n* = 503) in multivariate analysis for death or heart failure rehospitalisation at one year, with inclusion of renal indices.

Variable	HR	Lower CI	Upper CI	*p* Value
Age	1.02	1.00	1.03	0.045
Sex	1.00	0.72	1.37	0.983
PH heart failure	1.22	0.89	1.68	0.217
PH IHD	1.03	0.76	1.40	0.836
PH hypertension	0.77	0.57	1.05	0.095
PH diabetes	1.30	0.94	1.79	0.113
NYHA class	1.48	1.13	1.94	0.005
Current smoker	1.17	0.72	1.91	0.527
Oedema	0.81	0.60	1.10	0.179
Atrial Fibrillation	0.88	0.65	1.19	0.410
Systolic BP	1.00	0.99	1.00	0.321
Heart rate	0.99	0.99	1.00	0.054
Heamoglobin	1.00	0.99	1.01	0.958
Respiratory rate	1.04	1.02	1.06	0.001
Sodium	0.97	0.95	1.00	0.059
NT-proBNP	1.05	0.85	1.30	0.650
TMAO	1.09	0.92	1.29	0.326
Cardiac troponin	0.97	0.91	1.03	0.250
Urea	1.03	1.00	1.06	0.023
eGFR	0.99	0.98	1.00	0.157

BP = blood pressure; eGFR = estimated glomerular filtration rate; IHD = ischemic heart disease; NT-proBNP = N-terminal pro B-type natriuretic peptide; NYHA = New York Heart Association; PH = past history; TMAO = trimethylamine N-oxide

Table S3. Cox hazard rations (HR) with 95 % confidence intervals (lower and upper CI) for cardiac risk factors (including cardiac troponin, *n* = 503) in multivariate analysis for death at one year, without inclusion of renal indices.

Variable	HR	Lower CI	Upper CI	*p* Value
Age	1.04	1.02	1.06	0.001
Sex	1.11	0.77	1.61	0.574
PH heart failure	1.07	0.73	1.57	0.722
PH IHD	0.85	0.60	1.22	0.390
PH hypertension	0.62	0.43	0.88	0.008
PH diabetes	1.38	0.94	2.04	0.101
NYHA class	1.60	1.15	2.22	0.005
Current smoker	1.18	0.65	2.12	0.587
Oedema	0.93	0.65	1.33	0.689
Atrial Fibrillation	0.84	0.59	1.21	0.352
Systolic BP	0.99	0.98	0.99	< 0.0005
Heart rate	1.00	0.99	1.00	0.132
Heamoglobin	0.99	0.98	1.00	0.116
Respiratory rate	1.05	1.02	1.08	< 0.0005
Sodium	0.95	0.92	0.98	0.003
NT-proBNP	1.29	0.97	1.72	0.077
TMAO	1.23	1.04	1.46	0.016
Cardiac troponin	0.98	0.92	1.03	0.372

BP = blood pressure; IHD = ischemic heart disease; NT-proBNP = N-terminal pro B-type natriuretic peptide; NYHA = New York Heart Association; PH = past history; TMAO = trimethylamine N-oxide

Table S4. Cox hazard rations (HR) with 95 % confidence intervals (lower and upper CI) for cardiac risk factors (including cardiac troponin, *n* = 503) in multivariate analysis for death at one year, with inclusion of renal indices.

Variable	HR	Lower CI	Upper CI	*p* Value
Age	1.03	1.01	1.06	0.002
Sex	1.12	0.76	1.66	0.577
PH heart failure	0.94	0.64	1.39	0.756
PH IHD	0.85	0.59	1.23	0.385
PH hypertension	0.61	0.43	0.88	0.007
PH diabetes	1.32	0.89	1.96	0.165
NYHA class	1.42	1.02	1.97	0.038
Current smoker	1.35	0.75	2.44	0.315
Oedema	0.91	0.63	1.31	0.603
Atrial Fibrillation	0.91	0.64	1.31	0.617
Systolic BP	0.99	0.98	1.00	0.004
Heart rate	0.99	0.99	1.00	0.124
Heamoglobin	1.00	0.99	1.01	0.360
Respiratory rate	1.05	1.02	1.08	< 0.0005
Sodium	0.95	0.92	0.98	0.001
NT-proBNP	1.15	0.87	1.53	0.339
TMAO	0.98	0.80	1.21	0.850
Cardiac troponin	0.97	0.92	1.03	0.302
Urea	1.05	1.01	1.08	0.006
eGFR	0.99	0.97	1.00	0.104

BP = blood pressure; eGFR = estimated glomerular filtration rate; IHD = ischemic heart disease; NT-proBNP = N-terminal pro B-type natriuretic peptide; NYHA = New York Heart Association; PH = past history; TMAO = trimethylamine N-oxide

Table S5. Logistic regression analysis for in-hospital mortality using clinical risk score algorithms as a base model, with addition of N-terminal pro B-type natriuretic peptide (NT-proBNP) and/or trimethylamine *N*-oxide (TMAO) and corresponding ROC curve areas with comparison to the univariable base model. Values refer to odds ratio (95 % confidence interval).

			Multivariable					
In-hospital mortality	**Univariable**	***p* Value**	**Model 1**	***p* Value**	**Model 2**	***p* Value**	**Model 3**	***p* Value**
ADHERE score 1	Reference		Reference		Reference		Reference	
ADHERE score 2	2.88 (1.46 - 5.66)	0.002	2.66 (1.34 - 5.26)	0.005	2.77 (1.40 - 5.47)	0.003	2.60 (1.31 - 5.17)	0.006
ADHERE score 3	6.68 (3.47 - 12.86)	< 0.0005	5.97 (3.05 - 11.69)	< 0.0005	4.76 (2.35 - 9.61)	< 0.0005	4.52 (2.21 - 9.24)	< 0.0005
ADHERE score 4	5.19 (2.18 - 12.36)	< 0.0005	3.99 (1.59 - 9.99)	0.003	3.93 (1.60 - 9.65)	0.003	3.22 (1.26 - 8.25)	0.015
ADHERE score 5	16.87 (4.45 - 63.91)	< 0.0005	13.15 (3.34 - 51.76)	< 0.0005	10.29 (2.55 - 41.46)	0.001	8.79 (2.11 - 36.53)	0.003
NT-proBNP	1.96 (1.28 - 3.00)	0.002	1.32 (0.87 - 1.99)	NS	Excluded		1.26 (0.83 - 1.90)	NS
TMAO	1.72 (1.37 - 2.16)	< 0.0005	Excluded		1.40 (1.07 - 1.83)	0.014	1.35 (1.03 - 1.78)	0.031
ROC curve area	0.704		0.720	NS	0.738	0.07	0.731	NS
ADHERE score (continuous)	2.24 (1.72 - 2.92)	< 0.0005	2.07 (1.56 - 2.74)	< 0.0005	1.93(1.45 - 2.56)	< 0.0005	1.83 (1.37 - 2.46)	< 0.0005
NT-proBNP			1.37 (0.89 - 2.10)	NS	Excluded		1.29 (0.84 - 1.98)	NS
TMAO			Excluded		1.13 (1.06 - 1.88)	0.004	1.41 (1.09 - 1.84)	0.01
ROC curve area	0.703		0.706	NS	0.719	NS	0.717	NS
OPTIMIZE-HF score	2.42 (1.86 - 3.14)	< 0.0005	2.26 (1.72 - 2.97)	< 0.0005	2.16 (1.64 - 2.84)	< 0.005	2.06 (1.55 - 2.74)	< 0.0005
NT-proBNP			1.29 (0.85 - 1.97	NS	Excluded		1.21 (0.80 - 1.84)	NS
TMAO			Excluded		1.43 (1.11 - 1.84)	0.006	1.39 (1.08 - 1.80)	0.012
ROC curve area	0.734		0.733	NS	0.752	NS	0.745	NS
GWTG-HF score	2.30 (1.76 - 3.00)	< 0.0005	2.13 (1.61 - 2.82)	< 0.0005	2.01 (1.52 - 2.66)	< 0. 0005	1.92 (1.44 - 2.56)	< 0.0005
NT-proBNP			1.36 (0.89 - 2.08)	NS	Excluded		1.28 (0.84 - 1.95)	NS
TMAO			Excluded		1.46 (1.13 - 1.88)	0.003	1.42 (1.10 - 1.83)	0.008
ROC curve area	0.714		0.713	NS	0.730	NS	0.725	NS

Model 1: Base model of clinical risk prediction score plus NT-proBNP
Model 2: Base model of clinical risk prediction score plus TMAO
Model 3: Base model of clinical risk prediction score plus NT-proBNP and TMAO

Table S6. Reclassification analysis, for the endpoint of in-hospital mortality, using continuous reclassification showing the net reclassification improvement of adding N-terminal pro B-type natriuretic peptide (NT-proBNP) or trimethylamine N-oxide (TMAO) to the classification using the individual ADHERE components, and for adding TMAO to the classification using the individual ADHERE components with NT-proBNP.

Adding NT-proBNP to ADHERE components

Endpoint	NRI (95 % CI)	*p* Value
No	-27.7 (-34.6 - -20.9)	< 0.0005
Yes	50.0 (26.2 - 73.8)	< 0.0005
Total	22.3 (-2.5 - 47.0)	0.077

Adding TMAO to ADHERE components

Endpoint	NRI (95 % CI)	*p* Value
No	14.3 (7.5 - 21.1)	< 0.0005
Yes	42.0 (18.4 - 65.6)	< 0.0005
Total	56.3 (31.8 - 80.9)	< 0.0005

Adding TMAO to ADHERE components plus NT-proBNP

Endpoint	NRI (95 % CI)	*p* Value
No	37.7 (30.9 - 44.6)	< 0.0005
Yes	-5.9 (-29.7 - 17.9)	NS
Total	31.8 (7.1 - 56.6)	0.012

References

Heaney LM, Jones DJL, Mbasu RJ, Ng LL, Suzuki T. High mass accuracy assay for trimethylamine N-oxide using stable-isotope dilution with liquid chromatography coupled to orthogonal acceleration time of flight mass spectrometry with multiple reaction monitoring. *Anal Bioanal Chem* 2015;DOI: 10.1007/s00216-015-9164-6 [Epub ahead of print]