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ORIGINAL ARTICLE

Plasma amino acid profiling improves predictive accuracy of adverse events in patients with heart failure

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Abstract

Aims The clinical outcome of heart failure (HF) is complicated by the presence of multiple comorbidities including malnutrition and cachexia, and prediction of the outcome is still difficult in each patient. Metabolomics including amino acid profiling enables detection of alterations in whole body metabolism. The aim of this study was to determine whether plasma amino acid profiling improves prediction of clinical outcomes in patients with HF.

Methods and results We retrospectively examined 301 HF patients (70 ± 15 years old; 59% male). Blood samples for measurements of amino acid concentrations were collected in a fasting state after stabilization of HF. Plasma amino acid concentrations were measured using ultraperformance liquid chromatography. Clinical endpoint of this study was adverse event defined as all-cause death and unscheduled readmission due to worsening HF or lethal arrhythmia. During a mean follow-up period of 380 ± 214 days, 40 patients (13%) had adverse events. Results of analyses of variable importance in projection score, a measure of a variable's importance in partial least squares–discriminant analysis (PLS-DA) showed that the top five amino acids being associated with adverse events were 3-methylhistidine (3-Me-His), β-alanine, valine, hydroxyproline, and tryptophan. Multivariate Cox-proportional hazard analyses indicated that a high 3-Me-His concentration and low β-alanine and valine concentrations were independently associated with adverse events. When HF patients were divided according to the cut-off values of amino acids calculated from receiver operating characteristic curves, Kaplan–Meier survival curves showed that event-free survival rates were lower in HF patients with high 3-Me-His than in HF patients with low 3-Me-His (68% vs. 91%, $P < 0.01$). In a subgroup with high 3-Me-His, HF patients with low β-alanine and those with low valine had significantly lower event-free survival rates than did HF patients with high β-alanine and those with high valine, respectively. On the other hand, Kaplan–Meier curves of event-free survival rates did not differ between HF patients with and those without low β-alanine and low valine in subgroups of patients with low 3-Me-His. Inclusion of both high 3-Me-His and low β-alanine or low valine into the adjustment model including N-terminal pro-brain natriuretic peptide improved the accuracy of prediction of adverse events after discharge. 3-Me-His concentration was associated with muscle mass and nutritional status.

Conclusions Simple measurement of 3-Me-His with either β-alanine or valine improved the predictive ability for adverse events, indicating the utility of plasma amino acid profiling in risk stratification of hospitalized HF patients.

Keywords Heart failure; Amino acids; Prognosis; Preventive cardiology; Cachexia; Metabolism

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Introduction

Heart failure (HF) is a major cause of morbidity and mortality worldwide, and the incidence of HF has been increasing despite recent therapeutic advances.[1] Considering the progressive nature of HF with repeated episodes of hospitalization due to worsening HF and arrhythmia, accurate prediction of adverse events such as death and rehospitalization is crucial for an appropriate decision regarding the treatment strategy for HF patients. Several attempts have been made to achieve accurate prediction of prognosis in HF patients. Natriuretic peptides such as brain natriuretic peptide (BNP) and N-terminal fragment of proBNP (NT-proBNP) and high-sensitivity troponins, indexes of cardiomyocyte injury, are established as markers for diagnosis, management, and prognostication of HF, and the markers have been incorporated into clinical practice guidelines for acute HF and chronic HF.[2] Prediction models of prognosis for HF have also been developed by using variables that were selected as potential prognostic markers in analyses of a large HF population including patients included in clinical trials of HF.[3–5] However, the predictive accuracy of these approaches, especially in elderly HF patients, has been questioned.[6–8] Elderly HF patients have higher rates of HF with preserved ejection fraction (HFpEF); atrial fibrillation; and multiple comorbidities such as chronic kidney disease (CKD), anaemia, malnutrition, and cachexia. All of these factors appear to contribute to poor clinical outcomes in elderly HF patients, leading to inaccurate prediction of mortality.[7,9]

Metabolomics, one of the omics approaches, is an approach useful not only for elucidation of cellular metabolism but also for an understanding of disease pathogenesis and response to treatment.[10] Amino acids serve as metabolic intermediates in the regulation of multiple cell functions, and their plasma levels are modulated by sensing alterations in whole body metabolism, which has been applied to early detection of diseases including cancers and neurodegenerative diseases.[11] Furthermore, the results of several studies have shown that assessment of plasma metabolites including amino acids is useful for the early diagnosis, individualization, and risk stratification of HF and other cardiovascular diseases.[12–18] However, the prognostic value of plasma amino acid profiling remains elusive for an HF population including elderly subjects with multiple comorbidities. Non-cardiac comorbidities are frequently present in elderly HF patients, and the comorbidities including metabolic disorders such as cachexia have profound impacts on plasma metabolites.[12,13,19] Although plasma metabolites in cancer patients with cachexia were analysed in several earlier studies, the association of plasma amino acids with cachexia in HF patients has not been examined. An attempt to examine the association may lead to the development of a plasma marker of cachexia.

The aim of this study was to determine the utility of amino acid profiling for risk prediction of adverse events in HF patients and to explore a novel marker for detection of HF-induced metabolic derangement. To achieve the aim, we performed systematic metabolomic analyses of the relationships between plasma amino acid levels and adverse events in elderly HF patients.

Methods

Study subjects

This study was a single-centre, retrospective, and observational study. We enrolled 436 consecutive patients who were admitted to our institute for diagnosis and management of HF during the period from 15 February 2018 to 31 July 2020. Inclusion criterion was HF that was diagnosed according to the 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic HF.[20] Exclusion criteria were pulmonary artery hypertension, acute myocarditis, and chronic kidney disease at stage 5 that was defined as estimated glomerular filtration rate (eGFR) $<$ 15 mL/min/1.73 m^2, missing data for amino acid profiling and a follow-up period of less than 30 days as shown in *Figure 1*. This study was conducted in strict adherence with the principles of the Declaration of Helsinki and was approved by the Clinical Investigation Ethics Committee of Sapporo Medical University Hospital (Number 302-243).

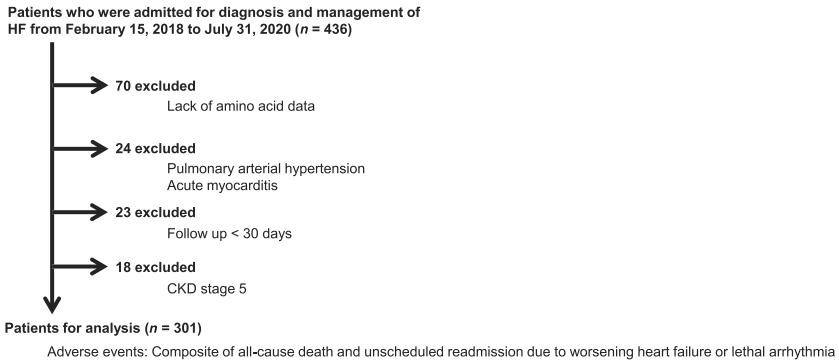
Biochemical analyses, echocardiography, body composition analyses, and assessment of nutritional status

Data for blood tests and left ventricular ejection fraction (LVEF), measured by the modified Simpson method, were retrieved from the patients' medical records. eGFR was calculated using equations developed for Japanese subjects as follows: eGFRcre (mL/min/1.73 m^2) = 194 × $Scr^{-1.094}$ × $age^{-0.287}$(×0.739 if female).[21] CKD was defined as eGFRcre of less than 60 mL/min/1.73 m^2.

Skeletal muscle mass (SSM) and appendicular skeletal muscle mass (ASM), which is the sum of bone-free lean masses in the arms and legs, were calculated by using a dual-energy X-ray absorptiometry scan (Horizon A DXA System, HOLOGIC, Waltham, MA, USA) as previously reported.[21] SSM index and ASM index (ASMI) were defined as SSM/$height^2$ and ASM/$height^2$, respectively.

Nutritional status was assessed using the Mini Nutritional Assessment-Short Form (MNA-SF) as previously described.[9] The MNA-SF consists of six questions about reduction in food intake over the past 3 months, weight loss during the past 3 months, mobility, psychological stress or acute disease in

Figure 1 Flow chart of inclusion of patients. CKD, chronic kidney disease; HF, heart failure.



the past 3 months, neuropsychological problems, and body mass index (BMI), and it is scored 0 to 14.

Plasma amino acid profiling

Blood samples for measurements of amino acid concentrations were collected in a fasting state after stabilization of HF. Plasma amino acid concentrations were measured using ultraperformance liquid chromatography (LSI Medience Corporation, Tokyo, Japan) as described in Supporting Information, *Detailed Methods* section.[22] The assay was validated as shown in *Table S1*. Among 34 metabolites measured, 10 essential amino acids (valine, leucine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, histidine, and tyrosine), 10 non-essential amino acids (glycine, alanine, arginine, cystine, asparagine, aspartic acid, glutamine, glutamic acid, serine, and proline), and 9 other amino acid metabolites (taurine, hydroxyproline, citrulline, α-amino-*n*-butyric acid, β-alanine, monoethanolamine, ornithine, 1-methylhistidine [1-Me-His], and 3-methylhistidine [3-Me-His]) were steadily detected and were subsequently analysed using MetaboAnalyst 4.0 (https://www.metaboanalyst.ca). A total of 44 (0.5%) missing values were detected because of low abundance metabolites (i.e. below the detection limit), and they were replaced by one-fifth of the minimum values of their corresponding metabolites. In addition, imputation of the missing values was performed using several other methods implemented in MetaboAnalyst 4.0: (i) replacement by *k*-nearest neighbours based on similar features, (ii) *k*-nearest neighbours based on similar samples, (iii) probabilistic principal component analysis, (iv) Bayesian principal component analysis, and (v) singular value decomposition. The concentrations of metabolites were normalized by auto scaling (mean-centred and divided by the standard deviation of each variable) in order to make features more comparable.

Clinical endpoints

Clinical endpoint in this study was adverse event that was defined as a composite of all-cause death and unscheduled readmission due to worsening HF or lethal arrhythmia after discharge. An episode of worsening HF was defined as either an unplanned scheduled hospitalization for HF or an urgent visit due to worsening HF symptoms. An episode of lethal arrhythmia was defined as either symptomatic arrhythmia or an appropriate implantable cardioverter defibrillator shock against ventricular arrhythmia. Data for clinical endpoints in the study patients were obtained for 2 months after enrolment of the last patient: the mean follow-up period was 380 ± 214 days.

Statistical analysis

Data are presented as means ± standard deviation or medians (interquartile range: 25th–75th percentile) and expressed as frequency and percentage. Student's *t*-test was used for a comparison of continuous variables in two groups. Differences in categorical variables between two groups were examined by the χ^2 test. Univariate and multivariate Cox-proportional hazard analyses were used to evaluate predictive ability. Multivariate Cox-proportional regression analyses were performed by incorporating four variables: age, sex, each amino acid, and each explanatory factor with $P < 0.10$ in the univariate analyses.

Multivariate analysis [partial least squares–discriminant analysis (PLS-DA)] was used to determine metabolites that showed a significant difference between patients with and those without adverse events. On the basis of a previous report,[23] we selected metabolites with a variable importance in projection (VIP) score ≥1.5 in PLS-DA in the first narrowing-down step. Correlation analysis and hierarchical clustering were then used in order to determine suitable

combinations of metabolites for reclassification of patients at risk.

Receiver operating characteristics analysis was performed to calculate the cut-off values of amino acid concentrations for prediction of adverse events. We divided the patients into two or four groups stratified by the calculated cut-off values and compared clinical outcomes. Survival curves were calculated by the Kaplan–Meier method, and statistical significance of differences between the curves was assessed by log-rank statistics. To examine the significance of the incremental discriminative value added by plasma amino acids, we constructed logistic models for adverse events based on the results obtained in the Cox regression models. Harrell's C-index, continuous net reclassification improvement (cNRI), and integrated discrimination improvement (IDI) were calculated and compared between the base model and the model with the addition of levels of amino acids according to our previous report.[9] A P value < 0.05 was considered statistically significant. Statistical analyses were carried out using JMP version 15.1.0 (SAS Institute Inc., Cary, NC, USA).

Results

Four hundred and thirty-six patients met the inclusion criterion, and 135 patients were excluded by the exclusion criteria. Thus, data for 301 patients were used for analyses as shown in *Figure 1*.

Baseline clinical characteristics

As shown in *Table 1*, the mean age of the patients was 70 ± 15 years, and 59% were male patients. The mean BMI of the patients was 23.2 ± 4.1 kg/m^2. Twenty-eight per cent of the patients were classified as New York Heart Association

Table 1 Baseline characteristics

Characteristic	All patients $N = 301$	Event (+) $N = 40$	Event (−) $N = 261$	P value
Age (years)	70.2 (14.5)	72.2 (15.7)	69.9 (14.3)	0.342
Male sex	177 (59)	23 (58)	154 (59)	0.857
BMI (kg/m^2)	23.2 (4.1)	22.1 (4.2)	23.3 (4.1)	0.087
NYHA III or IV	84 (28)	17 (43)	67 (26)	0.027
LVEF (%)	46.6 (16.0)	42.9 (17.3)	47.2 (15.7)	0.120
LVEF < 50%	165 (55)	25 (63)	140 (54)	0.294
Aetiology				0.873
Ischemic	43 (14)	7 (18)	36 (14)	
Cardiomyopathy	115 (38)	16 (40)	99 (38)	
Valvular heart disease	105 (35)	13 (32)	92 (35)	
Others	34 (13)	4 (10)	34 (13)	
Comorbidity				
Hypertension	185 (61)	23 (58)	162 (62)	0.580
Diabetes mellitus	107 (36)	16 (40)	91 (35)	0.528
Dyslipidaemia	163 (54)	22 (55)	141 (54)	0.908
CKD	174 (58)	35 (88)	139 (53)	<0.001
Atrial fibrillation	114 (38)	18 (45)	96 (37)	0.318
Laboratory data				
Haemoglobin (g/dL)	12.5 (2.1)	11.7 (1.9)	12.7 (2.1)	0.008
NT-proBNP (pg/mL)	995 (467–2150)	1387 (840–4227)	940 (416–2023)	0.016
BUN (mg/dL)	19 (15–25)	24 (18–34)	19 (14–24)	<0.001
Creatinine (mg/dL)	0.93 (0.77–1.21)	1.20 (0.92–1.75)	0.91 (0.76–1.16)	<0.001
eGFR (mL/min/1.73 m^2)	55.5 (20.5)	42.1 (16.9)	57.6 (20.2)	<0.001
Albumin (g/dL)	3.7 (0.5)	3.5 (0.4)	3.7 (0.5)	0.027
Medication				
ACE-I or ARB	174 (58)	17 (43)	157 (60)	0.035
β blocker	218 (72)	29 (73)	189 (72)	0.991
MRA	135 (45)	26 (65)	109 (42)	0.006
Loop diuretics	163 (54)	29 (73)	134 (51)	0.012
Device				<0.001
Pacemaker	24 (8)	6 (15)	18 (7)	
ICD	41 (14)	10 (25)	31 (12)	
CRT-P or CRT-D	30 (10)	9 (23)	21 (8)	

ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; BUN; blood urea nitrogen; CKD; chronic kidney disease; CRT-D, cardiac resynchronization therapy defibrillator; CRT-P, cardiac resynchronization therapy pacemaker; eGFR, estimated glomerular filtration rate; ICD, implantable cardioverter defibrillator; LVEF, left ventricular ejection fraction; MRA, mineralocorticoid receptor antagonist; NT-proBNP, N-terminal pro-brain natriuretic peptide; NYHA, New York Heart Association.
Values are presented as mean (± standard deviation), median (interquartile range), or *n* (%) wherever appropriate. *N* refers to the number of patients for whom the parameter was available.

(NYHA) III or IV. The mean LVEF was 47 ± 16%, and 55% of the patients was classified into HF with reduced ejection fraction (HFrEF) or HF with mid-range ejection fraction. The most frequent aetiology of HF was cardiomyopathy (38%) followed by valvular heart disease (35%) and ischemic cardiomyopathy (14%).

Clinical characteristics of heart failure patients with adverse events

During the mean follow-up period of 380 ± 214 days, 40 patients (13%) had adverse events (all-cause death, 11 patients; HF hospitalization, 17 patients; hospitalization for arrhythmia, 12 patients). As shown in *Table 1*, patients with adverse events tended to have lower BMI and had a higher prevalence of NYHA III symptoms than those in patients without adverse events, whereas age, sex and aetiologies of HF were not significantly different between the two groups. The proportion of patients who had CKD was higher in patients with adverse events than in patients without adverse events, but the proportions of patients with hypertension, dyslipidaemia, diabetes mellitus, and atrial fibrillation were similar in the two groups. Plasma level of NT-proBNP was higher, and levels of hemoglobin and albumin and eGFR were lower in patients with adverse events than in patients without adverse events. Angiotensin-converting enzyme inhibitors or angiotensin receptor blockers were less frequently used, and mineralocorticoid receptor antagonists (MRAs), loop diuretics, and device therapies including implantable cardioverter defibrillator therapy and cardiac resynchronization therapy were more frequently used in patients with adverse events.

Association of plasma amino acid profile with adverse events in heart failure patients

First, PLS-DA was performed to visualize the differences in serum amino acid levels between patients with adverse events and patients without adverse events. As shown in *Figure 2A*, Component 1, which accounted for 15.4% of the total variation, separated HF patients with adverse events from HF patients without adverse events. Analyses of VIP score, a measure of a variable's importance in the PLS-DA model, showed that the top five amino acids were 3-Me-His, β-alanine, valine, hydroxyproline, and tryptophan (*Figure 2B*). Similar top five amino acids were selected in analyses using several methods of the missing values imputation except for a case of replacement of the missing values by *k*-nearest neighbours based on similar features (*Figure S1*). As shown in *Figure 3*, a correlation heatmap and a hierarchical clustering showed that the five amino acids were separated into three clusters: a cluster associated with increased adverse events (hydroxyproline and 3-Me-His) and clusters associated with decreased adverse events (a cluster with valine and tryptophan and a cluster with β-alanine). The difference in each plasma amino acid concentration between patients with adverse events and patients without adverse events is shown in *Table S2*.

Prognostic impacts of amino acids in heart failure patients

Results of univariate Cox-proportional regression analyses showed that the top five amino acids in addition to NYHA functional class, CKD, haemoglobin, NT-proBNP, albumin, and use of MRAs, loop diuretics, and device therapy were

Figure 2 Multivariate analyses to identify amino acids associated with adverse events in patients with heart failure. (A) 2D-score plot between Components 1 and 2 derived from partial least squares–discriminant analysis (PLS-DA). The explained variance is shown in brackets. (B) *X*-axis indicates the variable importance in projection (VIP) scores corresponding to each variable on the *Y*-axis. Amino acids that indicate a VIP score of ≥0.7 are shown.

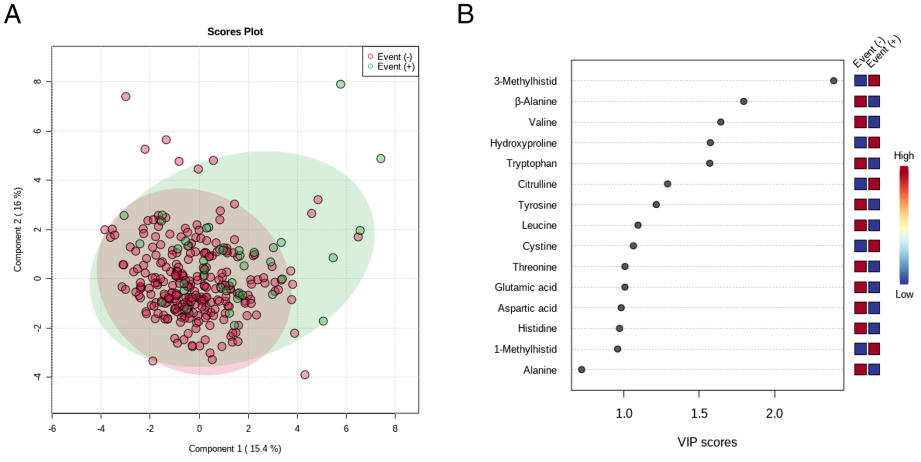
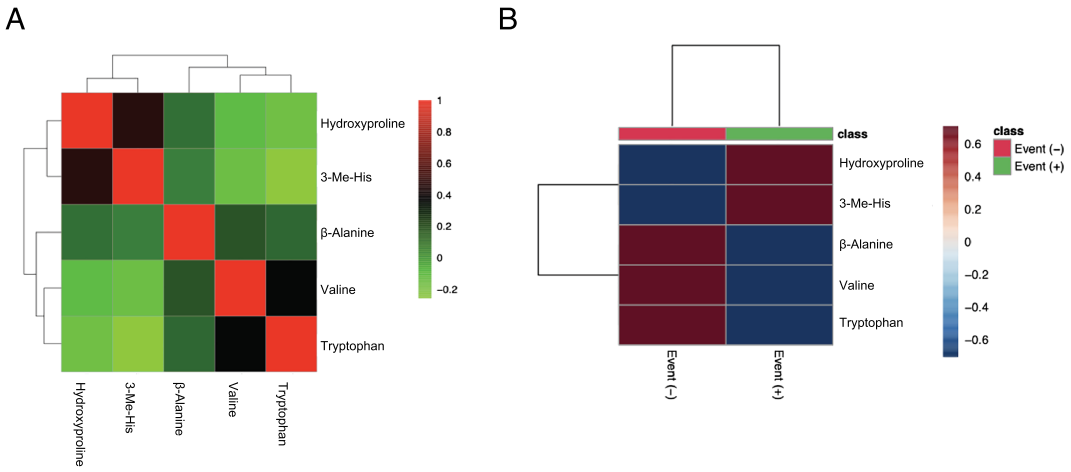


Figure 3 (A) A correlation heatmap of the top 5 adverse events-predictive amino acids identified by partial least squares–discriminant analysis (PLS-DA) in patients with heart failure. (B) A hierarchical clustering heatmap of the top 5 amino acids according to the presence or absence of adverse events. Dark beige denotes up-regulation, whereas dark blue denotes down-regulation.



predictors of adverse events in HF patients (*Table 2*). The top three amino acids, that is, 3-Me-His, β-alanine and valine, but not tryptophan and hydroxyproline, were selected as explanatory factors of adverse events in multivariate Cox-proportional hazard analyses that incorporated each variable with $P < 0.10$ in the univariate analyses in addition to age and sex (*Table 3*).

Table 2 Univariate Cox-proportional regression analyses identifying predictors for adverse outcomes

Characteristic	HR (95% CI)	*P* value
Age (years)	1.01 (0.99–1.04)	0.273
Male sex	0.93 (0.50–1.75)	0.834
BMI (kg/m^2)	0.92 (0.85–1.00)	0.056
NYHA III or IV	2.21 (1.18–4.13)	0.017
LVEF (%)	0.98 (0.96–1.00)	0.078
Hypertension	0.85 (0.46–1.60)	0.621
Diabetes mellitus	1.26 (0.67–2.37)	0.477
Dyslipidaemia	1.01 (0.54–1.88)	0.974
CKD	5.87 (2.30–14.99)	<0.001
Atrial fibrillation	1.37 (0.74–2.56)	0.324
Haemoglobin (g/dL)	0.79 (0.66–0.93)	0.004
Log NT-proBNP (pg/mL)	1.38 (1.10–1.71)	0.006
Albumin (g/dL)	0.47 (0.25–0.89)	0.020
ACE-I or ARB	0.56 (0.30–1.04)	0.066
β blocker	1.05 (0.53–2.11)	0.885
MRA	2.25 (1.17–4.31)	0.012
Loop diuretics	2.45 (1.23–4.92)	0.008
Device therapy	3.96 (2.09–7.51)	<0.001
Log 3-methylhistidine	2.98 (1.92–4.47)	<0.001
β-Alanine (nmol/mL)	0.85 (0.73–0.97)	0.020
Valine (nmol/mL)	0.992 (0987–0.998)	0.007
Hydroxyproline (nmol/mL)	1.06 (1.01–1.10)	0.010
Tryptophan (nmol/mL)	0.96 (0.93–0.99)	0.004

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; CI, confidence interval; CKD; chronic kidney disease; HR, hazard ratio; LVEF, left ventricular ejection fraction; MRA, mineralocorticoid receptor antagonists; NT-proBNP, N-terminal pro-brain natriuretic peptide; NYHA, New York Heart Association.

Receiver operating characteristics curve analyses were performed to calculate AUC and cut-off values of amino acid levels for prediction of adverse events (*Figure S2*); cut-off values of 3-Me-His, β-alanine, and valine were 9.3, 6.2, and 222.1 nmol/mL, respectively. When HF patients were divided into groups according to the cut-off values, Kaplan–Meier survival curves showed that event-free survival rate was lower in HF patients with high 3-Me-His than in HF patients with low 3-Me-His (68% vs. 91%, $P < 0.01$, *Figure S3A-C*), whereas the rates were lower in HF patients with low β-alanine and low valine than in HF patients with high β-alanine and high valine (β-alanine, 75% vs. 88%, $P = 0.01$; valine, 72% vs. 89%, $P < 0.01$, *Figure S3A-C*). In a subgroup with high 3-Me-His, HF patients with low β-alanine and those with low valine had significantly lower event-free survival rates than did HF patients with high β-alanine and those with high valine, respectively (*Figure 4A,B*). On the other hand, Kaplan–Meier curves of event-free survival rates did not differ between HF patients with and those without low β-alanine and low valine in subgroups of patients with low 3-Me-His (*Figure 4A,B*). In a *post hoc* analysis using a validation cohort (*Detailed Methods* in supporting information), associations of high 3-Me-His and low valine, but not low β-alanine, with lower event-free survival rate was observed (*Figure S3D–F*), although the difference did not reach statistical significance presumably due to the small sample size ($n = 53$).

The results showing that the top three amino acids in PLS-DA are independent predictors of adverse events in HF patients (*Figure 4*) led us to examine whether the addition of amino acids to known prognostic markers improves the accuracy of prediction of adverse events in HF patients by calculating C-index, cNRI, and IDI. Addition of 3-Me-His, β-alanine, and valine to the models (Model 1: age, sex, and NT-proBNP; Model 2: age, sex, NT-proBNP, and CKD; Model

Table 3 Multivariate Cox-proportional regression analyses identifying predictive amino acids for adverse outcomes

Variable	Log 3-methylhistidine		β-Alanine		Valine		Tryptophan		Hydroxyproline	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age + sex + NYHA III or IV	2.71 (1.68–4.35)	<0.001	0.85 (0.73–0.99)	0.032	0.992 (0.986–0.998)	0.008	0.96 (0.93–0.99)	0.017	1.05 (1.01–1.09)	0.016
Age + sex + CKD	2.22 (1.30–3.66)	0.004	0.82 (0.71–0.92)	0.001	0.993 (0.988–0.999)	0.026	0.97 (0.94–1.00)	0.057	1.04 (0.99–1.08)	0.135
Age + sex + haemoglobin	2.67 (1.65–4.24)	<0.001	0.86 (0.76–0.97)	0.018	0.993 (0.987–0.999)	0.043	0.97 (0.93–0.99)	0.034	1.04 (0.99–1.08)	0.061
Age + sex + log NT-proBNP	2.80 (1.64–4.57)	<0.001	0.85 (0.75–0.96)	0.009	0.993 (0.988–0.999)	0.042	0.96 (0.93–0.99)	0.027	1.05 (0.99–1.09)	0.057
Age + sex + albumin	2.84 (1.78–4.45)	<0.001	0.87 (0.77–0.98)	0.024	0.993 (0.987–0.999)	0.023	0.96 (0.93–0.99)	0.036	1.05 (1.00–1.09)	0.033
Age + sex + use of ACEI/ARB	3.23 (2.01–5.07)	<0.001	0.88 (0.79–0.99)	0.034	0.992 (0.986–0.998)	0.009	0.96 (0.93–0.99)	0.008	1.06 (1.02–1.10)	0.009
Age + sex + use of MRA	2.87 (1.76–4.56)	<0.001	0.89 (0.79–0.99)	0.040	0.992 (0.987–0.998)	0.013	0.96 (0.93–0.99)	0.011	1.06 (1.01–1.10)	0.014
Age + sex + use of loop diuretics	2.72 (1.65–4.38)	<0.001	0.86 (0.76–0.97)	0.013	0.993 (0.987–0.999)	0.019	0.96 (0.93–0.99)	0.008	1.06 (1.01–1.10)	0.018
Age + sex + device therapy	2.85 (1.76–4.53)	<0.001	0.89 (0.79–0.99)	0.047	0.991 (0.985–0.997)	0.006	0.95 (0.92–0.98)	0.001	1.05 (1.01–1.09)	0.014
Age + sex + BMI	2.89 (1.82–4.50)	<0.001	0.88 (0.78–0.99)	0.028	0.992 (0.987–0.998)	0.014	0.96 (0.93–0.99)	0.015	1.06 (1.01–1.09)	0.015

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; CI, confidence interval; CKD; chronic kidney disease; HR, hazard ratio; LVEF, left ventricular ejection fraction; MRA, mineralocorticoid receptor antagonists; NT-proBNP, N-terminal pro-brain natriuretic peptide; NYHA, New York Heart Association.

3: age, sex, NT-proBNP, CKD, haemoglobin, angiotensin-converting enzyme inhibitors/angiotensin receptor blockers, MRA, loop diuretics, and device therapy) tended to improve the C-index, and it significantly improved cNRI and IDI, although addition of 3-Me-His to Model 3 and addition of valine to Model 3 did not significantly improve IDI and cNRI, respectively (*Table 4*). On the other hand, inclusion of the combination of 3-Me-His with β-alanine or valine to Models 1 and 2 significantly improved the C-index in addition to cNRI and IDI (*Table 4*). These results suggest that an addition of amino acids to the baseline models improves the prediction of adverse events in HF patients.

Association of 3-Me-His with markers of cachexia

As shown in *Figure 5A–E*, log 3-Me-His was negatively correlated with ASMI in males ($r = -0.149$), MNA-SF scores ($r = -0.247$), and concentrations of albumin ($r = -0.166$) and haemoglobin ($r = -0.280$), although the correlation between log 3-Me-His and ASMI did not achieve statistical significance in females ($r = -0.168$, $P = 0.063$). When male and female patients were combined after subdivision into ASMI tertiles within sex to avoid sex differences in ASMI, there was a significant trend for lower log 3-Me-His in higher tertile of ASMI (*Figure 5F*).

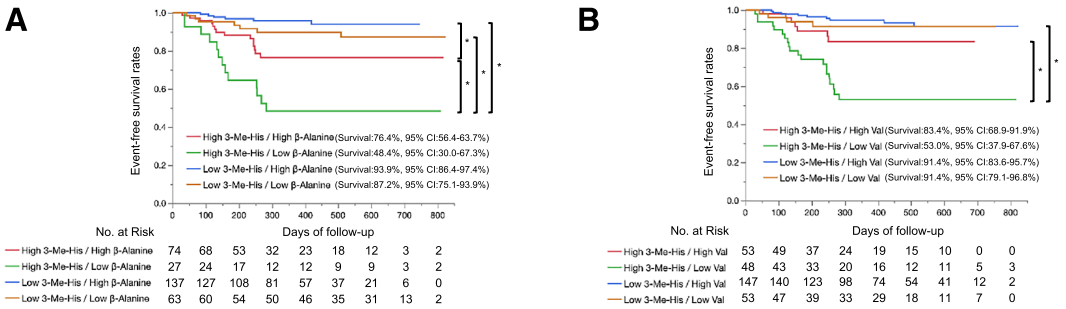
Discussion

The novel findings obtained from this study were as follows: (i) 3-Me-His, β-alanine, valine, tryptophan, and hydroxyproline were the top five metabolites that were associated with adverse events in HF patients (*Figures 2 and 3*); (ii) 3-Me-His, β-alanine, and valine were independent predictors of adverse events after adjustment for each prognostic marker including NT-proBNP and CKD (*Table 3*); and (iii) combined assessment of 3-Me-His with either β-alanine or valine improved the predictive ability for adverse events (*Figure 4* and *Table 4*).

Plasma amino acid profile as an easy-to-use predictor of clinical outcome in heart failure patients

Several earlier studies analysed the association of plasma metabolomic profiling with poor clinical outcomes.[12–15] Results of an unbiased metabolomics study by Cheng *et al*. showed that amino acids and phospholipids are important discriminators between non-HF controls ($n = 51$) and symptomatic HF patients ($n = 73$).[12] Their subsequent targeted metabolomic analyses of 140 HF patients (mean age of 64.1 years) indicated that combination of four metabolite components (i.e. the asymmetric dimethyarginine/arginine

Figure 4 Kaplan–Meier event-free survival curves in patients with heart failure. HF patients were divided into groups according to cut-off values calculated from receiver-operating curve analyses, that is, 9.3 nmol/mL of 3-Me-His group, 6.2 nmol/mL of β-alanine group, and 222.1 nmol/mL of valine group. (A, B) High 3-Me-His and high β-alanine or valine (red line), high 3-Me-His and low β-alanine or valine (green line), low 3-Me-His and high β-alanine or valine (blue line), vs. low 3-Me-His and low β-alanine or valine (orange line).



ratio, butyrylcarnitine, spermidine, and total essential amino acid amount) predicted the first defined events (death or HF-related rehospitalization) during a mean follow-up period of 1.3 years independently of BNP and traditional risk factors.[12] By using plasma metabolomic profiling, Lanfear *et al*. identified 13 plasma metabolites associated with survival in patients with HFrEF (mean age, 67.9 years; median follow-up period, 34 months).[13] The score that was developed using the 13 plasma metabolites including several amino acids predicted survival after adjustment for BNP and an established prognostic model of HF.[13] Thereafter, panels consisting of plasma metabolites that predict clinical outcomes were developed for patients with dilated cardiomyopathy and patients with advanced stages of HF.[14,15] However, those earlier studies in which relatively young HF patients and patients with HFrEF were enrolled may not be applicable for elderly HF patients because plasma metabolomic profiles have been shown to vary according to age, sex, HF aetiologies (ischemic vs. non-ischemic), LVEF categories of HF (HFrEF vs. HFpEF), and presence of comorbidities.[13,19] In addition, measurement of multiple kinds of metabolites including lipid metabolism is costly and not easy to use in a clinical setting. Therefore, plasma metabolomic profiling for prediction of clinical outcomes in an HF population including elderly subjects with multiple comorbidities has not been be established.

In the presented study, we focused on amino acids in plasma metabolomes because systematic and unbiased analyses of the relationships between plasma amino acid levels and adverse events were not performed in earlier studies[16–18] despite some reports of prognosis predictive values of amino acids such as essential branched-chain amino acids (BCAAs), phenylalanine, histidine, and ornithine. The results of the present study, which enrolled more elderly patients and non-HFrEF patients, demonstrated the top three amino acids closely associated with poor clinical outcomes, that is, 3-Me-His, β-alanine, and valine. Furthermore, measurement of 3-Me-His with either β-alanine or valine improved the predictability of adverse events (*Figure 4* and *Table 4*). These findings indicate that addition of simple measurement of specific amino acids to conventional biomarkers significantly improves assessment of prognosis in HF patients with comorbidities. The non-inferiority of the plasma amino acid profiling to plasma metabolome profiling by use of panels reported by earlier studies is an interesting issue that remains to be investigated.

3-Me-His as a predictor of increased adverse events in heart failure: association with tissue wasting

To our knowledge, this study is the first study showing a close association between 3-Me-His and adverse event rates in HF patients. 3-Me-His, a histidine derivative, is formed by post-translational methylation of histidine residues of the myofibrillar proteins including those of ingested meat and is a marker of myofibrillar breakdown.[24] HF promotes long-lasting systemic catabolism, eventually resulting in cardiac cachexia characterized by tissue wasting.[25] The presence of non-intentional weight loss, a hallmark of cachexia, is an independent predictor for mortality,[26] and presence of tissue wasting including sarcopenia has been shown to be independently associated with poor clinical outcomes in HF patients.[27–30] Importantly, spillover of 3-Me-His from leg skeletal muscle has been shown to be increased in patients with cardiac cachexia compared with that in healthy control subjects.[31] Amount of meat intake is also a determinant of plasma levels of 3-Me-His level,[24] but VIP score of 3-Me-His level was clearly different from the score of 1-Me-His that derives from ingested meat but not from the human body (*Figure 2*). Thus, the contribution of meat intake amount to a close association between high 3-Me-His concentration and increased adverse events is likely to be small, if any.

Both of 3-Me-His and 1-Me-His released into blood are not reused for protein synthesis and are excreted in urine

Table 4 Incremental predictive value of amino acids for adverse events in patients with heart failure

Variable	C-index (95% CI)	*P* value	cNRI (95% CI)	*P* value	IDI (95% CI)	*P* value
Model 1	0.6297 (0.5384–0.7126)	Ref	—	Ref	—	Ref
+3-Methylhistidine ≧ 9.3 nmol/mL	0.6955 (0.5987–0.7777)	0.080	0.6986 (0.3868–1.0104)	<0.001	0.0613 (0.0334–0.0891)	<0.001
+β-Alanine ≦ 6.2 nmol/mL	0.6713 (0.5720–0.7573)	0.291	0.4636 (0.1355–0.7916)	0.006	0.0374 (0.0122–0.0625)	0.004
+Valine ≦ 222.1 nmol/mL	0.6942 (0.6018–0.7732)	0.061	0.6099 (0.2867–0.9331)	<0.001	0.0370 (0.0141–0.0599)	0.002
+3-Methylhistidine ≧ 9.3 nmol/mL + β-alanine ≦ 6.2 nmol/mL	0.7487 (0.6549–0.8238)	0.007	0.7603 (0.4605–1.0601)	<0.001	0.1161 (0.0650–0.1582)	<0.001
+3-Methylhistidine ≧ 9.3 nmol/mL + valine ≦ 222.1 nmol/mL	0.7420 (0.6402–0.8229)	0.035	0.6680 (0.3557–0.9802)	<0.001	0.1044 (0.0612–0.1476)	<0.001
Model 2	0.7054 (0.6203–0.7782)	Ref	—	Ref	—	Ref
+3-Methylhistidine ≧ 9.3 nmol/mL	0.7445 (0.6532–0.8185)	0.163	0.6183 (0.3106–0.9261)	<0.001	0.0377 (0.0145–0.0609)	0.002
+β-Alanine ≦ 6.2 nmol/mL	0.7518 (0.6700–0.8188)	0.040	0.4636 (0.1355–0.7916)	0.006	0.0452 (0.0117–0.0788)	0.009
+Valine ≦ 222.1 nmol/mL	0.7462 (0.6587–0.8174)	0.047	0.6023 (0.2789–0.9256)	<0.001	0.0335 (0.0103–0.0566)	0.006
+3-Methylhistidine ≧ 9.3 nmol/mL + β-alanine ≦ 6.2 nmol/mL	0.7855 (0.7028–0.8501)	0.010	0.6950 (0.3882–1.0017)	<0.001	0.0923 (0.0447–0.1399)	<0.001
+3-Methylhistidine ≧ 9.3 nmol/mL + valine ≦ 222.1 nmol/mL	0.7724 (0.6772–0.8459)	0.035	0.5986 (0.2777–0.9195)	<0.001	0.0782 (0.0391–0.1173)	<0.001
Model 3	0.8267 (0.7450–0.8863)	Ref	—	Ref	—	Ref
+3-Methylhistidine ≧ 9.3 nmol/mL	0.8382 (0.7585–0.8953)	0.278	0.4038 (0.0949–0.7126)	0.017	0.0162 (−0.0047–0.0371)	0.124
+β-Alanine ≦ 6.2 nmol/mL	0.8414 (0.7577–0.9000)	0.209	0.5059 (0.1781–0.8337)	0.003	0.0360 (0.0046–0.0673)	0.025
+Valine ≦ 222.1 nmol/mL	0.8333 (0.7498–0.8930)	0.509	0.3260 (−0.0042–0.6562)	0.055	0.0268 (0.0038–0.0497)	0.023
+3-Methylhistidine ≧ 9.3 nmol/mL + β-alanine ≦ 6.2 nmol/mL	0.8478 (0.7681–0.9035)	0.131	0.6067 (0.2936–0.9197)	<0.001	0.0527 (0.0137–0.0918)	0.009
+3-Methylhistidine ≧ 9.3 nmol/mL + valine ≦ 222.1 nmol/mL	0.8448 (0.7634–0.9019)	0.195	0.3570 (0.0306–0.6835)	0.036	0.0445 (0.0114–0.0776)	0.010

BMI, body mass index; CI, confidence interval; CKD; chronic kidney disease; cNRI, continuous net reclassification improvement; IDI, integrated discrimination improvement; LVEF, left ventricular ejection fraction; MRA, mineralocorticoid receptor antagonists; NT-proBNP, N-terminal pro-brain natriuretic peptide; NYHA, New York Heart Association; Ref, reference. Model 1: age, sex, and NT-proBNP. Model 2: age, sex, NT-proBNP, and CKD. Model 3: age, sex, NT-proBNP, BMI, NYHA 3 or 4, LVEF, CKD, haemoglobin, albumin, RASI, MRA, loop diuretics, and device.

without reabsorption,[24] the behaviour of which is similar to that of serum creatinine and cystatin C. Importantly, however, an independent association between 3-Me-His concentration and adverse event rates remained after adjustment for CKD (*Table 4*). Taken together, the results of the present study suggest that plasma 3-Me-His level is a promising marker of sarcopenia and/or cachexia and also a CKD-independent predictor of adverse event in HF patients. The time-dependent changes of the relationship between plasma 3-Me-His level and body composition may warrant investigation for roles of plasma 3-Me-His as a biomarker.

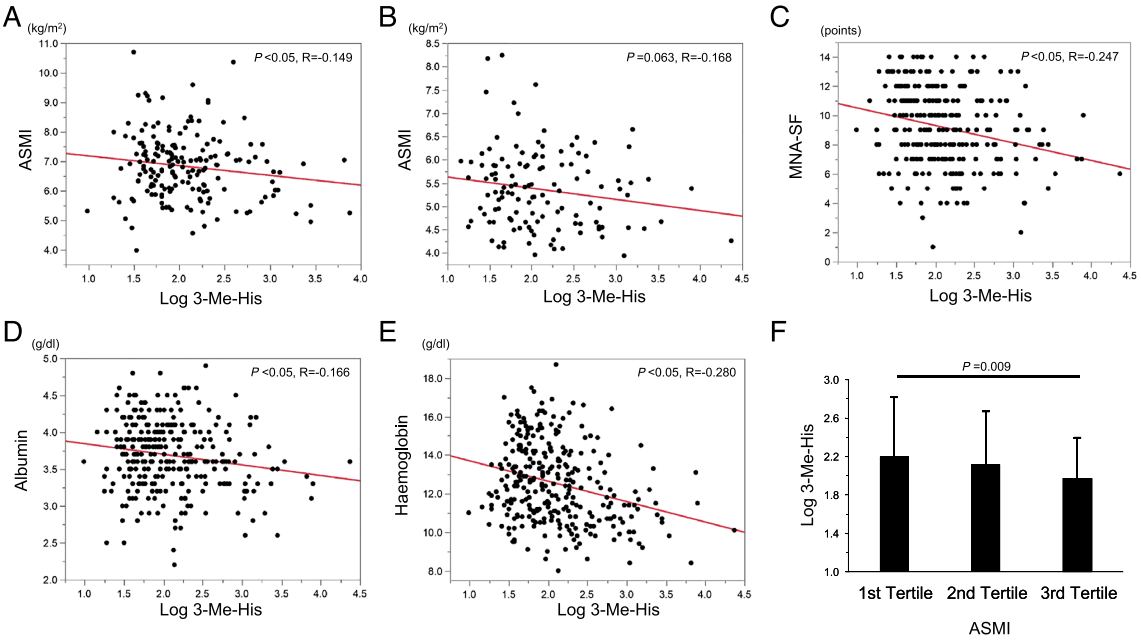
Low β-alanine as a predictor of adverse events in heart failure

Regulation of plasma β-alanine concentration is highly complex,[32,33] and we do not have clear explanation for the mechanism of reduction in plasma β-alanine level, associating with adverse events, in HF patients. However, a possible speculation is loss of ergogenic effect of tissue carnosine due to low level of β-alanine. Circulating β-alanine is a rate-limiting precursor for synthesis of carnosine in skeletal and heart muscles.[32] In experimental studies, exogenous carnosine afforded protection against ischemia/reperfusion injury and doxorubicin-induced cardiomyopathy through multiple mechanisms including pH-buffering, antioxidant properties, and handling modulation.[32] Ergogenic effect of β-alanine supplementation has been reported in the field of sports medicine.[34] Thus, there is the possibility that a low plasma β-alanine concentration hampers skeletal and cardiac muscle functions in HF patients.

Low valine and increased adverse events in heart failure: an epiphenomenal marker of malnutrition or a potential therapeutic target

Low plasma concentration of valine, a BCAA, was found to be an independent predictor for adverse events in HF patients (*Table 3*). Inadequate protein/calorie intake, intestinal protein loss due to malabsorption, altered gut microbiota and metabolite rewiring for vital organs have been suggested for the mechanisms of low plasma concentrations of essential amino acids in HF patients.[35] Recent experimental studies have suggested that BCAA regulate muscle fatty acid oxidation in both cell autonomous and paracrine manners.[36,37] It was shown in mice that BCAA catabolism in skeletal muscle promotes fatty acid oxidation via an increase in mitochondrial oxidative capacity, resulting in augmented ergogenic function.[36] A study by Jang *et al*. showed that 3-hydroxyisobutyrate, an intermediate metabolite of valine catabolism, is secreted from myocytes and promotes trans-endothelial fatty acid transport.[37]

Figure 5 Association of 3-methylhistidine (3-Me-His) with markers of cachexia. Simple regression analyses of log 3-Me-His with appendicular skeletal muscle mass index (ASMI) in men (A), ASMI in women (B), the Mini Nutritional assessment-Short Form (C), albumin (D), and haemoglobin (E). (F) Because there are obvious differences in ASMI between men and women, heart failure patients were subdivided into tertiles within sex as follows: first tertile, <6.30 kg/m^2 in men and <4.87 kg/m^2 in women; second tertile, 6.30 to <7.18 kg/m^2 in men and 4.87 to <5.70 kg/m^2 in women; and third tertile, ≥7.18 kg/m^2 in men and ≥5.70 kg/m^2 in women.



Further studies are needed to elucidate whether a low valine concentration is an epiphenomenal marker of malnutrition or a potential therapeutic target to modulate muscle energy metabolism in HF patients.

Limitations

There are limitations in the present study. First, there might have been selection bias in the study subjects because subjects were retrospectively enrolled in a single centre. Second, the present study might have insufficient statistical power. The number of patients in the validation cohort was much smaller than that in the variation cohort (*N* = 53 vs. 301). Results of the validation cohort (*Figure S3*) were consistent with those of the derivation cohort (*Figure 4*), though difference in the validation cohort did not reach statistical significance. The number of patients appears to be insufficient also for detection of differences among the groups with different aetiologies and LVEF categories of HF (HFrEF, HF with mid-range ejection fraction, and HFpEF). Third, comparative analyses of plasma amino acid profiles in HF patients and age-matched non-HF controls were not performed in the present study, although change in plasma β-alanine concentration by HF has been reported.[38,39] Fourth, changes in treatments such as medication, device, and ambulatory cardiac rehabilitation were not incorporated into the mortality analysis. Fifth, time-dependent changes in body composition such as muscle mass were not analysed in the present study, although the top 5 amino acids predicted adverse events even after adjustment with BMI. Finally, an earlier study showed race-dependent variation in plasma metabolic profiles.[13] The findings in the present study may not be extrapolated to other races with HF.

Conclusions

Simple measurement of 3-Me-His with either β-alanine or valine improved the predictive ability for adverse events, indicating the utility of plasma amino acid profiling in risk stratification of hospitalized HF patients. Association of co-existence of high 3-Me-His and low β-alanine or low valine with adverse events in HF patients suggest possible roles of tissue wasting and malnutrition in the poor prognosis, which requires further investigation.

Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Multivariate analyses to identify amino acids associated with adverse events in patients with heart failure. Imputation of the missing values was performed using several methods implemented in MetaboAnalyst 4.0 to confirm reproducibility of the data: (1) replacement by k-nearest neighbours based on similar features (KNN feature-wise); (2) k-nearest neighbours based on similar samples (KNN sample-wise); (3) probabilistic principal component analysis (PPCA); (4) Bayesian principal component analysis (BPCA); (5) singular value decomposition (SVD). X axis indicates the variable importance in projection (VIP) scores corresponding to each variable on the Y-axis. Amino acids that indicate a VIP score of $\geq$0.7 are shown.

Figure S2. Receiver operating characteristic curves to identify optimal cutoff values for prediction of adverse events. Abbreviations: 3-Me-His, 3-methylhistidine; AUC, area under the curve.

Figure S3. (a-c) Kaplan-Meier event-free survival curves in patients with heart failure. HF patients were divided into groups according to cutoff values calculated from receiver-operating curve analyses, i.e., 9.3 nmol/ml of 3-Me-His group, 6.2 nmol/ml of β-Alanine group, and 222.1 nmol/ml of Valine group. (**a**) Low 3-Methylhistidine (3-Me-His, red line) vs. High 3-Me-His (blue line). (**b**) High β-alanine (red line) vs. Low β-alanine (blue line). (**c**) High Valine (red line) vs. Low Valine (blue line). (**d-f**) Kaplan-Meier event-free survival curves in a validation cohort. (**d**) Low 3-Methylhistidine (3-Me-His, red line) vs. High 3-Me-His (blue line). (**e**) High β-alanine (red line) vs. Low β-alanine (blue line). (**f**) High Valine (red line) vs. Low Valine (blue line).

Table S1. Levels of limit of detection and intra- and interday precisions of amino acid measurements.

Table S2. Plasama concentration of amino acids.

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