Surrogate markers of gut dysfunction are related to heart failure severity and outcome – from the BIOSTAT-CHF consortium

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Full title: Surrogate markers of gut dysfunction are related to heart failure severity and outcome – from the BIOSTAT-CHF consortium

Short title: Gut dysfunction, severity and outcomes in heart failure

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ABSTRACT

Background: The contribution of gut dysfunction to heart failure (HF) pathophysiology is not routinely assessed. We sought to investigate whether biomarkers of gut dysfunction would be useful in assessment of HF (e.g., severity, adverse outcomes) and risk stratification.

Methods: A panel of gut-related biomarkers including metabolites of the choline/carnitine-pathway [acetyl-L-carnitine, betaine, choline, γ-butyrobetaine, L-carnitine and trimethylamine-N-oxide (TMAO)] and the gut peptide, Trefoil Factor-3 (TFF-3), were investigated in 1,783 patients with worsening HF enrolled in the systems BIOlogy Study to TAilored Treatment in Chronic Heart Failure (BIOSTAT-CHF) cohort and associations with HF severity and outcomes, and use in risk stratification were assessed.

Results: Metabolites of the carnitine-TMAO pathway (acetyl-L-carnitine, γ-butyrobetaine, L-carnitine and TMAO) and TFF-3 were associated with the composite outcome of HF hospitalisation or all-cause mortality at 3 years [HR 2.04-2.93 (95% CI 1.30-4.71) $p \leq 0.002$]. Combining the carnitine-TMAO metabolites with TFF-3, as a gut dysfunction panel, showed a graded association; a greater number of elevated markers was associated with higher New York Heart Association class ($p<0.001$), higher plasma concentrations of B-type natriuretic peptide ($p<0.001$), and worse outcome [HR 1.90-4.58 (95% CI 1.19-6.74) $p \leq 0.008$]. Addition of gut dysfunction biomarkers to the contemporary BIOSTAT HF risk model also improved prediction for the aforementioned composite outcome [C-statistics $p \leq 0.011$, NRI 13.5-21.1 (95% CI 2.7-31.9) $p \leq 0.014$].

Conclusions: A panel of biomarkers of gut dysfunction showed graded association with severity of HF and adverse outcomes. Biomarkers as surrogate markers are potentially useful for assessment of gut dysfunction to HF pathophysiology and in risk stratification.

INTRODUCTION

Heart failure (HF) pathophysiology involves complex regulation by multiple systemic conditions (i.e. neuroendocrine activation, metabolic impairment, iron deficiency/anemia, etc.) [1] compounded on to cardiac dysfunction. However, the contribution of gut dysfunction to HF pathophysiology is not routinely assessed. Bowel perfusion, gut permeability [2], and the gastro-intestinal (GI) microbiome [3] contribute to HF pathophysiology, and their assessment may aid in better understanding disease severity and adverse outcomes [4]. We sought to investigate whether biomarkers as surrogate markers of gut dysfunction would be useful to assess contribution of gut dysfunction to HF pathophysiology (e.g., severity, adverse outcomes) and risk stratification.

Gut-derived metabolites of the carnitine/choline metabolic pathway, reflecting alterations of the gut microbial flora, have recently been shown to exert toxic effects on the heart and blood vessels [5], and promote inflammation [6] that contributes to HF severity [7] and adverse outcomes in acute [8,9] and chronic [10-13] HF. This metabolic pathway of choline/carnitine links cardiovascular disease risk and the Western diet which is rich in red meat and eggs [14-16]. While there has been increasing interest in a pivotal molecule of this pathway, trimethylamine-N-oxide (TMAO), TMAO is only one component of a complex metabolic pathway and is generated from two pathways; 1) betaine -> choline -> TMAO and 2) acetyl-L-carnitine/γ-butyrobetaine -> carnitine -> TMAO [17]. Recent evidence suggests that multiple metabolites of the choline/carnitine-TMAO pathway also contribute to outcomes of HF [14]. A more comprehensive panel of gut-related metabolites might therefore provide further insight.

In addition, a peptide biomarker of gut dysfunction, Trefoil Factor-3 (TFF-3), is part of a family of peptides expressed in mucous membranes [18], including the GI tract, and involved

in repair and protection of epithelial surfaces [18,19]. TFF-3 has been shown to predict the risk of cardiovascular events outcome in HF [20], and might add value to gut-derived metabolites.

This report investigates the association of a panel of biomarkers as surrogate markers of gut dysfunction with HF pathophysiology (e.g., severity, adverse outcomes) and identifies a graded association that is potentially useful for risk stratification of the condition.

METHODS

Study Population

The BIOlogy Study to TAilored Treatment in Chronic Heart Failure (BIOSTAT-CHF) study was a multicentre, prospective, observational study that enrolled patients in 69 centres from 12 European countries that was designed to characterise biological pathways related to response to HF guideline recommended therapy [21]. Patients were enrolled between 2010-2014 with progressive worsening or new-onset symptoms of HF, confirmed by either left ventricular EF of ≤40% or B-type natriuretic peptide (BNP) and/or NT-proBNP plasma concentrations >400pg/ml or >2000pg/ml, respectively. All patients had to require a dose of furosemide ≥ 40 mg/day or equivalent for the control of congestion and received ≤ 50% of target doses of angiotensin-converting enzyme inhibitors or angiotensin II receptors (ACEi/ARBs) and beta-blockers at enrolment. Informed consent was obtained from each patient. This study was approved by the local ethics committee and adhered to the Declaration of Helsinki.

The primary outcomes were all-cause mortality and a composite of mortality with rehospitalisation due to HF (mortality/HF) at 3 years from enrolment.

Biomarker measurements

Plasma was aliquoted and stored at -80°C until analysis. At the time of analysis, samples were thawed at room temperature, prepared and analysed immediately. One-thousand seven hundred and eighty-three (n=1783) patients had available baseline plasma samples and were therefore used in this study.

The gut microbiome-related metabolites, of the choline (choline and betaine), and carnitine (acetyl-L-carnitine, γ-butyrobetaine, L-carnitine) metabolic pathway of TMAO were extracted from plasma using stable-isotope dilution and analysed by ultra-performance liquid

chromatography-tandem mass spectrometry (UPLC-MS/MS), using a recently developed method with amendments followed by validation (see Supplementary Material for amended method) [22].

TFF-3 levels were measured using a high-throughput technique using the Olink Proseek Multiplex Cardiovascular (CVD) III96x96 kit (Olink Proteomics, Uppsala, Sweden) [23]. Normalised protein expression (NPX) values were converted to the linear scale for use in this study (i.e., NPX values can be converted into linear scale: 2^{NPX}= linear NPX).

All other clinical biomarker measurements were done at a local hospital site or within the BIOSTAT-CHF central laboratory. BNP was measured using Luminex multiplexed bead-based immunoassays (Alere, San Diego, CA, USA) [21].

Statistical analyses

Analyses used a non-imputed BIOSTAT-CHF database as described elsewhere [11,24,25]. Association with outcomes was performed using Cox proportional hazards regression analyses. Outcome prediction accuracies were assessed by calculating the area under the curve (AUC) for the receiver operator characteristics (ROC) curve analysis and using net reclassification index (NRI) for the markers across end-points, after adjustment for the compact and extended risk models made from previously defined BIOSTAT-CHF models [26]. Kaplan-Meier survival curves were generated to demonstrate cumulative incidences of events for tertile groupings of gut dysfunction markers with the Mantel-Cox log rank tests used to report the significance of stratification. Kaplan-Meier survival analyses were conducted using graded response of gut dysfunction markers (i.e., the number of elevated metabolites above the median concentration for each particular metabolite).

Statistical analyses were performed using IBM SPSS Statistics (V26, IBM Corp., Armonk, New York, USA). A p-value <0.05 was considered statistically significant.

RESULTS

Study population

From the total BIOSTAT cohort (n=2,516), baseline acetyl-L-carnitine, betaine, choline, γ-butyrobetaine, L-carnitine, TMAO and TFF-3 were analysed in 1,783 patients (71%) based on the availability of adequate volume of sample. Baseline demographics are shown in Table 1. Most patients were men (74%) with a median age of 70 years and in New York Heart Association (NYHA) class III-IV (62%).

Association of gut markers with adverse outcomes of HF

Measured gut biomarkers were all associated with mortality and the composite outcome (HF hospitalisation or death) at 3 years on univariate analysis ($p<0.001$), with the exception of betaine (Table 2). A logistic risk prediction model (backward) showed that for death that acetyl-L-carnitine, TMAO and TFF-3 remained in the final model ($p\leq0.006$), whereas for death/HF hospitalization that the carnitine metabolites (acetyl-L-carnitine, L-carnitine and γ-butyrobetaine) remained alongside TFF-3 (Supplementary Table 1) but not the choline pathway metabolites (choline, betaine). Based on this, the carnitine pathway metabolites (acetyl-L-carnitine, L-carnitine and γ-butyrobetaine), TMAO and TFF-3 were combined using logistic regression to develop a composite variable to assess their association with the composite outcome of HF hospitalisation or death at 3 years. On univariate analysis, the hazard ratio of the gut dysfunction panel was >5-fold higher than for individual markers [HR 16.67-27.79 (95% CI 10.84-46.15) $p<0.001$] (Table 2).

Kaplan-Meier survival analysis was conducted by splitting the variable into tertiles. Results showed that elevated plasma concentrations of both individual and the panel of markers was associated with poor survival ($p<0.001$) (Supplementary Figures 1 & 2).

Association of graded/combined contribution of gut dysfunction markers to adverse outcomes

Kaplan-Meier survival analysis showed that patients with ≤1 marker elevated had the best prognosis and a graded relationship for 2, 3, 4 and 5 elevated markers, with those who had increases in all five metabolites having the worst outcome (Figure 1). Patients with only one elevated metabolite did not show any significant differences compared to the reference group ($p \geq 0.582$) (Figure 2). The number of increased biomarkers of gut dysfunction was also associated with worse NYHA class (chi-square $p<0.001$) and higher plasma concentrations of BNP ($p<0.001$) (Supplementary Figures 3A and B).

Patient demographics with respect to the groupings of elevated gut markers showed that patients with an increasing number of elevated markers were likely to be older, ischaemic aetiology, COPD and had previous HF hospitalisation ($p \leq 0.003$). They were also likely to have reduced diastolic BP, heart rate, haemoglobin, eGFR and sodium levels ($p \leq 0.034$) (Table 1).

Risk stratification using gut dysfunction markers with BNP

A biomarker risk score was constructed using the six biomarkers of BNP, TFF-3, acetyl-L-carnitine, γ-butyrobetaine, L-carnitine and TMAO, with each independent predictor assigned a value of 1 or 0 based on elevated levels above or below the median. Based on this, the BIOSTAT-CHF cohort attained an average risk prediction score of 2.99 points (Figure 3A). Logistic regression showed an association between biomarker score and the composite outcome at 3 years ($p \leq 0.018$), and the odds ratio increased progressively from an odds ratio of 2 for one biomarker to >10 when using all six biomarkers (Figure 3B).

Revised BIOSTAT risk prediction models with inclusion of gut dysfunction

Carnitine pathway metabolites (acetyl-L-carnitine, γ-butyrobetaine, L-carnitine), TMAO and TFF-3 showed associations after adjustment for the BIOSTAT compact and extended models [26] for mortality [HR 1.46-3.76 (95% CI 1.13-6.63) $p \leq 0.018$], with the

carnitine pathway metabolites and TFF-3 also associated with the composite outcome [HR 1.97-2.91 (95% CI 1.36-4.73) p≤0.001] (Table 2).

When adjusted for the compact and extended BIOSTAT models, the hazard ratios for the gut dysfunction model (carnitine pathway metabolites + TMAO + TFF-3) were greater than 2-fold higher than individual metabolites for death [HR 6.18-7.27 (95% CI 3.02-14.46) p<0.001] or the composite outcome [HR 4.28-4.90 (95% CI 2.22-8.50) p<0.001] (Table 2), resulting in improved C-statistics (p≤0.044). NRI analysis demonstrated total overall improvement for the gut dysfunction model when added to the compact and extended models for both mortality and the composite outcome at 3 years (p≤0.014) (Table 3).

DISCUSSION

The present study investigated whether biomarkers as surrogate markers of gut dysfunction could be used to assess contribution to HF pathophysiology and risk stratification. A panel of biomarkers including gut-derived metabolites of carnitine metabolism and the peptide biomarker, Trefoil Factor-3 (TFF-3), when used in combination showed a graded association with heart failure severity and worsening outcomes, and an additive role in risk stratification. Biomarker-based assessment of contribution of gut dysfunction to HF pathophysiology is a potentially promising method to allow routine assessment of this under-appreciated contribution of gut dysfunction to HF pathophysiology and risk stratification.

Pathophysiological implications of the gut-heart axis

There is increasing evidence of a 'gut-heart axis' in HF [7,15,16]. Systemic congestion and reduced cardiac output can trigger intestinal mucosal ischaemia/oedema and impaired barrier function resulting in increased bacterial translocation, with an increase in blood endotoxins contributing to the inflammatory responses seen in HF [2,7]. The microbiota is an important protective factor of the gut against disease with regards to bacterial translocation and products that affect the gut environment, while its perturbation affects the mucosal community which contributes to HF pathogenesis [27]. Alterations in the gut microbiota makes the gut susceptible to the growth of anaerobic bacteria [28] which affects the permeability to metabolites produced in the gut and subsequently on the functional and structural integrity of the mucosal barrier [8,12,15,22].

Surrogate markers of gut dysfunction in HF assessment

The present study investigated whether biomarkers as surrogate markers of gut dysfunction would be useful to assess contribution to HF pathophysiology (e.g., severity, adverse outcomes) and risk stratification.

Recent investigations have identified the role of gut-derived metabolites of the choline/carnitine pathway to HF pathophysiology [22]. Association of one of the metabolites of this pathway, TMAO, has received attention in HF pathophysiology and risk stratification [8,10,15]. Circulating levels of TMAO have been previously reported in the BIOSTAT-CHF cohort to be associated with HF adverse outcomes [11]; the present analysis investigated the extended metabolic pathway through carnitine/choline metabolism and shows that acetyl-L-carnitine, γ-butyrobetaine, and L-carnitine in addition to TMAO to be associated with adverse outcomes. Findings of contribution of the carnitine-TMAO pathway but not the choline-TMAO pathway is consistent with a previous single-center study that showed carnitine rather than the choline pathway contributes to HF outcomes [22] and validates findings in a larger real-world multi-center setting. Higher levels of carnitine derivatives (acetyl-carnitine, trimethyllysine, octanoyl-carnitine, and palmitoyl-carnitine) have been independently reported to be associated with the severity of HF as well [29].

Carnitine has an essential role in fatty acid and carbohydrate metabolism by transporting long-chain acyl groups from fatty acids into the mitochondrial matrix to be metabolised through β-oxidation to acetyl CoA via the citric acid cycle, and is ingested mainly through red meat as its dietary source [30]. Carnitine and its acyl-derivatives are disturbed in HF, and have been implicated in cardiac cachexia/sarcopenia which is common in advanced/severe HF [31,32], and carnitine insufficiency is commonly seen in HF patients and associated with reduced left ventricular diastolic function. Carnitine supplementation has been reported to be a

potential treatment of mitochondrial dysfunction in HF [33,34], and to lead to improvement in clinical symptoms, cardiac morphology/function, natriuretic peptide levels, and renal function; however, no clear effects on mortality have been demonstrated [35]. These beneficial effects are linked both to the metabolic effect on myocardial cells [36] through an increase in glucose utilisation (rather than a normalisation of the fatty acid metabolism), and to the anti-catabolic effect on skeletal muscle cells resulting in the L-carnitine anti-wasting effects [37].

Trefoil Factor-3 (TFF-3), another biomarker of gut dysfunction, is a thermostable and protease-resistant peptide that is expressed in the gastrointestinal tract and reported to play a role in mucosal protection against damage [38], showed added value when used alone or in combination with the aforementioned carnitine metabolites for assessment of HF severity and adverse outcomes. TFF-3 is involved in the reconstitution of epithelial barriers after injury; more specifically, it is required to maintain the integrity of the mucosal barrier to prevent environmental insult and promote wound repair [39], and has been previously reported to be associated with more severe HF and worse outcomes [40].

Of notable interest is the combined/graded manner of association of the aforementioned carnitine metabolites and TFF-3 with HF severity and adverse outcomes. This allowed for a scoring scale to assess the degree of contribution of biomarkers to HF assessment which will be useful for clinical application to quantify contribution of gut dysfunction. Added value to risk stratification was also shown in a revised contemporary model of HF outcomes (BIOSTAT risk model) when incorporating these biomarkers of gut dysfunction. The graded scoring shows that gut dysfunction is more involved with increasing number of gut-related biomarkers, and adds a new dimension of quantitative assessment of contribution of gut dysfunction to management of HF.

Further investigations to add additional biomarkers reflecting different pathophysiological facets of gut dysfunction to a surrogate biomarker panel are warranted to further develop/extend the concept of the 'gut-heart axis' in a comprehensive/systematic manner and to clinically translate assessment of gut dysfunction to HF management with the present investigation serving as an important first step (proof-of-concept) to this aim.

Study limitations

The observational design of the BIOSTAT-CHF study does not allow to infer a causative role of the gut biomarkers and outcome. In addition, information regarding diet and physical activity to adjust for these confounding factors were not available. All patients in this study had a recent HF hospitalisation that may limit the generalisability of the findings.

CONCLUSIONS

The present investigation showed that biomarkers of gut dysfunction, carnitine pathway metabolites and TFF-3, together were associated in a graded/combinatorial manner to adverse HF outcomes and disease severity, and add to current risk models of HF. Use of biomarkers as surrogate markers potentially allows for assessment of contribution of gut dysfunction to HF pathophysiology and risk stratification.

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CONFLICT OF INTEREST

SDA reports receiving fees from Abbott, Bayer, Boehringer Ingelheim, Cardiac Dimension, Cordio, Impulse Dynamics, Novartis, Occlutech, Servier, and Vifor Pharma, and grant support from Abbott and Vifor Pharma. J.G.C. has received consulting honoraria fees and/or research grants from Johnson & Johnson, Amgen, AstraZeneca, Bayer, Bristol Myers Squibb, GSK, Medtronic, Myokardia, Novartis, Philips, Pharmacosmos, PharmaNord, Sanofi, Servier, Stealth Biopharmaceuticals, Torrent Pharmaceuticals and Vifor. M.M. has received grants from the European Community, and participation to advisory boards with fees from Novartis and Bayer. L.L.N. has received grants from EU FP7. All other authors have no conflicts to report.

AUTHOR CONTRIBUTIONS

All authors listed in this manuscript have substantially contributed to the study's conception, design, analysis, drafting, reviewing and performance as per the journal guidelines.

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1 **TABLES**

2 **Table 1.** Patient characteristics for the total cohort and after grouping for the number of elevated gut dysfunction markers

			Elevated[#] number of gut dysfunction markers						
	Number of patients N (%)	Total cohort	0	1	2	3	4	5	p Value
Number of patients	1783 (100%)	1783	236	332	325	339	304	247	-
Age	1783 (100%)	70 [61-78]	65 [55-73]	66 [58-75]	69 [59-78]	71 [63-79]	74 [65-79]	74 [68-80]	<0.001
Male	1783 (100%)	74%	68%	72%	75%	73%	79%	76%	0.102
Current smoker	1780 (99.8%)	14%	19%	14%	15%	15%	12%	11%	0.200
Ischemic aetiology	1748 (98%)	53%	45%	51%	49%	59%	59%	62%	<0.001
Diabetes mellitus	1783 (100%)	32%	25%	32%	27%	34%	32%	44%	<0.001
COPD	1783 (100%)	18%	13%	15%	15%	19%	24%	21%	0.003
Previous HF hospitalisation	1783 (100%)	31%	25%	27%	30%	35%	30%	40%	0.003
NYHA class I	1729 (97%)	2%	2%	3%	1%	2%	1%	2%	
II		36%	42%	39%	42%	37%	32%	21%	
III		49%	47%	48%	47%	49%	50%	57%	<0.001
IV		13%	8%	10%	10%	12%	17%	20%	
LV ejection fraction (%)	1608 (90%)	30 [25-36]	30 [25-35]	30 [25-35]	30 [25-35]	30 [25-38]	30 [25-38]	30 [23-36]	0.424
Pulmonary congestion	1732 (97%)	52%	50%	48%	50%	56%	58%	59%	0.038
Peripheral oedema	1478 (83%)	58%	53%	49%	56%	61%	65%	66%	<0.001
Systolic blood pressure (mmHg)	1779 (99.8%)	120 [110-139]	125 [110-140]	120 [110-140]	125 [110-140]	120 [110-140]	120 [110-130]	120 [110-131]	<0.001
Diastolic blood pressure (mmHg)	1779 (99.8%)	73 [66-81]	80 [70-88]	80 [70-85]	75 [65-85]	74 [66-80]	70 [63-80]	70 [62-80]	<0.001
Heart rate (beat/min)	1778 (99.7%)	77 [67-90]	80 [70-95]	77 [67-90]	75 [65-85]	76 [66-90]	77 [69-88]	75 [66-85]	0.034
Beta-blocker	1783 (100%)	83%	90%	83%	81%	83%	84%	81%	0.078
ACE inhibitor or ARB	1783 (100%)	72%	78%	80%	73%	71%	67%	63%	<0.001
Haemoglobin (g/dL)	1720 (96%)	13.3 [11.9-14.5]	13.8 [12.6-14.9]	13.5 [12.4-14.5]	13.5 [12.1-14.9]	13.2 [11.9-14.4]	13.0 [11.6-14.2]	12.4 [11.2-13.8]	<0.001
Urea (mmol/L)	1567 (88%)	11.4 [7.6-18.2]	8.2 [6.0-12.8]	8.6 [6.3-13.5]	10.8 [7.6-15.7]	11.4 [8.0-17.9]	14.4 [9.6-21.8]	19.7 [12.3-28.9]	<0.001
eGFR* (ml/min/1.73m^2)	1782 (99.9%)	62 [47-78]	79 [68-94]	74 [61-87]	66 [54-81]	57 [45-72]	52.9 [42.9-67.8]	39.6 [30.0-51.3]	<0.001
Sodium (mmol/L)	1749 (98%)	140 [137-142]	140 [138-142]	140 [138-142]	140 [138-142]	139 [137-142]	139 [137-141]	139 [135-141]	<0.001
BNP (pg/mL)	1730 (97%)	237 [96-480]	201 [80-378]	178 [67-379]	189 [81-378]	239 [105-507]	284 [116-564]	370 [147-808]	<0.001
Protein intake (g/day)	1650 (93%)	54 [46-62]	56 [48-65]	56 [47-66]	54 [46-61]	53 [46-62]	52 [45-59]	52 [45-59]	<0.001

Gut dysfunction markers

Acetyl-L-carnitine (µmol/L)	1783 (100%)	8.4 [6.2-11.5]	5.5 [4.5-6.7]	6.6 [5.1-8.0]	7.2 [5.7-9.4]	9.2 [7.6-11.5]	10.8 [8.9-14.2]	14.7 [11.3-19.5]	<0.001
Betaine (µmol/L)	1783 (100%)	31.6 [24.0-42.6]	28.0 [21.5-36.5]	29.8 [23.6-38.1]	31.1 [23.4-41.9]	32.1 [24.2-45.1]	34.7 [26.2-48.6]	35.4 [26.6-47.6]	<0.001
Choline (µmol/L)	1783 (100%)	12.2 [9.9-15.3]	10.1 [8.4-12.1]	10.9 [9.2-13.4]	11.9 [9.5-14.3]	12.6 [10.6-15.3]	14.7 [11.2-17.3]	14.8 [12.1-19.6]	<0.001
γ-butyrobetaine (µmol/L)	1783 (100%)	1.2 [0.9-1.5]	0.9 [0.7-1.0]	0.9 [0.8-1.1]	1.1 [0.9-1.3]	1.3 [1.1-1.5]	1.4 [1.2-1.7]	1.7 [1.4-2.2]	<0.001
L-carnitine (µmol/L)	1783 (100%)	86.1 [68.3-110.4]	65.6 [52.8-75.9]	71.6 [56.5-82.1]	80.7 [66.0-98.1]	91.3 [74.8-108.7]	106.9 [92.3-127.6]	129.7 [109.2-158.0]	<0.001
Trefoil Factor-3	1783 (100%)	35 [24-54]	23 [17-29]	28 [21-36]	31 [23-47]	38.8 [27.5-54.3]	47.2 [33.6-69.2]	65.4 [46.8-102.7]	<0.001
Trimethylamine N-oxide (µmol/L)	1783 (100%)	6.4 [3.9-11.6]	3.4 [2.3-4.6]	4.5 [3.1-6.4]	5.7 [3.7-8.8]	7.3 [4.8-13.4]	9.1 [6.2-15.9]	14.7 [9.8-26.6]	<0.001
Endpoints									
3 years									
Death	1783 (100%)	468	14%	15%	22%	27%	34%	48%	<0.001
Death/HF	1783 (100%)	727	26%	26%	38%	47%	50%	61%	<0.001

Data are presented as median [interquartile range] for continuous variables and % for categorical values.

* Estimated by Chronic Kidney Disease Epidemiology Collaboration formula.

[#] Elevated is defined by those patients with biomarker levels above the median concentration

Groupings were compared using the independent samples Kruskal-Wallis test for continuous variables and the Fisher Exact test for categorical variables.

Table 2. Independent prediction abilities of gut-related metabolites for outcomes of all-cause mortality (death) and the composite endpoint of death and/or rehospitalisation due to HF (death/HF) at 3 years

	Univariate		Compact model		Extended model	
	HR [95% CI]	**p Value**	**HR [95% CI]**	**p Value**	**HR [95% CI]**	**p Value**
Death						
Acetyl-L-carnitine	6.85 [4.58-10.25]	<0.001	3.13 [1.96-4.99]	<0.001	2.74 [1.69-4.44]	<0.001
Betaine	1.44 [0.92-2.25]	0.113	1.25 [0.79-1.99]	0.345	1.26 [0.78-2.04]	0.338
Choline	3.08 [1.66-5.71]	<0.001	1.36 [0.70-2.67]	0.369	1.25 [0.63-2.48]	0.520
γ-butyrobetaine	6.80 [4.02-11.53]	<0.001	2.34 [1.27-4.32]	0.007	2.14 [1.14-4.02]	0.018
L-carnitine	6.47 [3.81-11.00]	<0.001	3.76 [2.13-6.63]	<0.001	3.06 [1.68-5.58]	<0.001
TMAO	2.35 [1.90-2.92]	<0.001	1.59 [1.24-2.04]	<0.001	1.46 [1.13-1.89]	0.003
TFF-3	5.60 [4.31-7.27]	<0.001	2.75 [1.93-3.92]	<0.001	2.51 [1.73-3.65]	<0.001
Gut dysfunction model	27.79 [16.74-46.15]	<0.001	7.27 [3.66-14.46]	<0.001	6.18 [3.02-12.62]	<0.001
Death/HF						
Acetyl-L-carnitine	4.20 [3.03-5.82]	<0.001	2.47 [1.73-3.53]	<0.001	2.21 [1.51-3.23]	<0.001
Betaine	1.57 [1.09-2.25]	0.015	1.38 [0.92-2.07]	0.115	1.34 [0.90-2.01]	0.155
Choline	2.84 [1.73-4.66]	<0.001	1.84 [1.07-3.16]	0.027	1.51 [0.86-2.63]	0.150
γ-butyrobetaine	5.68 [3.70-8.70]	<0.001	2.91 [1.79-4.73]	<0.001	2.37 [1.40-3.99]	0.001
L-carnitine	3.24 [2.12-4.94]	<0.001	2.66 [1.69-4.19]	<0.001	2.16 [1.36-3.43]	0.001
TMAO	1.84 [1.55-2.19]	<0.001	1.36 [1.12-1.66]	0.002	1.22 [0.98-1.50]	0.070
TFF-3	4.16 [3.35-5.17]	<0.001	2.20 [1.65-2.91]	<0.001	1.97 [1.42-2.75]	<0.001
Gut dysfunction model	16.67 [10.84-25.64]	<0.001	4.90 [2.82-8.50]	<0.001	4.28 [2.22-8.25]	<0.001

Compact model for all-cause mortality (mortality): age, blood urea (log-transformed), BNP (log-transformed), haemoglobin and use of beta-blockers at baseline.

Extended model for mortality: compact model plus ischaemic aetiology, COPD, diastolic blood pressure and sodium.

Compact model for mortality and/or rehospitalisation due to HF (mortality/HF): age, previous HF hospitalisation, peripheral oedema, systolic blood pressure, BNP (log-transformed), haemoglobin, sodium and use of beta-blockers at baseline.

Extended model for mortality/HF: compact model plus current smoker, COPD and eGFR.

Gut dysfunction combined- acetyl-L-carnitine + γ-butyrobetaine + L-carnitine + TMAO + TFF-3

Table 3. Reclassification analysis using continuous reclassification of adding gut-related metabolites to the BIOSTAT-CHF risk models

	C-statistic			NRI [95% CI]	
	without metabolite	with metabolite	p value		p value
Mortality at 3 years					
Compact	0.729				
Acetyl-L-carnitine		0.738	0.058	19.9 [8.3-31.4]	<0.001
Betaine		0.730	0.261	8.4 [-3.1-20.0]	0.152
Choline		0.729	0.968	5.7 [-5.9-17.2]	0.335
γ-butyrobetaine		0.732	0.244	15.2 [3.6-26.7]	0.010
L-carnitine		0.739	0.072	15.3 [3.8-26.9]	0.009
Trimethylamine N-oxide		0.734	0.166	16.5 [5.0-28.1]	0.005
Trefoil factor-3		0.740	0.032	29.2 [17.7-40.8]	<0.001
Gut dysfunction model		0.739	0.023	25.3 [13.7-36.8]	<0.001
Extended	0.745				
Acetyl-L-carnitine		0.751	0.129	16.6 [4.9-28.4]	0.005
Betaine		0.745	0.755	11.4 [-0.3-23.1]	0.057
Choline		0.745	0.835	7.7 [-4.1-19.4]	0.200
γ-butyrobetaine		0.747	0.459	14.5 [2.8-26.2]	0.015
L-carnitine		0.750	0.116	11.3 [-0.2-23.0]	0.058
Trimethylamine N-oxide		0.749	0.175	11.7 [0.0-23.5]	0.050
Trefoil factor-3		0.753	0.076	25.3 [13.6-37.0]	<0.001
Gut dysfunction model		0.753	0.044	23.7 [12.0-35.5]	<0.001
Mortality/HF at 3 years					
Compact	0.716				
Acetyl-L-carnitine		0.727	0.009	24.9 [14.1-35.6]	<0.001
Betaine		0.717	0.478	7.7 [-3.0-18.5]	0.160
Choline		0.718	0.366	0.0 [-10.8-10.8]	1.000
γ-butyrobetaine		0.726	0.012	19.2 [8.4-29.9]	<0.001
L-carnitine		0.723	0.066	17.9 [7.2-28.7]	0.001
Trimethylamine N-oxide		0.720	0.206	10.8 [0.1-21.6]	0.048
Trefoil factor-3		0.728	0.012	21.0 [10.3-31.8]	<0.001
Gut dysfunction model		0.730	0.001	21.1 [10.4-31.9]	<0.001
Extended	0.727				
Acetyl-L-carnitine		0.735	0.031	17.3 [6.5-28.0]	0.002
Betaine		0.728	0.615	5.8 [-5.0-16.5]	0.293
Choline		0.728	0.855	0.0 [-10.8-10.8]	1.000
γ-butyrobetaine		0.733	0.042	12.6 [1.8-23.4]	0.022
L-carnitine		0.731	0.223	10.2 [-0.6-20.9]	0.064
Trimethylamine N-oxide		0.728	0.687	9.8 [-1.0-20.5]	0.075
Trefoil factor-3		0.734	0.079	13.0 [2.2-23.7]	0.018
Gut dysfunction model		0.736	0.011	13.5 [2.7-24.3]	0.014

Compact model for all-cause mortality (mortality): age, blood urea (log-transformed), BNP (log-transformed), haemoglobin and use of beta-blockers at baseline.

Extended model for mortality: compact model plus ischaemic aetiology, COPD, diastolic blood pressure and sodium.

Compact model for mortality and/or rehospitalisation due to HF (mortality/HF): age, previous HF hospitalisation, peripheral oedema, systolic blood pressure, BNP (log-transformed), haemoglobin, sodium and use of beta-blockers at baseline.

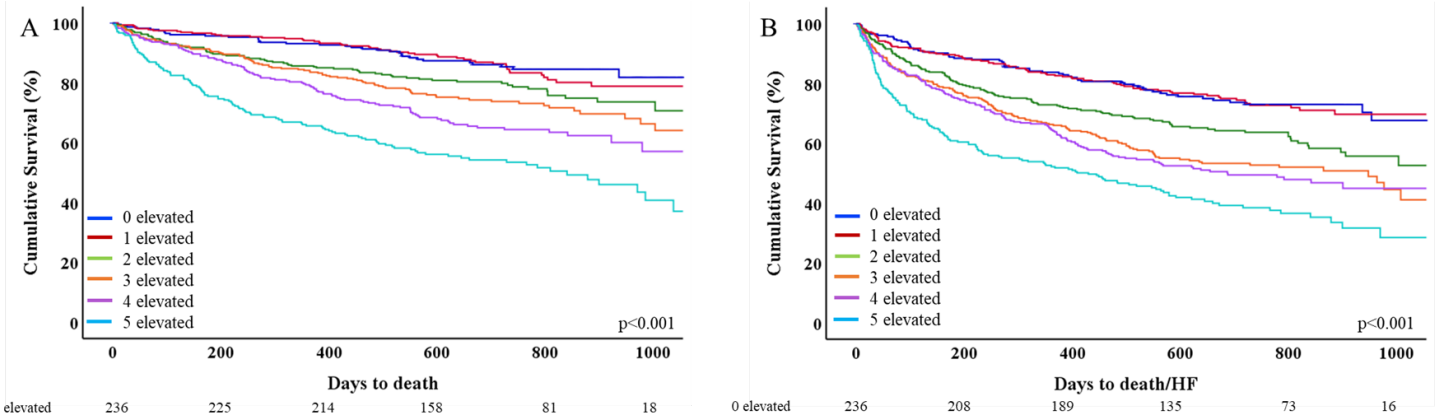
Extended model for mortality/HF: compact model plus current smoker, COPD and eGFR.

Data are presented as net reclassification index (NRI), and 95% confidence interval (CI).

Gut dysfunction model- acetyl-L-carnitine + γ-butyrobetaine + L-carnitine + TMAO + TFF-3

FIGURE TITLES

Figure 1. Kaplan-Meier survival curves for (A) death and (B) all cause death and/or rehospitalisation due to heart failure stratified by the number of elevated gut dysfunction markers

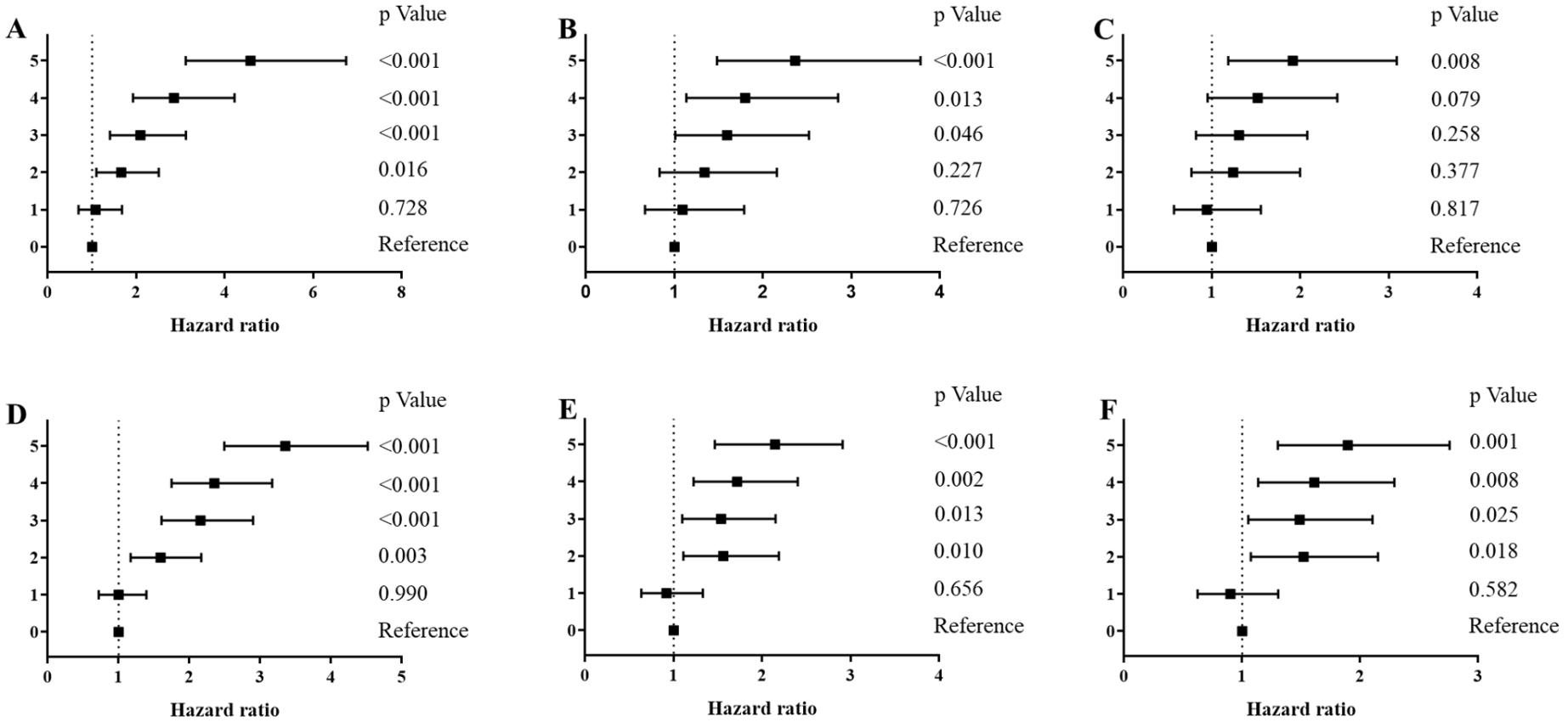


0 elevated	236	225	214	158	81	18
1 elevated	332	316	303	225	104	27
2 elevated	325	288	269	195	106	28
3 elevated	339	302	272	189	99	31
4 elevated	304	264	227	149	71	17
5 elevated	247	182	153	98	50	13

0 elevated	236	208	189	135	73	16
1 elevated	332	291	265	192	93	21
2 elevated	325	255	227	160	88	20
3 elevated	339	255	212	133	67	16
4 elevated	304	225	180	114	54	10
5 elevated	247	147	122	72	36	6

	1 elevated		2 elevated		3 elevated		4 elevated		5 elevated	
	Chi-square	p Value	Chi-square	p Value	Chi-square	p Value	Chi-square	p Value	Chi-square	p Value
(A) 0 elevated	0.166	0.684	5.946	0.015	13.941	<0.001	30.342	<0.001	69.470	<0.001
1 elevated			5.354	0.021	14.894	<0.001	35.165	<0.001	85.642	<0.001
2 elevated					2.133	0.144	12.250	<0.001	47.455	<0.001
3 elevated							4.645	0.031	32.217	<0.001
4 elevated									12.417	<0.001
(B) 0 elevated	0.001	0.980	9.072	0.003	27.642	<0.001	33.965	<0.001	69.558	<0.001
1 elevated			10.907	0.001	34.367	<0.001	41.990	<0.001	86.263	<0.001
2 elevated					6.383	0.012	10.254	0.001	38.157	<0.001
3 elevated							0.617	0.432	14.863	<0.001
4 elevated									9.271	0.002

Figure 2. Forest plot showing the association with outcome for patients with the number of elevated gut dysfunction markers.



Cox proportional hazards regression modelling was used to compare the risk of death at 3 years (A) unadjusted (B) adjusted compact model (C) adjusted extended model, and for death/HF (D), (E), (F). Data are presented as hazard ratio (HR) and 95% confidence interval (CI)

Figure 3. (A) Distribution of biomarker score in patients from the BIOSTAT-CHF cohort and (B) Logistic regression from the biomarker score for outcomes of death/HF at 3 years