Title: Trimethylamine N-oxide and risk stratification after acute myocardial infarction

Running title: TMAO and acute myocardial infarction

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ABSTRACT

Background: Risk stratification in acute myocardial infarction (MI) remains a clinical challenge. Trimethylamine \(N\)-oxide (TMAO), a gut-derived metabolite, was investigated for its ability to assist in risk stratification for acute MI hospitalizations.

Methods: TMAO was analyzed in 1079 acute MI patients. Associations with adverse outcome of all-cause mortality or reinfarction (death/MI) for shorter (6 months) and longer (2 years) terms were assessed and compared to other cohort-specific biomarkers. Added value in risk stratification by combined use with the GRACE (Global Registry of Acute Coronary Events) score was also investigated.

Results: TMAO independently predicted death/MI at 2 years [292 events, hazard ratio 1.21 (95% confidence intervals) (1.03-1.43), \(p=0.023\)] but was not able to predict death/MI at 6 months [161 events, \(p=0.119\)]. For death/MI at 2 years, TMAO retained independent prediction of risk (\(p=0.034\)) and improved stratification even after addition of multiple alternative and contemporary biomarkers previously shown to provide added prognostic value in this cohort. From these contemporary biomarkers, TMAO remained the only significant predictor of outcome. Further, TMAO improved risk stratification for death/MI at 6 months by down-classifying risk in patients with GRACE score >119 and plasma TMAO concentration \(\leq 3.7\mu mol/L\).

Conclusions: TMAO levels showed association with poor prognosis (death/MI) at 2 years and superiority over contemporary biomarkers for patients hospitalized due to acute MI. Furthermore, when used with the GRACE score for calculating risk at 6 months, TMAO re-identified patients at lower risk after initial categorization into a higher risk group and showed usefulness as a secondary risk stratification biomarker.
**Abbreviations list**

MI  myocardial infarction  
GRACE Global Registry for Acute Coronary Events  
STEMI ST elevated myocardial infarction  
PENK proenkephalin  
MR-proADM mid-regional pro-adrenomedullin  
Pro-SP pro-substance P  
TMAO trimethylamine N-oxide  
eGFR estimated glomerular filtration rate  
SD standard deviation  
Death/MI composite of all-cause mortality or reinfarction  
NT-proBNP N-terminal pro B-type natriuretic peptide  
BP blood pressure  
AUC area under the curve  
CHAID chi-square automatic interaction detection  
HR hazard ratio  
CI confidence intervals  
NRI net reclassification index
INTRODUCTION

Prediction of adverse cardiac events in patients with acute myocardial infarction (MI) remains a challenge. Clinical scoring algorithms have been developed to aid in risk stratification (e.g. GRACE,[1] TIMI,[2] PURSUIT,[3] etc.). Representatively, the GRACE (Global Registry for Acute Coronary Events) score is used to risk stratify patients with diagnosed acute coronary syndrome to estimate their in-hospital, 6 month and up to 3 year mortality or repeated MI rates, and is suitable for both non-ST and ST elevated MI (STEMI).[1]

Clinical algorithms are derived from population-based statistics. Additional personalized information can be contributed by biomarkers that often are a measure of underlying disease activity. Combining these to provide individualized risk stratification is a topic of interest for precision medicine. The discovery and evaluation of new biomarkers that provide added value to further improve and refine risk stratification is, therefore, of clinical interest.

To this end, the authors have made efforts in developing stratifying biomarkers to aid in the diagnostic and prognostic assessment of coronary artery disease which have included copeptin, [4] proenkephalin (PENK),[5] mid-regional pro-adrenomedullin (MR-proADM),[6] pro-substance P (pro-SP),[7] oxidized low density lipoprotein (oxidized phosphatidylcholine, malondialdehyde-modified)[8,9] and molecular forms of natriuretic peptide.[10]

Aside from the traditional protein-based biomarkers discussed above, newer metabolite-based biomarkers are a topic of recent interest. One which has shown promise is trimethylamine N-oxide (TMAO), which is an oxidized metabolite mediated by gut microbial metabolism of choline-containing lipids and carnitine-based molecules.[11-13] Production of TMAO, via gut microbe release of trimethylamine and liver oxidation to TMAO,[14] has
been shown to be associated with the promotion of atherosclerosis [13,15,16] and with mortality and hospitalization for cardio-renal disorders (e.g. heart failure [17,18] and chronic kidney disease [19,20]). More recently, TMAO has been reported to increase thrombotic risk (MI, stroke) with induced platelet hyperreactivity as the underlying mechanism,[21] linking elevated levels of circulating TMAO to a potential rise in risk of an acute ischemic event. The aim of the present study was to investigate the prognostic ability of TMAO to assist in risk stratification of patients hospitalized with acute MI.
MATERIALS AND METHODS

Study population

One thousand and seventy-nine patients with acute MI were admitted to University Hospitals of Leicester, UK between August 2004 and April 2007. Each patient consented (written and informed) to have blood samples taken and outcomes surveyed. The study complied with the Declaration of Helsinki and was approved by the local ethics committee.

All patients with a diagnosis of acute MI had a cardiac troponin I level above the 99th centile with at least one of the following: chest pain lasting >20 min or diagnostic serial electrocardiographic changes consisting of new pathological Q waves or ST-segment and T-wave changes,[22] excluding patients with malignancy, renal replacement therapy or surgery within 1 month. Estimated glomerular filtration rate (eGFR) was calculated from the simplified Modification of Diet in Renal Disease formula.[23] All patients received standard medical treatment and revascularization at the discretion of the attending physician.

Sample collection

Venipuncture was performed in recumbent patients during hospital stay following admittance with acute MI. An initial investigation into serial blood sampling of 34 patients at day 1, 3 and 5 post-admission was performed. Circulating TMAO levels were observed to rise between day 1 and 3 (p=0.036), and stabilize up to day 5 (see supplementary figure S1). Therefore, sample analysis was performed on secondary draw samples taken at 3.5 ± 1.1 days (mean ± standard deviation (SD)) post-admission. Blood was collected in pre-chilled tubes containing EDTA and aprotonin, and plasma was separated by centrifugation at 1500 xg for 20 min at 4 ºC. Plasma was aliquoted and stored at -80 ºC until analysis. At the time of
analysis, samples were defrosted at room temperature and analyzed immediately after preparation.

**Biomarker measurements**

Plasma TMAO was extracted using stable-isotope dilution and analyzed by ultra-performance liquid chromatography-high resolution mass spectrometry, with previously reported techniques.[24] Briefly, protein precipitation was performed by adding 80 µL deuterated TMAO (D9-, 10 µmol/L) to 20 µL of plasma. Extracted samples were analyzed using hydrophilic interaction liquid chromatography-time of flight mass spectrometry with multiple reaction monitoring. 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. N-terminal pro B type natriuretic peptide (NT-proBNP) was measured in all patients using a sandwich immunoassay as described previously.[25] Data for copeptin, PENK, MR-proADM and pro-SP were extracted from previous investigations.[4-7]

**Endpoints**

The primary outcome measured was a composite of all-cause mortality and reinfarction (death/MI). The primary outcome was assessed for shorter (6 months) and longer (2 years) term risk prediction. Secondary outcomes of all-cause mortality at 6 months and 2 years were also examined. Addition of TMAO to the GRACE score (for outcome at 6 months) was tested for the endpoint of death/MI at 6 months. Endpoints were obtained by
reviewing the local hospital databases and the Office of National Statistics Registry and by telephone calls to patients, and those data were verified by reviewing medical records. One hundred percent follow-up was achieved.

**Statistical Analyses**

Statistical analyses were performed using IBM SPSS Statistics (v22, IBM, Armonk, NY, USA) and Stata (v14, College Station, TX, USA). Patient demographics were compared using the Kruskal-Wallis H Test for continuous variables and Chi-squared tests for categorical variables after stratification for tertiles of TMAO levels. Independent predictors of log TMAO levels were assessed using backward removal general linear models. Cox proportional hazard analyses were used to identify independent predictors of the outcome endpoints. A base model was constructed using variables for mortality risk (age, heart rate, systolic blood pressure (BP), revascularization, ST-segment depression and troponin as a cardiac marker),[1] other traditional cardiovascular risk markers and MI specific markers (sex, past history of increased BP, MI/angina and diabetes and Killip score) medication at discharge (aspirin, beta blockers, ACE/ARB and statins), eGFR and blood urea as markers of renal dysfunction, and NT-proBNP and copeptin as established prognostic markers of MI.[4,25] For Cox models, troponin and copeptin levels were expressed as a continuous variable, log transformed and hazard ratios refer to a 10-fold rise in levels. TMAO, NT-proBNP, blood urea, PENK, MR-proADM and pro-SP levels were log transformed, and normalized to 1 SD, so that hazard ratios refer to the Z-transformed variables. Kaplan-Meier survival curves were generated to visualize the relationship between TMAO tertiles and death/MI at 2 years and for groups produced for risk of death/MI at 6 months by combination of GRACE and TMAO values. Mantel-Cox log rank tests were used to assess the significance of the curves of TMAO levels after stratification by tertiles. Comparisons of area under the curve (AUC) for the receiver operator characteristic were performed to assess
differences between prediction of the GRACE score at 6 months with and without TMAO as an additional variable. Continuous reclassification analyses [26] were performed to test the added value of TMAO to the GRACE score at 6 months. Decision tree analysis was performed using the chi-square automatic interaction detection (CHAID). An alpha value (p) of <0.05 was deemed statistically significant.
RESULTS

Patient characteristics

Clinical and demographic factors are shown in table 1 for the entire cohort and further categorized for tertiles of TMAO levels. Analysis of trends for elevated TMAO levels showed that patients were more likely to be older, male, have reduced renal function, decreased diastolic BP and increased heart rate along with increased risk factors of circulating NT-proBNP and copeptin levels and calculated GRACE score for mortality at 6 months (p≤0.040). More patients with elevated TMAO levels had past history of MI or angina, diabetes, increased BP, or were allocated a Killip score above 1 (p≤0.001). Fewer patients with elevated TMAO levels had STEMI, underwent revascularization, had history of smoking, or were allocated medication on discharge (p≤0.018). Fifty-three percent of patients were non-STEMI and 47% STEMI. Revascularization rates were 33.0% and 17.3% for non-STEMI and STEMI patients, respectively.

Correlation analyses

Spearman’s correlation analyses were performed to investigate the clinical correlates of TMAO. Spearman’s rank correlation coefficients (rs) showed that TMAO was significantly correlated to age (0.374), eGFR (-0.371), blood urea (0.358), systolic BP (0.068), heart rate (0.092), NT-proBNP (0.200) and copeptin (0.154, p≤0.035). TMAO levels were not correlated to size of infarct as measured by peak creatine phosphokinase levels (rs = -0.067, p=0.102).

Independent predictors of log TMAO levels were age, eGFR and blood urea (p≤0.001). The general linear model reported that the variation of TMAO as explained by all factors was 20.9%.
Survival analyses

TMAO was assessed for risk prediction of adverse outcome at 2 years using Cox proportional hazards survival analyses. TMAO was a univariate predictor of death/MI at 2 years [hazard ratio (HR) (95% confidence intervals (CI)) HR 1.40 (1.26 to 1.55), p<0.0005]. Independent predictive qualities of TMAO were tested by adding TMAO to a base model of traditional cardiovascular risk factors and variables included within the GRACE score for outcome at 6 months [including age, sex, systolic BP, heart rate, past histories of MI/angina, increased BP and diabetes, Killip score, STEMI class, revascularization, medication at discharge (aspirin, beta blockers, ACE/ARB and statins), renal function measurements (eGFR, blood urea) and cardiovascular biomarkers (troponin, NT-proBNP, copeptin)]. Multivariable analyses for death/MI at 2 years showed that TMAO was an independent predictor for event outcome [HR 1.21 (1.03 to 1.43), p=0.023]. Other independent predictors of death/MI at 2 years were age and copeptin levels (p≤0.029, figure 1). Addition of TMAO to the base model resulted in a significant increase in the likelihood ratio χ² (p=0.014).

Kaplan-Meier survival analysis was performed on death/MI rates at 2 years for patients stratified by TMAO tertiles. Results indicated that the highest tertile was significantly different to the middle and lowest tertile (p<0.0005), with no differences observed between the middle and lowest tertile (figure 1). TMAO was not an independent predictor of all-cause mortality alone at 2 years [HR 1.21 (0.98 to 1.48), p=0.074].

Further survival analyses were performed to assess the ability for TMAO to independently predict the endpoint of death/MI at 6 months. TMAO was a univariate predictor of death/MI at 6 months [HR 1.33 (1.17 to 1.51), p<0.0005]. Independent predictive qualities of TMAO were tested by adding TMAO to the same base model as used to analyze risk at 2 years. Multivariable analyses for death/MI at 6 months showed that TMAO was not an independent predictor for event outcome [HR 1.19 (0.96 to 1.48), p=0.119]. Independent predictors of death/MI at 6 months were age,
revascularization and NT-proBNP levels (p≤0.037). TMAO was not able to independently predict all-cause mortality alone at 6 months [HR 1.31 (0.99 to 1.72), p=0.059].

**Comparison with other biomarkers**

In order to test the risk prediction qualities of TMAO, Cox survival analyses were performed with the inclusion of previously reported biomarkers for this cohort. Included biomarkers were NT-proBNP,[5] copeptin,[4] PENK,[5] MR-proADM [6] and pro-SP.[7] A base model was constructed including the variables used in previous Cox multivariable models for prediction of death/MI at 2 years (including NT-proBNP and copeptin but excluding TMAO, model 1), and tested with addition of the 3 previously reported biomarkers (model 2) and with further addition of TMAO (model 3). TMAO in model 3 was able to independently predict death/MI at 2 years [HR 1.20 (1.01 to 1.42), p=0.034, table 2]. In the model for death/MI at 2 years, TMAO was the only biomarker that displayed a significant prediction of outcome. All other contemporary biomarkers were univariate predictors of death/MI at 2 years (HR 1.48 to 2.16, p<0.0005), but were unable to independently predict outcome at 2 years when combined with the base model and plasma TMAO levels (HR ≤1.32, p≥0.080).

**Reclassification analysis**

Category-free reclassification analysis was performed to assess the added value of TMAO to the established GRACE clinical risk score. Predictive qualities of the GRACE score for death/MI at 6 months were compared with those of the GRACE score with TMAO added. The addition of TMAO was able to down-classify individuals with low risk [net reclassification index (NRI) 29.0 (95% CI 19.8 to 38.3), p<0.0005], but not able to reclassify those at high risk [NRI -9.1 (-30.0 to 11.8), p>0.05] leading to an overall non-significant reclassification analysis (NRI 19.9 (-2.9 to 42.8), p=0.087). No differences were observed for AUC values between the two prediction sets (GRACE 0.691 (0.642 to 0.740) versus GRACE with TMAO 0.703 (0.654 to 0.752), p>0.05).
**Decision tree analysis**

Decision tree analysis was done to assess the clinical applicability of using TMAO as a secondary risk stratification biomarker after initial GRACE scoring for risk prediction (death/MI) at 6 months. In addition to GRACE and TMAO, troponin levels (as a continuous variable) were included in the analysis to allow for more contemporary use of this biomarker in risk stratification. Using GRACE as the initial classifier, a score of >119 and a TMAO plasma concentration of >3.7 µmol/L defined the highest risk group of patients \( n = 296, 31.5\% \) of the total, figure 2) who had a group-relative event risk of 24.0%, a cohort-relative event risk of 7.6% and accounted for 51.8% of total events measured. In addition to this, a GRACE score of >119 and a TMAO plasma concentration ≤3.7 µmol/L showed successful down-stratification of patient risk, with a group-relative risk of 15.6%. Patients with a GRACE score ≤119 showed a group-relative risk of 8.2%. Troponin levels were not included in the decision tree using the CHAID analysis method. For Kaplan-Meier analysis of the 3 groups and the outcome of death/MI at 6 months, the highest risk group was shown to be significantly higher than the middle and lowest risk groups \( p ≤ 0.031 \), and the middle risk group higher than the lowest risk group \( p = 0.005 \). These findings support TMAO’s ability to further identify patients at lower risk among those initially classified as high risk by the GRACE clinical scoring method, and potential use as a secondary risk stratification biomarker when used in combination with clinical risk algorithms.
DISCUSSION

The gut microbiome-related metabolite TMAO was demonstrated to be a predictive biomarker of death/MI in acute MI at 2 years, and retained independent predictive ability after adjustment for other biomarkers that had been previously assessed in this cohort (NT-proBNP, copeptin, PENK, MR-proADM and pro-SP). TMAO further showed utility for risk prediction when combined with an established clinical algorithm. Decision tree analysis and category-free reclassification highlighted the potential clinical application of TMAO as a secondary risk stratification biomarker when used in conjunction with the GRACE score calculated for adverse outcome at 6 months.

Previous investigations have assessed the prognostic implications of TMAO on overall cardiovascular disease risk (death, stroke or MI)[12] but no report, to date, has examined prognosis in patients hospitalized due to acute MI. TMAO levels were predictive of adverse outcome at 2 years after adjustment for clinical and demographic variables, including measurements of renal dysfunction.

TMAO was an independent predictor for death/MI at 2 years following hospitalization due to acute MI. Addition of TMAO to a comprehensive base model showed significant increases in the predictive qualities of the survival analyses, as evaluated by the likelihood ratio $\chi^2$ test for nested regression models. When stratified by TMAO tertile values, patients in the highest tertile showed significant risk increase for death/MI at 2 years, with the middle and lower tertile showing equal and reduced risk.

Clinically, TMAO showed potential usefulness as a secondary risk stratification biomarker when used in combination with the GRACE score. In patients with higher GRACE scores (>119), TMAO was able to further define patients with lower and higher risk among this group for death/MI at 6 months, and would contribute to risk prediction in patients with acute MI when used in this context. A GRACE score of 119 reflects patients in the high risk category for non-STEMI
and upper medium risk for STEMI (high risk defined as ≥119 for non-STEMI and ≥128 for STEMI).[27] The median GRACE score for the present cohort was 120, and therefore these analyses highlight a beneficial re-stratification to patients in the upper 50% of risk probability. Secondary stratification using TMAO defined a patient at lower risk with a plasma concentration of ≤3.7 µmol/L, a value equal to the cohort median and in-line with previous reports of healthy reference values (generally <5 µmol/L).[12,13,17,20,24]

Comparison of predictive ability against other biomarkers that had been previously assessed in this cohort (NT-proBNP, copeptin, PENK, MR-proADM, pro-SP),[4-7] showed that TMAO retained independent predictive ability after adjustment for them. Interestingly, NT-proBNP, as a golden standard benchmark biomarker for prediction of adverse outcomes following acute MI,[25] was not able to retain independent prediction for adverse outcome in this cohort at 2 years. Of importance, TMAO was the only significant biomarker for death/MI prediction at 2 years. As TMAO reflects different underlying pathophysiological processes as compared to other contemporary biomarkers such as NT-proBNP, with the latter representing mechanically induced neurohormonal stress processes and TMAO reflecting a previously unrecognized contribution of gut-mediated degradation of lipids; use of TMAO will be beneficial to understand additional contribution of these processes to cardiac outcomes.

Mechanistically, a causal association between TMAO and atherosclerosis remains to be established. Pro-atherosclerotic responses have been observed in mice fed with TMAO precursors (i.e. choline and L-carnitine) [11,13] and, in humans, TMAO levels have been reported to associate with atherosclerotic burden in coronary artery disease.[28] Recently, patients with elevated TMAO levels were shown to be at risk for thrombotic diseases including MI and stroke, with mechanistic investigation suggesting a possible mechanistic link with platelet hyperreactivity [21], but platelet dysfunction was not demonstrated in the patients with ischemic events. The present study shows that elevated TMAO levels at time of MI is further associated with poor outcome. Moreover, a non-lethal choline analogue to inhibit bacterial production of
trimethylamine, in turn reducing the levels of circulating TMAO [29], has been recently described, and intervention studies using this or similar compounds will be important to address the causative role of TMAO on coronary artery disease and outcomes in the future. In an initial investigation into TMAO kinetics post-acute MI in a small cohort, circulating TMAO levels were observed to rise and stabilize between day 1 and 5 post-admission. The reasons for this rise and plateau are not known and warrant further investigation to understand the kinetics of circulating TMAO levels post-acute ischemic events.

On limitations of the present study, the findings are based on a population from a single center with 2 admitting hospitals. The low rate of early revascularization in this historical cohort does not reflect contemporary standard of care associated with invasive approaches to revascularization. Nevertheless, the models for have been corrected for revascularization rate and also presence of ST-elevation. In addition, serial measurements of troponin levels were not performed, and therefore the values incorporated into the prediction models may not reflect the plateau or peak levels.

Collectively, with beneficial prognostic information available for heart failure,[17,18] chronic kidney disease [19,20] and now acute MI, TMAO is proving to be an applicable biomarker across a range of cardio-renal pathological states.

In conclusion, TMAO levels show association with poor prognosis (death/MI) at 2 years for patients hospitalized due to acute MI, but not at 6 months. However, when used with the GRACE score for calculating risk at 6 months, TMAO showed usefulness as a secondary risk stratification biomarker by re-identifying patients at lower risk after initial categorization into a higher risk group by the GRACE clinical risk score. In addition, TMAO showed independent predictive ability when compared against other biomarkers that had been previously assessed in this cohort.
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REFERENCES


Table 1. Demographics for patients admitted to hospital with acute myocardial infarction.

<table>
<thead>
<tr>
<th></th>
<th>Combined</th>
<th>TMAO &lt; 2.9 µmol/L</th>
<th>TMAO 2.9 to 5.1 µmol/L</th>
<th>TMAO &gt; 5.1 µmol/L</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ages (years)</td>
<td>67 (57 to 77)</td>
<td>61 (53 to 71)</td>
<td>66 (57 to 75)</td>
<td>74 (64 to 81)</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>Male (%)</td>
<td>72</td>
<td>72</td>
<td>77</td>
<td>69</td>
<td>0.008</td>
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<td>Systolic BP (mmHg)</td>
<td>136 (120 to 151)</td>
<td>136 (120 to 153)</td>
<td>140 (121 to 156)</td>
<td>137 (123 to 151)</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>77 (68 to 88)</td>
<td>80 (70 to 90)</td>
<td>78 (68 to 90)</td>
<td>75 (67 to 86)</td>
<td>0.040</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>75 (63 to 93)</td>
<td>76 (65 to 93)</td>
<td>75 (63 to 90)</td>
<td>79 (67 to 86)</td>
<td>0.025</td>
</tr>
<tr>
<td>Past history MI or angina (%)</td>
<td>33</td>
<td>26</td>
<td>29</td>
<td>45</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>Past history increased BP (%)</td>
<td>52</td>
<td>46</td>
<td>50</td>
<td>60</td>
<td>0.001</td>
</tr>
<tr>
<td>Past history diabetes (%)</td>
<td>23</td>
<td>19</td>
<td>18</td>
<td>32</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>Past history smoking (%)</td>
<td>42</td>
<td>41</td>
<td>51</td>
<td>34</td>
<td>&lt; 0.0005</td>
</tr>
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<td>Past history HF (%)</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>5</td>
<td>NS</td>
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<tr>
<td>Aspirin on discharge (%)</td>
<td>85</td>
<td>88</td>
<td>84</td>
<td>82</td>
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<td>Beta blockers on discharge (%)</td>
<td>81</td>
<td>84</td>
<td>81</td>
<td>78</td>
<td>NS</td>
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<tr>
<td>ACE/ARB on discharge (%)</td>
<td>84</td>
<td>85</td>
<td>87</td>
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<td>0.013</td>
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<td>Statins on discharge (%)</td>
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<td>93</td>
<td>88</td>
<td>86</td>
<td>0.013</td>
</tr>
<tr>
<td>Killip score &gt;1 (%)</td>
<td>42</td>
<td>37</td>
<td>37</td>
<td>51</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>ST-elevation MI (%)</td>
<td>47</td>
<td>50</td>
<td>53</td>
<td>39</td>
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<td>Revascularization (%)</td>
<td>26</td>
<td>30</td>
<td>28</td>
<td>21</td>
<td>0.018</td>
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<td>Variable</td>
<td>Median (IQR)</td>
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<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td>NS</td>
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<td>-----------------------</td>
<td>-----------------------</td>
<td>----------</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>7.5 (6.3 to 9.9)</td>
<td>7.3 (6.2 to 9.3)</td>
<td>7.6 (6.3 to 9.6)</td>
<td>7.6 (6.3 to 10.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Troponin (ng/mL)</td>
<td>3.6 (1.0 to 12.1)</td>
<td>3.2 (1.2 to 10.5)</td>
<td>3.8 (0.9 to 11.1)</td>
<td>3.6 (1.2 to 12.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Peak CPK (U/L)</td>
<td>888 (325 to 1788)</td>
<td>949 (364 to 1813)</td>
<td>923 (360 to 1923)</td>
<td>702 (266 to 1628)</td>
<td>NS</td>
</tr>
<tr>
<td>Na+ (mmol/L)</td>
<td>138 (136 to 140)</td>
<td>138 (136 to 140)</td>
<td>138 (136 to 140)</td>
<td>138 (136 to 140)</td>
<td>NS</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73m²)</td>
<td>66 (53 to 78)</td>
<td>72 (61 to 84)</td>
<td>68 (58 to 82)</td>
<td>57 (40 to 70)</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>6.1 (4.9 to 7.9)</td>
<td>5.4 (4.4 to 6.7)</td>
<td>5.9 (4.8 to 7.3)</td>
<td>7.3 (5.4 to 9.9)</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>NT-proBNP (pmol/L)</td>
<td>812 (259 to 2199)</td>
<td>370 (128 to 1090)</td>
<td>413 (112 to 1455)</td>
<td>794 (332 to 2336)</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>Copeptin (pmol/L)</td>
<td>10.7 (5.4 to 29.7)</td>
<td>8.3 (4.5 to 22.5)</td>
<td>10.7 (5.4 to 26.3)</td>
<td>15.1 (6.8 to 40.7)</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>TMAO (µmol/L)</td>
<td>3.7 (4.6 to 6.4)</td>
<td>2.3 (2.0 to 2.6)</td>
<td>3.8 (3.2 to 4.5)</td>
<td>8.5 (6.2 to 15.0)</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>GRACE*[^1] score</td>
<td>120 (96 to 143)</td>
<td>107 (87 to 130)</td>
<td>117 (98 to 139)</td>
<td>136 (112 to 158)</td>
<td>&lt; 0.0005</td>
</tr>
</tbody>
</table>

**Endpoints** (*n*)

6 months

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Median (IQR)</th>
<th>Median (IQR)</th>
<th>Median (IQR)</th>
<th>Median (IQR)</th>
<th>&lt; 0.0005</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-cause mortality</td>
<td>79</td>
<td>11</td>
<td>25</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Death/MI</td>
<td>161</td>
<td>35</td>
<td>51</td>
<td>75</td>
<td></td>
</tr>
</tbody>
</table>

2 years

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Median (IQR)</th>
<th>Median (IQR)</th>
<th>Median (IQR)</th>
<th>Median (IQR)</th>
<th>&lt; 0.0005</th>
</tr>
</thead>
<tbody>
<tr>
<td>All-cause mortality</td>
<td>119</td>
<td>19</td>
<td>32</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>Death/MI</td>
<td>232</td>
<td>49</td>
<td>68</td>
<td>115</td>
<td></td>
</tr>
</tbody>
</table>

Data expressed as median (interquartile range) for continuous variables.
* denotes GRACE score at 6 months.
BP = blood pressure, CPK = creatine phosphokinase, death/MI = all-cause mortality or reinfarction, eGFR = estimated glomerular filtration rate, HF = heart failure, MI = myocardial infarction, NT-proBNP = N-terminal pro-B type natriuretic peptide, TMAO = trimethylamine N-oxide
Table 2. Cox regression analyses for all-cause mortality or reinfarction (death/MI) at 2 years for the base model (model 1) with the addition of alternative biomarkers (model 2) and a comparison with trimethylamine N-oxide (TMAO, model 3).

**Death/MI at 2 years**

<table>
<thead>
<tr>
<th>Independent predictors</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
<td>p Value</td>
</tr>
<tr>
<td>Age</td>
<td>1.03</td>
<td>1.01 to 1.05</td>
<td>0.010</td>
</tr>
<tr>
<td>eGFR</td>
<td>1.01</td>
<td>1.00 to 1.03</td>
<td>0.048</td>
</tr>
<tr>
<td>Copeptin</td>
<td>1.54</td>
<td>1.02 to 2.32</td>
<td>0.041</td>
</tr>
<tr>
<td>NT-proBNP</td>
<td>1.24</td>
<td>0.94 to 1.62</td>
<td>NS</td>
</tr>
<tr>
<td>PENK</td>
<td>1.39</td>
<td>1.02 to 1.90</td>
<td>0.040</td>
</tr>
<tr>
<td><strong>Log likelihood ratio</strong></td>
<td>78.52</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
<td>p Value</td>
</tr>
<tr>
<td>Copeptin</td>
<td>1.26</td>
<td>0.93 to 1.72</td>
<td>NS</td>
</tr>
<tr>
<td>NT-proBNP</td>
<td>1.24</td>
<td>0.96 to 1.60</td>
<td>NS</td>
</tr>
<tr>
<td>PENK</td>
<td>1.39</td>
<td>1.02 to 1.90</td>
<td>0.040</td>
</tr>
<tr>
<td>MR-proADM</td>
<td>1.26</td>
<td>0.93 to 1.72</td>
<td>NS</td>
</tr>
<tr>
<td>Pro-SP</td>
<td>1.26</td>
<td>0.93 to 1.72</td>
<td>NS</td>
</tr>
<tr>
<td>TMAO</td>
<td>1.24</td>
<td>0.96 to 1.60</td>
<td>NS</td>
</tr>
</tbody>
</table>

Model 1 = Age, sex, past histories of myocardial infarction/angina, blood pressure and diabetes, Killip score >1, ST-elevation (STEMI), revascularization, discharge medication (aspirin, beta blockers ACE/ARB and statins), estimated glomerular filtration rate (eGFR), blood urea, systolic blood pressure, heart rate, cardiac troponin, N-terminal B-type natriuretic peptide (NT-proBNP) and copeptin; Model 2 = as model 1 plus proenkephalin (PENK), mid-region pro-adrenomedullin (MR-proADM) and pro-substance P (pro-SP); Model 3 = as model 2 plus TMAO; CI = confidence intervals; HR = hazard ratio
FIGURE LEGENDS

**Figure 1.** Forest plot to show cox hazard ratios (squares) and 95% confidence intervals (horizontal bars) for a multivariable model including cardiac risk factors and trimethylamine N-oxide (TMAO) for all-cause mortality or reinfarction (death/MI) at 2 years (left), and Kaplan-Meier survival curves to show event-free survival stratified by TMAO tertiles for death/MI at 2 years (right). Curves represent tertile 1 (solid line), tertile 2 (dashed line) and tertile 3 (dotted line). # p<0.0005 compared to tertile 1 and 2.

BP = blood pressure, Dx = discharge, eGFR = estimated glomerular filtration rate, MI = myocardial infarction, NS = p>0.100, NT-proBNP = N-terminal pro B-type natriuretic peptide, PH = past history.

**Figure 2.** Classification tree to show risk stratification for the combined use of the GRACE score for all-cause mortality or reinfarction (death/MI) at 6 months, troponin (as a continuous variable) and trimethylamine N-oxide (TMAO) (main body), and cumulative event incidence of risk groups A-C (inset).

GRACE = Global Registry for Acute Coronary Events, OR = odds ratio

* p≤0.005 compared to group A, † p=0.031 compared to group B.
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BP = blood pressure, Dx = discharge, eGFR = estimated glomerular filtration rate, MI = myocardial infarction, NS = p>0.100, NT-proBNP = N-terminal pro B-type natriuretic peptide, PH = past history.
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GRACE = Global Registry for Acute Coronary Events, OR = odds ratio

* p≤0.005 compared to group A, † p=0.031 compared to group B.
**Figure S1.** Box and whisker plot to show an initial investigation into serial blood sampling in a small cohort (n=34) of acute myocardial infarction patients. Samples were collected at day 1, 3 and 5 post-admission.

Note: Boxes represent median and interquartile range, and whiskers the 5th and 95th percentiles. NS = non-significant (p>0.100)