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Association of gut-related metabolites with outcome in acute heart failure

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Short title: Gut-related metabolites and outcomes in heart failure

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1 ABSTRACT

2 **Background:** Trimethylamine N-oxide (TMAO), a gut-related metabolite, is associated with
3 heart failure (HF) outcomes. However, TMAO is the final product of a complex metabolic
4 pathway (i.e. choline/carnitine) that has never been entirely investigated in HF. The present
5 study investigates a panel of metabolites involved in the TMAO-choline/carnitine metabolic
6 pathway for their associations with outcome in acute HF patients.

7 **Methods:** In total, 806 plasma samples from acute HF patients were analysed for TMAO,
8 trimethyllysine, L-carnitine, acetyl-L-carnitine, γ -butyrobetaine, crotonobetaine,
9 trimethylamine, betaine aldehyde, choline, and betaine using a developed liquid
10 chromatography-tandem mass spectrometry method. Associations with outcome of all-cause
11 mortality (death) and a composite of all-cause mortality and/or rehospitalization due to HF
12 (death/HF) at 30 days and 1 year were investigated.

13 **Results:** TMAO, trimethyllysine, L-carnitine, acetyl-L-carnitine, and γ -butyrobetaine were
14 associated with death and death/HF at 30 days (short-term) (HR 1.30-1.49, $p \leq 0.021$) and at 1
15 year (long-term) (HR 1.15-1.25, $p \leq 0.026$) when adjusted for cardiac risk factors. L-carnitine
16 and acetyl-L-carnitine were superior for short-term outcomes whereas TMAO was the
17 superior metabolite for association with long-term outcomes. Furthermore, acetyl-L-carnitine
18 and L-carnitine were superior for in-hospital mortality and improved risk stratification when
19 combined with current clinical risk scores (i.e. ADHERE, OPTIMIZE-HF, GWTG-HF)
20 ($OR \geq 1.52$, $p \leq 0.020$).

21 **Conclusions:** Carnitine-related metabolites show associations with adverse outcomes in
22 acute HF, in particular L-carnitine and acetyl-L-carnitine for short-term outcomes, and
23 TMAO for long-term outcomes. Further studies are warranted to investigate the role and
24 implications of carnitine metabolites including intervention in the pathogenesis of HF.

1 INTRODUCTION

2 A growing body of evidence supports that the gut microbiota plays an important role
3 in the pathogenesis of heart failure (HF) (1-3). In particular, trimethylamine N-oxide
4 (TMAO), a gut-derived metabolite of the choline/carnitine metabolic pathway (4,5), has been
5 highlighted as a novel risk marker for outcomes of death in cardiovascular disease (6).
6 Several studies have shown that elevated TMAO levels are associated with poor prognosis in
7 both acute (6) and chronic HF patients (7-10). More recently, TMAO levels did not respond
8 to guideline-based therapy in a large multicentre HF cohort despite being associated with
9 death due to HF (11,12). Furthermore, the combination of TMAO to the current gold-
10 standard HF biomarker, B-type natriuretic peptide (BNP), showed improvements in risk
11 stratification when BNP levels were less elevated in patients with preserved ejection fraction
12 (13).

13 Investigations assessing the role of gut-related metabolites in HF have focused
14 primarily on the role of TMAO. However, other metabolites within the TMAO-
15 choline/carnitine gut microbiota pathway have also demonstrated independent associations
16 with cardiovascular risk, such as trimethyllysine (TML), γ -butyrobetaine, and L-carnitine
17 (14,15). Therefore, the hypothesis of the present study was that the measurement of a full
18 panel of metabolites involved in the TMAO-choline/carnitine pathway, individually and in
19 combination, would improve the risk stratification in HF patients.

20 The aims of the present study were to investigate the association of circulating levels
21 of a panel of metabolites within the TMAO-choline/carnitine metabolic pathway (Figure 1):
22 trimethylamine (TMA), choline, betaine, betaine aldehyde, crotonobetaine, TMAO, acetyl-L-
23 carnitine, L-carnitine, γ -butyrobetaine, and TML, individually and in combination for their
24 short- and long-term associations with acute HF outcomes.

METHODS

Study population

Eight hundred and six patients, with available plasma sample volume, admitted with acute HF to the University Hospitals of Leicester, UK between February 2006 and August 2011 were enrolled, as previously described (6,16). Each patient consented to have blood samples taken and outcomes surveyed. This study was approved by the local ethics committee and complied with the Declaration of Helsinki. Diagnosis of acute HF was made according to clinical signs and symptoms, following ESC guidelines (17). Exclusion criteria included patients with previous history of cancer or renal replacement therapy, surgical procedure within the previous month, presence of cardiogenic shock, sepsis, pneumonia, or acute coronary syndromes, and inability to consent (e.g. dementia).

The primary endpoints were all-cause mortality (death) and a composite of death and/or rehospitalization due to HF (death/HF) at 30 days and at 1 year. Where multiple events occurred, the time to the first event was counted as the outcome. Determinations of inclusion and endpoints were performed by an independent cardiologist.

Sample collection and measurement of biomarkers

Peripheral intravenous blood samples were obtained within the 24h period after admission to hospital with acute HF and collected in pre-chilled tubes containing EDTA and aprotinin. Plasma was separated via centrifugation at 1500g for 20 min at 4°C and stored in aliquots at -80°C until analysis. For analysis, samples were thawed at room temperature and analyzed immediately following preparation.

N-terminal pro B-type natriuretic peptide (NT-proBNP) was measured in all patients using a sandwich immunoassay as described previously (18).

The gut-related metabolites, TMAO, TMA, TML, γ -butyrobetaine, acetyl-L-carnitine, choline, L-carnitine, betaine, betaine aldehyde and crotonobetaine were extracted from 50 μ l of plasma using stable-isotope dilution and derivatization. Extracted samples were analysed by UHPLC-MS/MS with multiple reaction monitoring (MRM) using a Shimadzu UPLC Nexera X2 LC30AD with an Acquity UPLC BEH HILIC 100 mm analytical column coupled to a Shimadzu 8050 triple quadrupole mass spectrometer (Shimadzu Limited, Kyoto Japan). Detailed description of the methods is described in the Supplementary Material.

Statistical analyses

Spearman correlations were calculated for the metabolites and clinical parameters. Cox proportional hazards regression analyses were performed to identify associations with death/HF at 30 days and 1 year. Metabolite levels were log transformed, and normalised to 1-standard deviation, so that hazard ratios refer to Z-transformed variables. Kaplan-Meier survival curves were generated to show the relationship between the metabolites and the outcomes of death/HF. Mantel-Cox log rank tests were used to report the significance of stratification by tertiles for the metabolites. To analyse the predictive power of the metabolites for in-hospital mortality, binary logistic regression analyses were performed and included the Acute Decompensated HEart Failure National Registry (ADHERE), Organized Program To Initiate Lifesaving Treatment In Hospitalized Patients With Heart Failure (OPTIMIZE-HF), and (Get With The Guidelines-Heart Failure) GWTG-HF scores (19-21). The ADHERE risk score was categorised against the first group (reference) and patients were assigned to risk groups using the ADHERE registry model. Outcome prediction accuracies were assessed by calculating the area under the curve (AUC) for the receiver operator characteristics (ROC) curve analysis and reclassification analyses were used to assess the clinical utility of adding individual metabolites and combination of metabolites to a basic model of clinical cardiovascular risk. A p-value of <0.05 was deemed to be statistically

significant. Statistical analyses were performed using IBM SPSS Statistics (V26, IBM Corp., Armonk, New York, USA).

RESULTS

Patient demographics

Eight hundred and six patients, admitted with acute HF, were investigated for the described metabolites. The majority of patients were of NYHA class III/IV (95%), male (61%) with a median age of 78 years. A total of 313 events were recorded during the 1-year follow-up period with 57 deaths during in-hospital care, 154 deaths post-discharge and 100 readmissions to hospital due to HF. Clinical demographics and measured endpoints are shown in Table 1.

Associations with clinical parameters

Spearman's correlation analysis was performed to assess the correlations between the metabolites and clinical parameters. Of the metabolites, acetyl-L-carnitine, betaine, choline, γ -butyrobetaine, L-carnitine, TMA, TMAO and TML were quantifiable in our cohort and showed positive correlations with each other ($r_s = 0.099$ - 0.737 , $p \leq 0.005$) and negative correlations with systolic blood pressure ($r_s = -0.103$ - -0.167 , $p \leq 0.005$). Apart from betaine, all other metabolites showed correlations with renal markers, eGFR ($r_s = -0.123$ - -0.437 , $p \leq 0.001$), and urea ($r_s = 0.142$ - 0.392 , $p < 0.001$). Correlations with available clinical parameters are detailed in Supplementary Table 6.

The association between metabolites and clinical characteristics was also investigated. Only TMA showed differences in distribution across severity for NYHA class III and IV patients (mean rank 350.6 pmol/L and 401.8 pmol/L respectively, $p = 0.001$), whereas betaine alone showed differences based on ejection fraction ($<40\%$ and $\geq 40\%$, mean rank 323.3 pmol/L and 265.69 pmol/L respectively, $p < 0.001$). For distribution with treatment, apart from betaine, all other metabolites showed lower levels in patients who were given beta-blockers ($p \leq 0.036$), with only TML showing differences for angiotensin-converting enzyme (ACE)

inhibitor or angiotensin receptor blocker (ARBs), with patients given ACE/ARBs reporting higher levels of TML ($p=0.034$).

Associations with death/HF

Cox survival analyses were conducted to investigate the association of independent metabolites with death/HF at 30 days and 1 year. Models were adjusted for sex, age, previous medical history (HF, ischaemic heart disease, hypertension, and diabetes mellitus), New York Heart Association (NYHA) class, smoking status, oedema, atrial fibrillation, systolic blood pressure, diastolic blood pressure, heart rate, haemoglobin, respiratory rate, blood sodium and (log) NT-proBNP. Of the metabolites analysed, univariate analysis showed that TMAO, TML, L-carnitine, γ -butyrobetaine, and acetyl-L-carnitine were all associated with death/HF across both timepoints [HR 1.20-1.79 (95% CI 1.07-2.16) $p\leq 0.002$]. After adjustment, acetyl-L-carnitine, γ -butyrobetaine, L-carnitine, TMAO and TML showed independent associations at 30 days [HR 1.30-1.49 (95% CI 1.04-1.88) $p\leq 0.021$] and 1 year [HR 1.15-1.25 (95% CI 1.02-1.42) $p\leq 0.026$] (Table 2). Betaine, choline, and TMA did not show independent associations with outcome across either timepoint as univariate or multivariate markers.

For short-term outcome at 30 days, elevated acetyl-L-carnitine levels were associated with an increase in death/HF [HR 1.49 (95% CI 1.19-1.88) $p=0.001$], whereas for long-term outcomes TMAO was associated with an increase in death/HF after adjustment [HR 1.25 (95% CI 1.09-1.42) $p=0.001$].

Additionally, after the inclusion of renal markers (urea and eGFR), none of the metabolites showed significant associations with the outcome of death/HF across all endpoints, indicating a strong confounding influence from biomarkers of renal function (Supplementary Table 7). Furthermore, Cox survival analysis were performed by splitting the population into ischaemic HF and non-ischaemic HF. Results showed that in patients with

1 ischaemic HF, none of the metabolites were significant across both timepoints. However, in
2 patients with non-ischaemic HF, only TMAO was significant at both timepoints [HR 1.24-
3 1.48 (95% CI 1.04-1.95) $p \leq 0.017$] (Supplementary Table 8).

4 For Kaplan-Meier survival analysis, patients were stratified based on tertile levels of
5 each metabolite and analysed for association with events of death/HF. Results showed that
6 TMAO, TML, L-carnitine, acetyl-L-carnitine, and γ -butyrobetaine showed association of
7 reduced survival with elevated levels at 1 year ($p \leq 0.005$) (Supplementary Figure 2). Receiver
8 operating characteristics (ROC) analysis showed incremental decreases over time from 30
9 days to 1 year for acetyl-L-carnitine, γ -butyrobetaine, L-carnitine, TMAO and TML [AUC
10 (95% CI) at 30 days were 0.606-0.675 (0.548-0.730) $p < 0.001$; and at 1 year were 0.563-0.602
11 (0.523-0.643) $p \leq 0.002$]. Betaine, choline and TMA were not significant across any timepoint
12 ($p \geq 0.073$) (Table 3).

13 **Associations with mortality**

14 Cox survival analyses were performed for death and adjusted using the same
15 aforementioned model. Univariate analysis showed that TMAO, TML, L-carnitine, γ -
16 butyrobetaine, and acetyl-L-carnitine were associated with death at 30 days [HR 1.43-2.24
17 (95% CI 1.11-2.81) $p \leq 0.005$], and betaine, TMA, TMAO, TML, L-carnitine, γ -butyrobetaine,
18 and acetyl-L-carnitine at 1 year [HR 1.17-1.50 (95% CI 1.03-1.50) $p \leq 0.018$]. When adjusted,
19 betaine, choline, and TMA were not associated with mortality at 30 days nor at 1 year. The
20 remaining metabolites showed associations with death at 30 days [HR 1.39-1.81 (95% CI
21 1.05-2.42) $p \leq 0.022$] and at 1 year [HR 1.26-1.34 (95% CI 1.08-1.58) $p \leq 0.004$] after
22 adjustment (Table 2).

1 Additionally, after the inclusion of renal markers (urea and eGFR), acetyl-L-carnitine
2 was associated with outcome at 30 days [HR 1.37 (95% CI 1.01-1.85) $p=0.045$], as was γ -
3 butyrobetaine at 1 year [HR 1.19 (95% CI 1.01-1.40) $p=0.042$] (Supplementary Table 7).

4 Kaplan-Meier survival analysis for death when stratified by tertiles showed
5 association of reduced survival with elevated levels of acetyl-L-carnitine, γ -butyrobetaine, L-
6 carnitine, TMA, TMAO and TML at 1 year ($p\leq 0.003$) (Supplementary Figure 3).

7 8 **Associations with in-hospital mortality**

9 For ROC analysis, acetyl-L-carnitine reported the highest AUC for association with
10 in-hospital mortality [AUC 0.744 (95% CI 0.67-0.82) $p<0.001$]. Betaine, γ -butyrobetaine, L-
11 carnitine, TMA, TMAO and TML also showed associations with in-hospital mortality [AUC
12 0.586-0.684 (95% CI 0.513-0.758) $p\leq 0.022$] (Table 3).

13 Analyses were performed to evaluate the added value of the metabolites with clinical
14 risk scores as either a categorical (ADHERE) or continuous variable (ADHERE, OPTIMIZE-
15 HF and GWTG-HF). The levels of each metabolite showed differences when stratified by the
16 ADHERE categorical score ($p\leq 0.002$). Pairwise comparison against the reference group
17 (ADHERE group 1) showed significant differences and elevations in groups 3-5 for TMAO
18 (adj. $p\leq 0.008$), and group 3 and 4 for acetyl-L-carnitine, choline, γ -butyrobetaine, L-
19 carnitine, TMA, and TML (adj. $p\leq 0.035$) (Figure 2). When the metabolites were added to the
20 base model of the clinical risk scores, only acetyl-L-carnitine, L-carnitine and γ -butyrobetaine
21 ($p\leq 0.020$) were associated with in-hospital mortality for all three clinical risk scores, with a
22 strong influence from renal function biomarkers, eGFR, and urea (Figure 3).

Net reclassification analyses were performed by adding each metabolite to each of the three scoring systems (ADHERE, OPTIMIZE-HF and GWTG-HF). The results showed that in the present cohort, only acetyl-L-carnitine, L-carnitine, γ -butyrobetaine, and TMA showed overall improvements in reclassification when added to the ADHERE score ($p \leq 0.037$). Furthermore, when added to the OPTIMIZE-HF score, improvements were seen with the addition of choline, acetyl-L-carnitine, L-carnitine, and γ -butyrobetaine ($p \leq 0.035$). Overall improvements were also seen with betaine, acetyl-L-carnitine, γ -butyrobetaine, and L-carnitine when added to the GWTG-HF score ($p \leq 0.007$) (Table 4).

Associations of combined metabolites with outcomes

As TMA, choline, and betaine did not show associations as univariate or multivariate markers with death/HF, they were discounted from combinatorial statistical analysis.

Logistic regression analysis for associations with death/HF at 30 days (short-term) and 1 year (long-term) identified TMAO and acetyl-L-carnitine as prospective markers in a combinatorial model. Furthermore, these metabolites were also confirmed as those with the highest area under the curve (AUC) on ROC curve analysis (Table 3). When combined, the ROC curve analysis did not show additional advantages for combined metabolites at short-term ($p \geq 0.086$) nor long-term ($p = 0.934$) outcomes of death/HF for continuous analysis.

For binary analysis, the Youden's Index for optimal cut off point from the ROC curve for death/HF at 1 year was applied (TMAO 16.7 $\mu\text{mol/L}$ and acetyl-L-carnitine 18.5 $\mu\text{mol/L}$), with both metabolites yielding approximately identical categories (71% of patients below the cut-off for TMAO and 74% for acetyl-L-carnitine) (22,23). Kaplan-Meier survival curve analysis was performed for death/HF at 30 days and at 1 year using the same cut-off point. Results showed that TMAO and acetyl-L-carnitine showed association of reduced survival

1 with elevated levels at 30 days ($p<0.001$) and at 1 year ($p<0.001$). The metabolites were
2 combined as a risk score of four categories (both below-cut off, TMAO above-cut off and
3 acetyl-L-carnitine below-cut off, TMAO-below cut off and acetyl-L-carnitine above-cut off,
4 and both above-cut off). Results for death/HF at 30 days showed that elevated levels of both
5 metabolites showed reduced survival, superior to elevations of either metabolite or no
6 metabolite elevated ($p\leq 0.033$, chi-square ≥ 4.526). Similarly, for death/HF at 1 year, results
7 showed reduced survival when both metabolites were elevated when compared to other
8 groupings ($p\leq 0.022$, chi-square ≥ 5.281) (Supplementary Figure 4). Interestingly, for short-
9 term outcomes, low levels of both metabolites and high TMAO-low acetyl-L-carnitine
10 showed no significant difference ($p=0.212$) whereas at long-term outcomes, low levels of
11 both metabolites and low TMAO-high acetyl-L-carnitine showed no significant difference
12 ($p=0.062$), suggesting that acetyl-L-carnitine is the weighing factor for short-term outcomes
13 and TMAO for long-term.

DISCUSSION

The present study analysed a panel of TMAO-choline/carnitine pathway metabolites, with several novel findings. Firstly, carnitine-related TMAO precursors (trimethyllysine, L-carnitine, acetyl-L-carnitine, and γ -butyrobetaine) showed association with HF outcomes [death and death/HF at 30 days (short-term) and at 1 year (long-term)], whilst choline-derived metabolites (choline and betaine) were not associated with outcomes. Secondly, metabolites showed different association with outcomes; specifically, acetyl-L-carnitine and L-carnitine both showed superiority over the other metabolites for short-term associations and in-hospital mortality in combination with current clinical risk scoring methods, whilst TMAO was not only associated with outcome, as previously reported, but was also the most performant for long-term outcomes, suggesting that the different metabolites are useful for risk stratification at different stages (short-/long-term outcomes).

Previous studies have identified the gut-derived metabolite, TMAO, and associated metabolites, TML and γ -butyrobetaine, to show associations with outcomes of HF and other cardiovascular diseases (CVD) (6-10,24). Mechanistically, TMAO is derived from two principal pathways that involve choline and/or carnitine (1,4,5). Metabolites of the choline pathway, such as choline and its downstream betaine, showed elevated circulating levels in patients with chronic HF and correlations with clinical characteristics (e.g. haemodynamic status, disease severity); however, as in our report, associations with outcomes of death/HF during follow-up have not been reported (25). In contrast, metabolites associated with the carnitine pathway (L-carnitine, acetyl-L-carnitine, γ -butyrobetaine, and trimethyllysine) have shown associations with adverse outcomes of death/HF. Specifically, trimethyllysine has been reported to show associations with short- and long-term cardiovascular events (15), and its derivative, γ -butyrobetaine, has been identified to be a CVD risk marker (14).

L-carnitine and acetyl-L-carnitine play important roles in HF pathophysiology. Firstly, L-carnitine has an essential role in fatty acid metabolism and carbohydrate metabolism via acetyl-L-carnitine. Mechanistically, within the mitochondrial matrix, fatty acid oxidation is dependent on esterification of fatty acyl coA esters together with acyl-carnitines to allow transport into the mitochondria (26). Under physiological conditions, in the normal heart ATP is generated from mitochondrial oxidative phosphorylation by glucose and fatty acid oxidation, with the former as the most energy efficient. By contrast, in the pathological heart of HF, there is a shift in cardiac energy metabolism associated with decreased mitochondrial oxidative phosphorylation and chronic activation of the renin-angiotensin-aldosterone and adrenergic systems, resulting in a shift in energy metabolism and an overall net reduction in energy efficiency (27). In HF, when left ventricular dysfunction occurs, there is a subsequent reduction in myocardial intra-cellular carnitine levels (28), which is associated with a shift from glucose to fatty acid oxidation as the main source of oxidative metabolism with an increased production of reactive oxygen species, that contribute to cardiac damage (29,30), together with the reduction of the phosphocreatine/ATP ratio which determines cardiac energy loss and dysfunction (31). In this context, increased circulating carnitine levels have been proposed to represent a compensatory mechanism in HF, in the attempt of improving fatty acid transport for metabolism and oxidation, thereby improving myocardial function (32). In line with this concept, our observation that circulating levels of L-carnitine and acetyl-L-carnitine are the most performant for short-term outcomes, suggests that their measurement may identify patients with a more severe impairment of cardiac bioenergetics which results in outcomes of death/HF.

Collectively, our results suggest that the TMAO-choline/carnitine pathway is affected differently during phases of HF; for instance, patients with a more impaired mitochondrial function have increased L-carnitine levels with a very poor outcome, whereas patients with

less impaired mitochondrial bioenergetics and a longer survival show increased levels of TMAO, which were most associated with poor outcome in the long term. Notably, in our cohort, apart from betaine, all metabolites showed lower levels in patients who were given beta-blockers, suggesting the effects of adrenergic modulation on the metabolic pathway. Further, L-carnitine has also been implicated in muscle wasting conditions (i.e. sarcopenia), via the same mechanism discussed above and demonstrated in skeletal wasting/cardiac cachexia, a typical feature of advanced HF (33,34). In addition, carnitine insufficiency is common amongst HF patients, and has been associated with reduced left ventricular diastolic function. Carnitine supplementation has been reported as a possible treatment of mitochondrial dysfunction in HF (35,36), leading to improvement in clinical symptoms, cardiac morphology and function, natriuretic peptides, and renal function; however, no clear effects on mortality have been demonstrated (37,38). These beneficial findings are linked both to the metabolic effect on myocardial cells (39), through an increase in glucose utilisation rather than a normalisation of the fatty acid metabolism (39), and to the anti-catabolic effect on skeletal muscle cells, resulting in the L-carnitine anti-wasting effect (40). Our results, showing that patients with higher levels of L-carnitine and acetyl-L-carnitine (i.e. patients with a more severe impairment of mitochondrial bioenergetics) have the poorest short-term prognosis, further support the important role of the carnitine pathway in HF, suggesting that further studies on the role of carnitine are needed.

Study Limitations

As study limitations, patients were recruited from a single centre with the majority being Caucasians, and data regarding dietary intake that could influence metabolite levels were not available. Furthermore, crotonobetaine and betaine aldehyde were below the limit of quantitation for this method. Considering that these metabolites were quantified in previous studies at very low concentrations (41), it is reasonable to hypothesise that the reportedly low

1 concentrations as well as the lack of quantification in our cohort are due to these metabolites
2 being transient intermediates. This method was not developed to detect acyl-L-carnitine and
3 therefore acetyl-CoA (product of beta-oxidation) and acetyl-L-carnitine:free L-carnitine ratio
4 was not reported in the present investigation.

6 **CONCLUSIONS**

7 In conclusion, the present study demonstrates the role of metabolites of the TMAO-
8 carnitine/choline metabolic pathway in risk stratification of acute HF, with a preponderant
9 role played by carnitine-related metabolites. In addition to risk stratification, carnitine
10 intervention may potentially have a therapeutic role which needs to be further investigated.

Conflicts of interest

The authors report no conflict of interest.

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1 TABLES

2 **Table 1** Patient demographics for acute HF patients at the time of admission to hospital.

Age (yrs)	78 (69-84)
Male	61%
Systolic BP (mmHg)	133 (115-150)
Diastolic BP (mmHg)	75 (65-85)
Heart rate (beats/min)	90 (74-107)
de novo HF	32%
Past history IHD	31%
Past history diabetes	34%
Past history HTN	58%
Past history COPD	9%
Past history hyperlipidemia	24%
Current smoker	10%
Orthopnea	52%
Oedema	60%
Raised JVP	56%
Pulmonary oedema	32%
HFrEF (%)*	58%
Beta-blocker (%)	41%
ACE/ARBs (%)	54%
NYHA Class IV	53%
III	42%
II	5%
I	<1%
Atrial fibrillation	46%
Respiratory rate (breaths/min)	22 (18-26)
Urea (mmol/L)	8.9 (6.6-12.8)
Creatinine (μmol/L)	112 (91-141)
eGFR (mL/min/1.73m²)	52 (39-67)
Na⁺ (mmol/L)	138 (135-141)
K⁺ (mmol/L)	4.4 (4.0-4.7)
Hemoglobin (g/L)	123 (108-137)
NT-proBNP (pmol/L)	2160 (1018-3726)
ADHERE group 3-5	17%

OPTIMIZE-HF score	35 (29-39)
GWTG-HF score	43 (38-48)

Metabolites (μmol/L)

Acetyl-L-carnitine	12.1 (8.1-19.4)
Betaine	45.7 (33.0-67.1)
Choline	19.9 (14.5-28.7)
γ-butyrobetaine	0.51 (0.86-1.49)
L-carnitine	52.1 (39.2-77.4)
Trimethylamine	0.27 (0.21-0.35)
Trimethylamine N-oxide	10.2 (5.8-18.7)
Trimethyllysine	0.84 (0.51-1.39)

Endpoints

In-hospital death	57
Death at 30 days	62
Death at 1 year	213
Death/HF at 30 days	98
Death/HF at 1 year	313

Data are reported as median (interquartile range) for continuous variables and as a % for categorical.

BP, blood pressure; COPD, chronic obstructive pulmonary disease; eGFR, estimated glomerular filtration rate; HF, heart failure; HFrEF, heart failure with reduced ejection fraction (<40%); HTN, hypertension; IHD, ischemic heart disease; JVP, jugular venous pressure; NT-proBNP, N-terminal pro B-type natriuretic peptide; ADHERE; Acute Decompensated Heart Failure National Registry; CI, confidence interval; GWTG-HF, Get With The Guidelines®-Heart Failure, NRI, net reclassification index; OPTIMIZE-HF, Organized Program to Initiate Lifesaving Treatment in Hospitalized Patients with Heart Failure; NYHA, New York Heart Association.

*597 patients only

Table 2 Multivariate analysis for death and death/HF

	Death					
	30 days			1 year		
	HR	95% CI	p Value	HR	95% CI	p Value
Acetyl-L-carnitine	1.81	1.36-2.42	<0.001	1.33	1.14-1.54	<0.001
Betaine	1.31	0.96-1.78	0.086	1.13	0.96-1.34	0.150
Choline	1.07	0.81-1.41	0.648	1.12	0.96-1.30	0.165
γ-butyrobetaine	1.59	1.17-2.17	0.003	1.34	1.13-1.58	0.001
L-carnitine	1.63	1.25-2.11	<0.001	1.31	1.14-1.51	<0.001
TMA	1.03	0.75-1.39	0.874	1.08	0.93-1.25	0.305
TMAO	1.39	1.05-1.84	0.022	1.26	1.08-1.47	0.004
TML	1.40	1.07-1.85	0.015	1.31	1.12-1.52	0.001

	Death/HF					
	30 days			1 year		
	HR	95% CI	p Value	HR	95% CI	p Value
Acetyl-L-carnitine	1.49	1.19-1.88	0.001	1.15	1.02-1.31	0.026
Betaine	1.24	0.97-1.58	0.093	1.08	0.95-1.23	0.264
Choline	1.14	0.91-1.42	0.254	1.06	0.94-1.21	0.324
γ-butyrobetaine	1.45	1.13-1.85	0.003	1.17	1.02-1.33	0.023
L-carnitine	1.43	1.15-1.78	0.001	1.16	1.03-1.31	0.015
TMA	0.93	0.72-1.21	0.592	1.03	0.91-1.17	0.619
TMAO	1.38	1.10-1.73	0.006	1.25	1.09-1.42	0.001
TML	1.30	1.04-1.63	0.021	1.19	1.05-1.35	0.006

Models were adjusted for sex, age, previous medical history (HF, ischaemic heart disease, hypertension, and diabetes mellitus), New York Heart Association (NYHA) class, smoking status, oedema, atrial fibrillation, systolic blood pressure, diastolic blood pressure, heart rate, haemoglobin, respiratory rate, blood sodium and (log) NT-proBNP.

CI, confidence interval; HR, hazard ratio; TMA, trimethylamine; TMAO, trimethylamine N-oxide; TML, trimethyllysine.

Table 3 Receiver operator characteristics for metabolites at 30 days and 1-year outcomes of death/HF and, in-patient mortality

	30 days			1 year			In-patient mortality		
	AUC	95% CI	p Value	AUC	95% CI	p Value	AUC	95% CI	p Value
Acetyl-L-carnitine	0.675	0.621-0.730	<0.001	0.575	0.535-0.616	<0.001	0.744	0.673-0.816	<0.001
Betaine	0.543	0.483-0.603	0.156	0.535	0.494-0.576	0.092	0.586	0.513-0.660	0.022
Choline	0.541	0.479-0.603	0.193	0.525	0.483-0.566	0.241	0.563	0.483-0.643	0.121
γ-butyrobetaine	0.627	0.568-0.686	<0.001	0.568	0.527-0.608	0.001	0.680	0.604-0.756	<0.001
L-carnitine	0.639	0.580-0.697	<0.001	0.563	0.523-0.604	0.002	0.684	0.610-0.758	<0.001
TMA	0.495	0.435-0.555	0.865	0.538	0.497-0.579	0.073	0.609	0.533-0.684	0.005
TMAO	0.636	0.579-0.693	<0.001	0.602	0.562-0.643	<0.001	0.658	0.581-0.736	<0.001
TML	0.606	0.548-0.665	<0.001	0.568	0.528-0.609	0.001	0.621	0.544-0.698	0.002

3

4 AUC, area under curve; CI, confidence interval; TMA, trimethylamine; TMAO,
5 trimethylamine N-oxide; TML, trimethyllysine.

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Table 4 Net reclassification index for in-patient mortality

ADHERE	NRI	95% CI	p Value
Acetyl-L-carnitine	56.9	29.4-84.3	<0.001*
Betaine	26.8	-0.7-54.3	0.056
Choline	23.0	-4.4-50.4	0.100
γ-butyrobetaine	39.3	11.8-66.8	0.005*
L-carnitine	40.6	13.1-68.0	0.004*
TMA	29.3	1.8-56.8	0.037*
TMAO	18.4	-9.1-45.9	0.189
TML	15.2	-12.3-42.7	0.278

OPTIMIZE-HF	NRI	95% CI	p Value
Acetyl-L-carnitine	52.8	19.0-86.6	0.002*
Betaine	25.6	-8.2-59.4	0.138
Choline	36.3	2.5-70.1	0.035*
γ-butyrobetaine	46.6	12.8-80.5	0.007*
L-carnitine	37.8	4.0-71.6	0.028*
TMA	16.9	-16.9-50.7	0.326
TMAO	12.7	-21.1-46.5	0.461
TML	10.0	-23.8-43.8	0.561

GWTG-HF	NRI	95% CI	p Value
Acetyl-L-carnitine	52.8	25.3-80.3	<0.001*
Betaine	37.5	10.0-65.0	0.007*
Choline	16.1	-11.4-43.6	0.251
γ-butyrobetaine	38.0	10.5-65.5	0.007*
L-carnitine	44.1	16.6-71.5	0.002*
TMA	22.4	-5.0-49.9	0.110
TMAO	25.9	-1.6-53.3	0.065
TML	21.8	-5.7-49.3	0.120

ADHERE; Acute Decompensated Heart Failure National Registry; CI, confidence interval; GWTG-HF, Get With The Guidelines®-Heart Failure; NRI, net reclassification index; OPTIMIZE-HF, Organized Program to Initiate Lifesaving Treatment in Hospitalized Patients with Heart Failure; TMA, trimethylamine; TMAO, trimethylamine N-oxide; TML, trimethyllysine. * p<0.05

FIGURES

Figure 1. Depiction of the TMAO-Choline/Carnitine metabolic pathway

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3 **Figure 2.** Box and whisker plot to show the distribution of gut-mediated metabolites in patients stratified by ADHERE score grouping.

4 ADHERE; Acute Decompensated Heart Failure National Registry. TML, trimethyllysine; TMA, trimethylamine; TMAO, trimethylamine N-oxide

Figure 3. Forest plots to show binary logistic ORs (dots) and 95% CIs (horizontal bars) for cardiac risk factors for multivariate models for the individual components of in-hospital mortality prediction algorithms of ADHERE, OPTIMIZE-HF and GWTG-HF registries with the addition of metabolites.

TMA, trimethylamine; TMAO, trimethylamine N-oxide; TML, trimethyllysine; ADHERE; Acute Decompensated Heart Failure National Registry; CI, confidence interval; GWTG-HF, Get With The Guidelines®-Heart Failure, NRI, net reclassification index; OPTIMIZE-HF, Organized Program to Initiate Lifesaving Treatment in Hospitalized Patients with Heart Failure TML, trimethyllysine; TMA, trimethylamine; TMAO, trimethylamine N-oxide

SUPPLEMENTARY MATERIAL

Association of gut-related metabolites with outcome in acute heart failure

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METHOD DEVELOPMENT AND VALIDATION

Chemical and reagents

Reference metabolite samples: O-acetyl-L-carnitine hydrochloride, betaine aldehyde chloride, betaine hydrochloride, choline chloride, crotonobetaine hydrochloride, gamma butyrobetaine hydrochloride, L-carnitine hydrochloride, trimethylamine hydrochloride, TMAO (98.9% purity), trimethyllysine hydrochloride were all purchased from Sigma Aldrich UK. Acetyl-d₃-L-carnitine hydrochloride, betaine - (d₁₁, 98%), choline chloride (trimethyl-d₉, 98%), L-carnitine-(trimethyl – d₉, 98%), d₉-TMAO (≥98% purity, 99.9% enrichment), trimethylamine DCL (TMA-d₁₀, 98%) were purchased from Cambridge Isotopes USA. Formic acid, water, acetonitrile and methanol all Optima™ LC/MS Grade were obtained from Fisher Scientific UK. Ammonium hydroxide, extra pure, 25% solution in water was purchased from ACROS Organics™. Stripped plasma (4× charcoal, EDTA gender pooled: product code HMPLEDTA2STRPD-HEV-53432) was purchased from Seralab (Haywards Heath, UK).

Preparation of calibrators and quality control samples

Stock solutions for calibrators and QC samples of each metabolite were prepared separately. Standard stock solutions of each of the 10 metabolites were prepared in ultrapure water at a concentration of 5 mmol/L. For betaine aldehyde, crotonobetaine, γ-BB and TML, no deuterated standards were commercially available, therefore betaine-d₁₁ was used as the internal standard (I.S.) for quantitation of betaine aldehyde, crotonobetaine and γ-BB with TMAO-d₉ successfully used for quantitation of TML. Stock solutions of the six internal standards were prepared in methanol at a concentration of 1 mmol/L. The internal standard working solution was prepared at a final concentration of 10 μmol/L for acetyl-L-carnitine d₃,

betaine-d₁₁, choline-d₉, L-carnitine-d₉, TMA-d₁₀, and TMAO-d₉, before it was stored at – 80°C in aliquots.

Multicomponent working solutions for each metabolite were prepared freshly by appropriately diluting the stock solutions with water. The calibration ranges were chosen to cover the concentration ranges for the ten metabolites. A minimum of 8-point calibration curve was prepared by spiking charcoal stripped plasma with the correct concentration of multicomponent solution. These metabolites are endogenous compounds and are naturally present in human plasma, hence, it is difficult to find an analyte free matrix. It has been reported that in the absence of an analyte free matrix, an appropriate surrogate matrix (1,2) such as pure water (3,4), bovine serum albumin (5) and charcoal stripped plasma (6), can be used. Quality control (QC) samples at four concentration levels were prepared separately by spiking charcoal stripped plasma samples with multicomponent solutions of the ten metabolites.

Sample extraction and derivatization

Sample preparation involves a derivatization step where 20 µL of methanol containing I.S. was added to 50 µl of calibrators, QC or patient plasma samples, followed by the addition of 75 µl of 50 mmol/L tert-butyl bromoacetate in acetonitrile and 25 µL of 25% ammonium hydroxide. Samples were incubated at room temperature for 30 minutes, followed by addition of 50 µL of acetonitrile containing 1% formic acid to each extract. The extract was vortexed for one minute and centrifuged at 16,900 rpm for 20 minutes. The supernatant was collected into LC vials and analysed by LC-MS/MS. Derivatization was necessary due to TMA's low MS sensitivity and low blood levels (4).

UHPLC-MS/MS analysis

Analyses were achieved on a Shimadzu Nexera X2 LC-30AD online to a Shimadzu 8050 triple quadrupole mass spectrometer (TQ-MS). Separation of the ten metabolites and IS was performed using an Acquity UPLC[®] BEH HILIC (130Å, 100 mm × 2.1 mm, 1.7 µm pore size column, Waters Corporation, USA) which was preceded by an Acquity UPLC[®] BEH HILIC VanGuard pre-column (130 Å, 1.7 µm, 2.1×5 mm, Waters Corporation, USA). Separation was performed using gradient elution mobile phase composed of a binary solvent mixture comprising of water containing 0.1% (v/v) formic acid and 0.025% (v/v) ammonium hydroxide (eluent A) and acetonitrile (eluent B). The gradient started with 95% solvent B for 3 mins and was dropped to 60% solvent B over 1.5 mins, where it was maintained for 1.5 mins before ramping back to 95% in 8 mins. It was held at 95% solvent B for 2 mins before initiating the next injection. Chromatography was performed at 50 °C with a flow rate of 0.6 mL/min and a run time of 10 min. The autosampler was maintained at 4°C with an injection volume of 0.5 µL for each sample.

MS/MS acquisition was performed in multiple reaction monitoring (MRM) mode using electrospray positive ionization for detection. The optimum MS source and chamber conditions were as follows. Nebuliser gas flow: 3 L/min; heating gas flow: 10 L/min; drying gas flow: 10 L/min; interface temperature: 300 °C; desolvation line (DL) temperature: 300 °C; heat block temperature: 400 °C; ion gauge (IG) vacuum: 2.0E – 003Pa; pirani gauge (PG) vacuum: 1.5E + 002Pa and CID gas: 270KPa. Detection of the ten metabolites and IS were achieved by monitoring the precursor to product ion transitions of each metabolite (Supplementary Table 1). Data acquisition and quantitative processing were accomplished using Labsolutions Software version 5.93 and Labsolutions Insight LCMS version 3.2 (Shimadzu Limited, Kyoto Japan).

1 **Method validation**

2 For the purposes of validation studies, four concentrations were chosen for the separate
3 preparation of quality control samples (QCs) at the LLOQ, low, medium and high
4 concentration levels for the ten metabolites and run alongside calibrators. To demonstrate that
5 the developed assay was fit for purpose, validation was conducted based upon international
6 guidelines (7). The selectivity, linearity, sensitivity (lower limit of quantitation (LLOQ)),
7 imprecision (intra and inter-assay accuracy and precision), matrix effects and stability were
8 determined for acetyl-L-carnitine, betaine, betaine aldehyde, choline, crotonobetaine, γ -BB,
9 L-carnitine, TMA, TMAO, and TML.

10 **Linearity**

11 Linearity was determined using a linear regression model. Calibrators of the ten metabolites
12 were prepared in charcoal stripped plasma matrix and used to generate standard curves over
13 the following concentration ranges: 0.1– 100 $\mu\text{mol/L}$ for acetyl-L-carnitine, crotonobetaine,
14 γ -BB, TMA and TML; 0.5 – 200 $\mu\text{mol/L}$ for betaine, choline, L-carnitine, and TMAO; 1.0 –
15 400 $\mu\text{mol/L}$ for betaine aldehyde. The concentrations of the ten metabolites in the plasma
16 sample was determined from each metabolite/IS peak area ratio against nominal analyte
17 concentration and a $1/x^2$ weighted linear regression was applied. The LLOQ was calculated
18 based on a response $\geq$ five times the response of the zero calibrator with a signal to noise
19 ratio of 10 (7).

20 For the investigated dynamic range for each of the ten metabolites in plasma, $r^2 > 0.99$ was
21 obtained. The data (slope, intercept and mean correlation coefficient (r^2)) for each metabolite
22 is presented in Supplementary Table 2. The LLOQ with a signal to noise ratio of ≥ 10 and the
23 required assay precision and accuracy was 0.1 $\mu\text{mol/L}$ for acetyl-L-carnitine, crotonobetaine,

1 γ -BB, TMA and TML; 0.5 μ mol/L for betaine, choline, L-carnitine, and TMAO; 1.0 μ mol/L
2 for betaine aldehyde.

3 **Assay imprecision (accuracy and precision)**

4 Replicate (n = 6) analyses of (QC) samples, prepared at the LLOQ, low, medium and high
5 concentration levels of the ten metabolites were analysed to evaluate the inter- and intra-day
6 accuracy and precision. This involved three daily runs over a six-day period. Accuracy was
7 expressed as the relative error (%RE) and precision as the relative standard deviation
8 (%RSD). In line with FDA guidelines, a RE and RSD of $\leq 20\%$ at the LLOQ and $\leq 15\%$ at
9 other tested concentrations were considered acceptable.

10 Data obtained for within and between run variations for both accuracy and precision were
11 within the defined $\leq 20\%$ limit at the LLOQ and $\leq 15\%$ limit at all other concentrations in each
12 run for the 10 metabolites. A summary of the results is presented in Supplementary Table 3.

13 **Carryover and selectivity**

14 Carryover was assessed by analysing blank samples consisting of methanol injections
15 interspersed between QC samples and calibrators. Selectivity was achieved by using the most
16 abundant product ion of each metabolite and IS for quantitation (Supplementary Table 1).

17 EICs from blank methanol injections interspersed between QC samples and calibrators
18 showed that there was no carryover effect. Analysis of samples revealed there were no
19 interfering endogenous compounds in plasma that coeluted with the quantitation of the ten
20 metabolites when monitoring the selected product ions for the 10 metabolites (Supplementary
21 Figure 1).

Recovery and matrix effect

The efficiency of the extraction procedure was determined using replicate ($n = 6$) samples prepared at the QC (low, medium and high) concentrations of the ten metabolites spiked into plasma. Recovery was assessed by comparing the ratio of analyte to I.S. response from plasma-spiked metabolite extracts, with those obtained from blank plasma extracts spiked with metabolite solution standards of equal concentration.

Matrix effects were assessed with spiked plasma from blood samples obtained from 6 healthy volunteers. Replicate ($n = 6$) samples of the ten metabolites and I.S. spiked in plasma extracts to represent low, medium and high concentrations were prepared to evaluate ion suppression or enhancement. The prepared samples were compared with standards of equal concentration spiked into acetonitrile. The matrix effect was calculated using the formula $(B/A - 1) \times 100$. Where A represents the ratio of the metabolite/I.S response from metabolite spiked into pure solvent and B represents the ratio of metabolite/I.S response from metabolite spiked into extracted plasma.

Recoveries for the ten metabolites were consistent and reproducible, with values between 70% and 100% for charcoal stripped and human plasma. The high extraction efficiencies indicate good extraction and metabolite stability under the applied extraction conditions. Recovery and matrix effect data for each metabolite at the QC concentrations investigated are summarised in Supplementary Table 4. There were no significant ($<20\%$) matrix effects on metabolite signal due to coelutents from plasma at all concentrations tested for each metabolite. These results demonstrated robustness of the extraction procedure and mechanism of ionisation for these metabolites.

Stability

The stability of the 10 metabolites in plasma extracts was evaluated by analysing medium QC samples by comparing the concentrations of the metabolites determined in samples prepared freshly, with the same samples stored at 4°C, -20°C, and -80°C after storage for 1, 2 and 4 weeks. The freeze thaw samples were analysed with freshly prepared calibrators and QC samples.

The ten metabolites were found to be stable when stored at temperatures of 4°C, -20 °C and -80 °C for up to 4 weeks in processed samples. No substantial changes in concentrations were seen at the QC concentrations investigated for the 10 metabolites (Supplementary Table 5). These findings indicate that, once samples are prepared, they can be stored in the fridge for 4 weeks or frozen to ensure stability over time. Additionally, pre-processed samples stored at temperatures of 4°C were stable for up to 16 weeks.

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Supplementary Table 1. Retention times and MRM transitions for the metabolites and internal standards

Analyte	Retention time (mins)	Transitions, m/z		Collision energy (V)
		Precursor ion	Product ions	
Acetyl-L-carnitine	6.50	204.0	85.05 /60.20	-18
Betaine Aldehyde	3.63	102.0	58.10 /59.15	-23
Betaine	5.13	118.0	58.10 /59.15	-25
Choline	4.16	104.1	58.10 /60.15	-28
Crotonobetaine	6.80	144.1	58.10 /59.15	-25
γ -butyrobetaine	6.74	146.1	87.05 /64.10	-16
L-carnitine	6.68	162.1	60.15 /103.10	-16
TMA	3.10	174.1	59.05 /118.10	-24
TMAO	6.42	76.2	58.10 /59.15	-24
TML	7.20	189.1	84.10 /130.15	-22
Acetyl-L-carnitine – d ₃	6.50	207.1	85.10 /166.15	-19
Betaine – d ₁₁	5.13	129.3	66.25 /58.10	-28
Choline – d ₉	4.16	113.1	66.10 /69.20	-32
L-carnitine – d ₉	6.68	171.1	69.20 /103.20	-18
TMAO – d ₉	6.41	85.2	66.15 /68.20	-22
TMA – d ₁₀	3.10	183.1	66.05 /127.1	-39

*The product ions in bold were used for quantitation

Reported collision energies are for product ions used for quantitation

m/z, mass-to-charge ratio; TMA, trimethylamine; TMAO, trimethylamine N-oxide; TML, trimethyllysine; V, voltage

Supplementary Table 2. Linearity and sensitivity data for the ten metabolites in plasma

Metabolite	Range (μmol/L)	Slope	Intercept	R ²	LOQ (μmol/L)
Acetyl-L-carnitine	0.1 - 100	0.352 ± 0.024	0.265 ± 0.115	0.998 ± 0.0084	0.1
Betaine Aldehyde	1.0 - 400	0.156 ± 0.013	0.150 ± 0.091	0.997 ± 0.0035	1.0
Betaine	0.5 - 200	0.562 ± 0.101	0.367 ± 0.143	0.994 ± 0.0006	0.5
Choline	0.5 - 200	0.228 ± 0.016	0.127 ± 0.096	0.997 ± 0.0074	0.5
Crotonobetaine	0.1 - 100	0.348 ± 0.028	0.315 ± 0.052	0.994 ± 0.0034	0.1
γ-butyrobetaine	0.1 - 100	0.296 ± 0.019	0.760 ± 0.125	0.997 ± 0.0015	0.1
L-carnitine	0.5 - 200	0.260 ± 0.014	0.185 ± 0.080	0.997 ± 0.0032	0.5
TMA	0.1 - 100	0.072 ± 0.002	0.077 ± 0.021	0.999 ± 0.0017	0.1
TMAO	0.5 - 200	0.114 ± 0.005	0.069 ± 0.017	0.997 ± 0.0021	0.5
TML	0.1 - 100	0.123 ± 0.016	0.335 ± 0.023	0.997 ± 0.0037	0.1

LOQ, limit of quantitation; R², coefficient of determination; TMA, trimethylamine; TMAO, trimethylamine N-oxide; TML, trimethyllysine

1 **Supplementary Table 3.** Intra- and inter-day accuracy and precision data for the ten
2 metabolites in plasma samples (n = 6 at all concentration levels)

Metabolite	Nominal conc. ($\mu\text{mol/L}$)	Intra-day ^a		Inter-day ^b	
		Measured conc. ($\mu\text{mol/L}$)	RSD (%)	Measured conc. ($\mu\text{mol/L}$)	RSD (%)
Acetyl-L-carnitine	0.1	0.12	6.25	0.11	4.65
	0.3	0.35	8.70	0.36	0.94
	5	5.04	0.85	5.85	1.72
	50	50.14	4.71	49.03	2.00
Betaine Aldehyde	1	1.06	7.29	1.07	1.19
	3	3.02	4.87	3.08	1.91
	25	25.04	6.24	25.92	1.80
	200	195.55	1.36	196.29	0.49
Betaine	0.5	0.51	7.26	0.51	1.32
	1.5	1.49	4.16	1.52	1.72
	25	24.73	12.92	24.86	0.55
	100	94.39	7.92	98.45	3.65
Choline	0.5	0.53	3.35	0.52	2.29
	1.5	1.53	2.88	1.50	3.26
	25	23.83	4.19	24.35	2.40
	100	97.90	3.56	99.48	1.94
Crotonobetaine	0.1	0.12	6.50	0.12	4.44
	0.3	1.50	1.52	1.51	0.49
	5	5.32	8.43	5.46	5.56
	50	51.10	5.07	52.35	0.24
γ -butyrobetaine	0.1	0.12	6.14	0.13	2.28
	0.3	0.31	2.80	0.32	0.54
	5	5.18	3.40	5.61	4.65
	50	51.57	2.16	51.82	1.57
L-carnitine	0.5	0.53	6.47	0.53	1.08
	1.5	1.51	2.21	1.51	0.57
	25	25.45	5.00	25.10	2.99
	100	98.38	6.08	99.20	1.20
TMA	0.1	0.13	9.08	0.13	2.19
	0.3	0.31	3.11	0.32	0.38
	5	5.27	0.49	5.51	4.37
	50	51.57	4.73	52.33	0.61
TMAO	0.5	0.55	5.45	0.53	4.42
	1.5	1.56	1.92	1.53	1.54
	25	24.17	1.77	24.18	2.43
	100	96.05	3.17	99.45	3.00
TML	0.1	0.10	6.76	0.12	2.92
	0.3	0.32	2.03	0.32	1.00
	5	5.47	3.22	5.32	3.36
	50	53.52	4.64	51.40	1.85

- 1 RSD, relative standard deviation; TMA, trimethylamine; TMAO, trimethylamine N-oxide;
- 2 TML, trimethyllysine
- 3 ^aCalculated for six replicates and three experiments conducted within a period of 24 h
- 4 ^bCalculated for six replicates and three experiments conducted over a six day period

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Supplementary Table 4. Recovery and matrix effects data for the ten metabolites extracted from plasma samples at the low, medium and high QC concentration levels (n = 6)

Metabolite	Nominal conc. (μM)	Recovery (%)	Standard Deviation	Mean Matrix Effects (%)	Standard Deviation
Acetyl-L-carnitine	0.3	78.04	4.11	98.02	5.82
	5	76.82	0.63	99.50	1.10
	50	82.73	5.12	100.73	1.99
Betaine Aldehyde	3	92.64	9.40	100.73	10.56
	50	79.56	7.84	97.45	13.39
	200	72.43	7.42	93.91	11.09
Betaine	1.5	88.45	7.26	92.41	9.61
	25	74.50	5.15	88.17	6.03
	100	77.01	0.89	90.77	3.81
Choline	1.5	87.11	10.58	102.76	7.71
	25	86.78	2.32	93.14	4.17
	100	87.69	1.33	101.13	4.94
Crotonobetaine	0.3	82.01	5.95	90.33	5.36
	5	75.71	3.47	98.70	12.12
	50	81.80	2.42	102.33	6.29
γ-butyrobetaine	0.3	92.03	4.28	103.70	9.10
	5	84.62	3.61	98.53	9.34
	50	85.41	1.71	101.92	6.84
L-carnitine	1.5	88.83	6.65	97.54	7.27
	25	83.08	11.77	91.08	1.26
	100	79.79	3.58	97.10	2.86
TMA	0.3	102.83	7.47	89.58	5.66
	5	105.85	5.69	85.19	7.34
	50	99.10	3.03	84.61	4.62
TMAO	1.5	78.61	2.32	101.83	4.82
	25	87.66	0.69	95.11	1.52
	100	88.68	1.71	99.41	2.12
TML	0.3	91.51	8.07	84.40	4.30
	5	92.07	1.60	84.45	6.03
	50	91.46	3.50	97.50	7.12

QC, quality control; TMA, trimethylamine; TMAO, trimethylamine N-oxide; TML, trimethyllysine

Supplementary Table 5. Effect of temperature and storage duration on metabolite concentrations measured from processed samples at the medium QC concentration range

Metabolite	Storage temperature (°C)	Nominal conc. (µM)	T = 1 week	T = 2 weeks	T = 4 weeks
Acetyl-L-carnitine	4	5	5.00 ± 0.12	5.32 ± 0.17	5.19 ± 0.04
	-20		4.94 ± 0.15	5.12 ± 0.05	5.05 ± 0.10
	-80		5.21 ± 0.05	5.08 ± 0.11	4.99 ± 0.11
Betaine Aldehyde	4	50	48.25 ± 1.05	52.42 ± 1.22	52.73 ± 0.92
	-20		47.98 ± 2.96	46.33 ± 3.82	48.10 ± 2.76
	-80		50.89 ± 3.88	50.05 ± 1.99	49.88 ± 3.55
Betaine	4	25	25.93 ± 0.86	25.20 ± 0.78	27.05 ± 0.01
	-20		27.01 ± 0.22	26.88 ± 1.30	25.04 ± 0.28
	-80		24.98 ± 1.76	26.32 ± 0.84	23.94 ± 2.83
Choline	4	25	24.97 ± 1.14	24.72 ± 0.49	24.01 ± 0.11
	-20		23.20 ± 2.94	22.93 ± 1.66	24.85 ± 0.27
	-80		23.80 ± 1.40	25.80 ± 0.86	23.55 ± 0.45
Crotonobetaine	4	5	5.22 ± 0.02	4.97 ± 0.03	5.10 ± 0.03
	-20		5.03 ± 0.05	5.11 ± 0.06	5.05 ± 0.20
	-80		4.79 ± 0.15	5.02 ± 0.11	4.83 ± 0.55
γ-butyrobetaine	4	5	4.78 ± 0.25	4.84 ± 0.08	4.92 ± 0.14
	-20		4.66 ± 0.55	5.51 ± 0.12	5.30 ± 0.09
	-80		4.97 ± 0.36	5.02 ± 0.44	5.11 ± 0.34
L-carnitine	4	25	26.88 ± 0.64	26.37 ± 0.71	24.69 ± 0.43
	-20		24.47 ± 4.33	23.97 ± 2.56	25.55 ± 0.84
	-80		24.85 ± 2.91	24.86 ± 3.85	24.32 ± 0.10
TMA	4	5	5.19 ± 0.19	4.97 ± 0.04	4.96 ± 0.11
	-20		5.06 ± 0.14	4.95 ± 0.33	5.06 ± 0.50
	-80		4.93 ± 0.22	4.84 ± 0.26	5.08 ± 0.04
TMAO	4	25	25.80 ± 0.94	24.65 ± 0.04	25.45 ± 0.45
	-20		24.39 ± 0.47	25.05 ± 0.65	24.91 ± 0.77
	-80		27.11 ± 0.39	26.08 ± 0.54	23.15 ± 0.54
TML	4	5	4.93 ± 0.06	5.33 ± 0.06	5.11 ± 0.21
	-20		5.22 ± 0.32	5.05 ± 0.22	4.99 ± 0.16
	-80		5.10 ± 0.40	5.09 ± 0.08	5.06 ± 0.42

QC, quality control; T, time; TMA, trimethylamine; TMAO, trimethylamine N-oxide; TML, trimethyllysine

1 **Supplementary Figure 1.** Overlaid extracted ion chromatogram from liquid
2 chromatography-tandem mass spectrometry with multiple reaction monitoring of 1)
3 trimethylamine, 2) betaine aldehyde, 3) choline, 4) betaine, 5) trimethylamine N-oxide, 6)
4 acetyl-L-carnitine, 7) L-carnitine, 8) γ -butyrobetaine, 9) crotonobetaine and 10)
5 trimethyllysine at the lower limit of quantitation

6

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Supplementary Table 6. Correlations between gut microbiota metabolites and associated clinical factors

	Acetyl-L-carnitine		Betaine		Choline		γ -butyrobetaine		L-carnitine		TMA		TMAO		TML	
	r_s	p Value	r_s	p Value	r_s	p Value	r_s	p Value	r_s	p Value	r_s	p Value	r_s	p Value	r_s	p Value
eGFR	-0.242	<0.001	0.008	0.816	-0.215	<0.001	-0.208	<0.001	-0.162	<0.001	-0.123	0.001	-0.437	<0.001	-0.313	<0.001
Sodium	-0.213	<0.001	-0.089	0.014	-0.031	0.401	-0.124	0.001	-0.197	<0.001	0.011	0.772	-0.004	0.909	-0.019	0.603
Urea	0.196	<0.001	0.025	0.485	0.184	<0.001	0.216	<0.001	0.142	<0.001	0.193	<0.001	0.392	<0.001	0.290	<0.001
Systolic BP	-0.143	<0.001	-0.167	<0.001	-0.103	0.005	-0.111	0.002	-0.134	<0.001	-0.135	<0.001	-0.150	<0.001	-0.104	0.004
Potassium	0.139	<0.001	-0.010	0.792	0.123	0.001	0.113	0.002	0.112	0.002	0.037	0.304	0.150	<0.001	0.147	<0.001
Respiratory Rate	0.134	<0.001	-0.079	0.039	-0.133	0.001	0.013	0.745	0.158	0.188	-0.001	0.971	-0.048	0.210	0.034	0.381
Diastolic BP	-0.118	0.001	0.008	0.829	-0.032	0.379	-0.036	0.323	-0.082	0.026	-0.051	0.166	-0.136	<0.001	-0.093	0.011
Age	0.104	0.003	-0.168	<0.001	-0.017	0.628	0.080	0.023	0.014	0.701	0.006	0.861	0.177	<0.001	0.043	0.222
NT-proBNP	0.052	0.141	0.118	0.001	0.035	0.325	0.145	<0.001	-0.054	0.128	0.101	0.004	0.123	<0.001	0.010	0.781
HR	-0.004	0.923	0.031	0.393	-0.067	0.066	-0.039	0.289	-0.004	0.913	0.005	0.902	-0.070	0.055	-0.018	0.622
Acetyl-L-carnitine			0.220	<0.001	0.384	<0.001	0.519	<0.001	0.737	<0.001	0.219	<0.001	0.347	<0.001	0.489	<0.001
Betaine					0.470	<0.001	0.406	<0.001	0.394	<0.001	0.340	<0.001	0.099	0.005	0.164	<0.001
Choline							0.505	<0.001	0.510	<0.001	0.355	<0.001	0.381	<0.001	0.476	<0.001
γ-butyrobetaine									0.590	<0.001	0.229	<0.001	0.324	<0.001	0.645	<0.001
L-Carnitine											0.323	<0.001	0.363	<0.001	0.513	<0.001
TMA													0.306	<0.001	0.235	<0.001
TMAO															0.475	<0.001
TML																

BP, blood pressure; eGFR, estimated glomerular filtration rate; HR, hazard ratio; NT-proBNP, N-terminal pro B-type natriuretic peptide; TMA, trimethylamine; TMAO, trimethylamine N-oxide; TML, trimethyllysine

Supplementary Table 7. Multivariate analysis for death and death/HF with renal marker influence

Death						
	30 days			1 year		
	HR	95% CI	p Value	HR	95% CI	p Value
Acetyl-L-carnitine	1.37	1.01-1.85	0.045*	1.15	0.98-1.36	0.090
Betaine	1.24	0.92-1.69	0.159	1.13	0.95-1.33	0.166
Choline	0.82	0.61-1.10	0.191	0.98	0.84-1.16	0.843
γ-butyrobetaine	1.25	0.93-1.68	0.146	1.19	1.01-1.40	0.042*
L-carnitine	1.27	0.96-1.66	0.091	1.16	1.00-1.35	0.057
TMA	0.81	0.58-1.13	0.209	1.00	0.86-1.17	0.963
TMAO	0.93	0.69-1.26	0.638	1.07	0.89-1.27	0.469
TML	1.06	0.79-1.42	0.708	1.14	0.97-1.34	0.117

Death/HF						
	30 days			1 year		
	HR	95% CI	p Value	HR	95% CI	p Value
Acetyl-L-carnitine	1.20	0.95-1.53	0.131	1.02	0.89-1.17	0.751
Betaine	1.19	0.93-1.52	0.159	1.07	0.94-1.22	0.325
Choline	0.94	0.75-1.18	0.597	0.97	0.85-1.10	0.619
γ-butyrobetaine	1.20	0.94-1.52	0.139	1.06	0.93-1.23	0.402
L-carnitine	1.18	0.94-1.48	0.150	1.04	0.92-1.18	0.502
TMA	0.79	0.60-1.03	0.083	0.97	0.86-1.11	0.684
TMAO	1.05	0.82-1.34	0.688	1.11	0.96-1.28	0.174
TML	1.04	0.82-1.32	0.772	1.07	0.94-1.23	0.325

Adjusted for sex, age, PH (HF, IHD, HTN, DM), NYHA, smoker, oedema, atrial fibrillation, sBP, dBP, HR, Hb, respiratory rate, Na, (log)NT-proBNP, urea, eGFR; CI, confidence interval; HR, hazard ratio; TMA, trimethylamine; TMAO, trimethylamine N-oxide; TML, trimethyllysine.

Supplementary Table 8. Multivariate analysis for death/HF in ischaemic and non-ischaemic HF

30 days	Ischaemic HF		Non-ischaemic HF	
	HR (95% CI)	p Value	HR (95% CI)	p Value
Acetyl-L-carnitine	1.38 (0.89-2.14)	0.146	1.49 (1.11-1.99)	0.008*
Betaine	1.28 (0.86-1.91)	0.230	1.10 (0.79-1.54)	0.563
Choline	0.89 (0.59-1.35)	0.591	1.21 (0.92-1.58)	0.180
γ -butyrobetaine	1.52 (0.96-2.42)	0.076	1.35 (0.99-1.84)	0.062
L-carnitine	1.14 (0.77-1.68)	0.527	1.55 (1.16-2.09)	0.003*
TMA	1.02 (0.62-1.69)	0.933	0.91 (0.66-1.24)	0.538
TMAO	1.04 (0.66-1.64)	0.858	1.48 (1.12-1.95)	0.006*
TML	1.36 (0.85-2.16)	0.200	1.24 (0.95-1.62)	0.119
1 year				
Acetyl-L-carnitine	1.22 (0.99-1.51)	0.057	1.11 (0.93-1.32)	0.235
Betaine	1.11 (0.91-1.36)	0.302	1.01 (0.84-1.22)	0.916
Choline	1.03 (0.84-1.25)	0.800	1.07 (0.91-1.26)	0.394
γ -butyrobetaine	1.09 (0.86-1.38)	0.485	1.23 (1.04-1.47)	0.019
L-carnitine	1.10 (0.91-1.34)	0.309	1.20 (1.01-1.43)	0.041*
TMA	1.10 (0.88-1.36)	0.407	1.04 (0.88-1.22)	0.660
TMAO	1.18 (0.93-1.51)	0.180	1.27 (1.08-1.49)	0.004*
TML	1.18 (0.94-1.49)	0.152	1.18 (1.00-1.39)	0.047*

Adjusted for sex, age, PH (HF, IHD, HTN, DM), NYHA, smoker, oedema, atrial fibrillation, sBP, dBP, HR, Hb, respiratory rate, Na, (log)NT-proBNP; CI, confidence interval; HR, hazard ratio; TMA, trimethylamine; TMAO, trimethylamine N-oxide; TML, trimethyllysine. * p<0.05.

Supplementary Figure 2. Kaplan-Meier survival curve for outcomes of death and/or rehospitalization (death/HF) stratified by tertiles

A- Acetyl-L-carnitine, B- Betaine, C- Choline, D- γ -butyrobetaine, E- L-carnitine, F- Trimethylamine, G- Trimethylamine N-oxide, H- Trimethyllysine

Supplementary Figure 3. Kaplan-Meier survival curve for outcome of death stratified by tertiles

A- Acetyl-L-carnitine, B- Betaine, C- Choline, D- γ -butyrobetaine, E- L-carnitine, F- Trimethylamine, G- Trimethylamine N-oxide, H- Trimethyllysine

Supplementary Figure 4. Mantel-Cox survival analysis for all cause death and/or rehospitalisation due to heart failure at (A) 30 days and (B) 1 year after combination of TMAO and acetyl-L-carnitine