Clinical Chemistry 63:4
880–886 (2017)

Prognostic Role of Molecular Forms of B-Type Natriuretic Peptide in Acute Heart Failure

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BACKGROUND: B-type natriuretic peptide (BNP) molecular forms 5-32, 4-32, and 3-32 are known to be present in the circulation of heart failure (HF) patients. This study investigated the prognostic role of circulating BNP molecular forms on risk prediction for patients with acute HF.

METHODS: BNP molecular forms were measured in plasma using an immunocapture MALDI-TOF–mass spectrometry (MS) method. Associations of molecular BNP forms with adverse outcome of all-cause mortality (death) and a composite of all-cause mortality and rehospitalization due to HF (death/HF) at 6 months and 1 year were investigated.

RESULTS: BNP molecular forms 5-32, 4-32, and 3-32 were detected in 838 out of 904 patient samples. BNP molecular forms were all able to independently predict death and death/HF at 6 months and 1 year. BNP 5-32 was the superior form with strongest predictive qualities for death at 6 months [adjusted hazard ratio (HR) 1.31, $P = 0.005$] and 1 year (adjusted HR 1.29, $P = 0.002$) and death/HF at 1 year (adjusted HR 1.18, $P = 0.011$). BNP 5-32, 4-32, and 3-32 showed decreased survival rates across increasing tertiles of circulating concentrations ($P \leq 0.004$). BNP molecular forms showed prognostic ability comparable with conventional BNP measurements across all end points ($P = 0.002–0.032$ vs $P = 0.014–0.039$, respectively) and reduced associations with renal dysfunction (blood urea; Spearman correlation $r_s = 0.187–0.246$ vs $r_s = 0.369$, respectively).

CONCLUSIONS: BNP molecular forms, notably BNP 5-32, showed association with poor prognosis at 6 months and 1 year in patients with acute HF. This is the first study reporting the prognostic ability of molecular BNP forms in HF patients and demonstrated comparable qualities to conventional BNP measurements.

Heart failure (HF)[3] is a major worldwide epidemic associated with high morbidity, mortality, and healthcare costs, with 1-year mortality rates as high as 45% for admitted patients *(1, 2)*. The pathophysiology of HF involves a multitude of factors and pathways (e.g., cardiac stress/injury, neurohormonal activation, etc.) *(3)*.

B-type natriuretic peptide (BNP) is released from cardiac tissue in response to neurohormonal stresses [i.e., hemodynamic stress and stretch regulated by left ventricle wall tension *(4)*], with circulating concentrations seen to be increased in line with severity of HF *(5)*, and is clinically used in diagnosis of the condition *(6)*. BNP functions are known to be central to cardiovascular homeostasis and include natriuresis, diuresis, and vasodilation *(7)*. Circulating BNP is present as 2 proteolytic forms, a physiologically active 32–amino acid peptide (known as BNP), and a physiologically inert 76–amino acid peptide from the N-terminal of the prohormone [NT-proBNP (N-terminal pro–B-type natriuretic peptide)]. NT-proBNP and BNP are synthesized in an equimolar ratio; however, NT-proBNP is considered as more stable with a longer half-life when present in the circulation *(8)*. Both of these markers are measured clinically for diagnosis of HF *(9)*.

BNP is further proteolyzed into molecular forms *(10, 11)*. Loss of end-chain amino acids truncates the peptide commonly by 2 (BNP 3-32), 3 (BNP 4-32), or 4 (BNP 5-32) residues. A multitude of BNP forms are known to be present in the circulation of patients with HF (e.g., BNP 3-29, 3-30, 4-29, 5-29, etc.) *(12)*. Among these, BNP 3-32, 4-32, and 5-32 have been shown in recent studies to be the major forms and BNP 5-32 was

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Received August 9, 2016; accepted December 1, 2016.
Previously published online at DOI: 10.1373/clinchem.2016.265140

[3] Nonstandard abbreviations: HF, heart failure; BNP, B-type natriuretic peptide; NT-proBNP, N-terminal pro–B-type natriuretic peptide; eGFR, estimated glomerular filtration rate; ACTH, adrenocorticotropic hormone; HR, hazard ratio; ADHERE, Acute Decompensated Heart Failure National Registry; OPTIMIZE-HF, Organized Program to Initiate Lifesaving Treatment in Hospitalized Patients With Heart Failure; GWTG-HF, Get With The Guidelines-HF; NYHA, New York Heart Association; ROC, receiver operating characteristics; AUC, area under the curve; OR, odds ratio.

recently reported to have the strongest association with BNP concentrations in chronic HF patients as measured using conventional commercial assays *(13)*. These data suggest that the combination of molecular forms in circulation are representative of BNP measurements by immunoassay, and not BNP 1-32 in isolation *(12, 13)*. Molecular forms of BNP have also been implicated in ischemic heart disease, with BNP 5-32 concentrations showing association with restenosis *(14)*.

Currently, there are no data available that indicate the association of BNP molecular forms and outcome in hospitalized patients with HF. Therefore, the aims of this study were to investigate the association of BNP molecular forms in patients admitted with acute HF and to assess the application of these measurements for use in prognostic risk prediction. To our knowledge, this is the first investigation to assess the prognostic ability of molecular BNP forms in HF patients and to directly compare the prognostic use of molecular BNP measurement with an established measurement procedure using circulating BNP through analysis of NT-proBNP.

Materials and Methods

STUDY POPULATION

Patients with acute HF admitted to the University Hospitals of Leicester, UK, between February 2006 and August 2011 were enrolled. Each patient consented to have blood samples taken and outcomes surveyed. This study was approved by the local ethics committee and adhered to the Declaration of Helsinki. Diagnosis of acute HF was made on the clinical signs and symptoms including pulmonary edema, peripheral edema or increased jugular venous pressure, and progressively worsening or new onset of shortness of breath *(15)*. Patients excluded from the study included those with previous history of cancer or renal replacement therapy; any surgical procedure within the previous month; presence of cardiogenic shock, sepsis, pneumonia, or acute coronary syndromes; and inability to consent (e.g., dementia).

For prognostic investigations, the primary end points were all-cause mortality (death) and a composite of death and rehospitalization due to HF (death/HF) at 6 months and 1 year. All surviving patients were followed up for a minimum of 1 year, with all outcome data obtained from hospital records. In cases where multiple events occurred, the time to the first event was counted as the outcome. Outcome data surpassing 1 year of follow-up are not included in this report. Inclusion and end-point evaluations were determined by an independent cardiologist. Estimated glomerular filtration (eGFR) was obtained by using the simplified modified diet in renal disease formula *(16)*.

SAMPLE COLLECTION

Peripheral intravenous blood samples were obtained within 24 h postadmission to hospital with acute HF. Blood was collected in prechilled tubes containing EDTA and aprotinin, and centrifuged at 1500*g* for 20 min at 4 °C. Plasma was aliquoted and stored at −80 °C until analysis. For analysis, samples were thawed at 37 °C, prepared, and analyzed immediately.

MEASUREMENT OF BNP MOLECULAR FORMS

Molecular forms of BNP were measured using a modified protocol adapted from an immunocapture assay described previously *(14)*. Briefly, BNP molecular forms were captured from 100 μL plasma using a monoclonal antibody raised against the ring region of BNP (antibodies were received as a gift from Sekisui Medical Co., Tokyo, Japan) bound to magnetic beads (Fisher Scientific). Captured BNP forms were then eluted in 3 μL 0.1% trifluoroacetic acid (TFA) and 0.75 μL spotted onto a metal target plate followed by 5 fmol/μL adrenocorticotropic hormone (ACTH) signal peptide and 10 g/L matrix [2,5-dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (α-CHCA), LaserBio Labs] in a 1:1:1 ratio. Samples were detected using an AXIMA Confidence MALDI-TOF-MS (Shimadzu Corporation) in positive ion mode. Identifications of molecular BNP forms were confirmed by comparison of mass-to-charge ratio of detected ions to those detected using synthetically produced BNP 5-32, 4-32, and 3-32 peptides. An example mass spectrum for the detection of molecular BNP forms in acute HF patient plasma can be seen in Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol63/issue4. The antibodies used in the present study allowed selective immunocapture of BNP molecular forms 5-32, 4-32, and 3-32. BNP 1-32, proBNP, and other alternative BNP molecular forms were not detected under our experimental conditions. The relative SD across the course of the study for synthetic BNP 5-32, BNP 4-32, and BNP 3-32 was 6.3%, 8.0%, and 10.3%, respectively. This is shown in online Supplemental Fig. 2. Modifications to the previously described protocol included the use of 5 fmol/μL ACTH signal peptide as an internal reference standard and Sekisui Medical Co. as an alternative supplier for the anti-BNP antibodies. NT-proBNP was measured in all patients using a sandwich immunoassay as described previously *(17)*.

STATISTICAL ANALYSES

Statistical analyses were performed using IBM SPSS Statistics (V22, IBM Corp.). Spearman correlations were calculated for molecular BNP forms against NT-proBNP values and other clinical variables. Cox proportional hazard regression analyses were performed to identify inde-

pendent predictors of death and death/HF at both 6 months and 1 year. Molecular BNP forms and NT-proBNP values were log transformed and normalized to 1 standard deviation, and therefore, the hazard ratios (HRs) refer to the Z-transformed values. Binary logistic regression was used to test the predictive value of log molecular BNP forms and log NT-proBNP values for in-hospital mortality and further adjusted for 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-HF (GWTG-HF) clinical risk scores *(18–20)*. The ADHERE score was assessed as both a categorical value against the first reference grouping and as a continuous variable from the formula for log odds mortality *(18)*. ADHERE groupings were allocated according to the ADHERE registry model ranging from 1 (lowest risk) to 5 (highest risk) *(18)*. Kaplan–Meier survival curves were generated to show the relationship between BNP forms, NT-proBNP and the outcome of death and death/HF at 6 months and 1 year. Mantel–Cox log-rank tests were used to compare event-free survival after stratification of biomarkers by tertiles. A *P*- value of <0.05 was considered to be statistically significant.

Results

PATIENT CHARACTERISTICS

Plasma samples from 904 patients admitted to the hospital with acute HF were analyzed for the presence of molecular BNP forms. Mass spectral peaks for BNP forms were detectable in a total of 838 samples, with the remaining 66 patient samples excluded from further analyses which is reflected in the statistical tests described herein. A total of 332 events were recorded during the 1-year follow-up period, with 60 deaths during in-hospital care, 167 deaths postdischarge and 105 readmissions to hospital due to HF. A breakdown of the measured end points, along with the clinical demographics for the patient cohort can be found in Table 1.

BNP MOLECULAR FORMS AND ASSOCIATED CLINICAL MEASUREMENTS

Univariate analyses showed clinical variables that correlated to one or more of the BNP forms to be blood urea, eGFR, age, systolic blood pressure, respiratory rate, and sodium as detailed in Table 2. All BNP forms were observed to be correlated to NT-proBNP (r_s = 0.438–0.557), and strongly correlated between themselves (r_s = 0.809–0.863). Correlations with markers of renal dysfunction (urea and eGFR) showed that BNP 5-32, 4-32, and 3-32 possessed a moderately reduced association than when compared against the relationship seen for NT-proBNP; for urea, r_s = 0.187–0.246, and 0.369,

Table 1. Patient demographics for acute HF patients at the time of admission to hospital.[a,b]

Age, years	78 (70–84)
Male	61%
Systolic BP, mmHg[c]	133 (115–150)
Diastolic BP, mmHg	75 (65–85)
Heart rate, beats/min	88 (73–105)
De novo HF	34%
Past history of IHD	27%
Past history of diabetes	34%
Past history of HTN	58%
Past history of COPD	10%
Past history of hyperlipidemia	25%
Current smoker	9%
Orthopnea	54%
Edema	64%
Raised JVP	56%
Pulmonary edema	32%
NYHA Class IV	55%
Atrial fibrillation	47%
Respiratory rate, breaths/min	22 (18–25)
Urea, mg/dL	25 (18–36)
mmol/L	8.9 (6.6–12.8)
Creatinine, mg/dL	1.27 (1.03–1.59)
μmol/L	112 (91–141)
eGFR, mL · min^{-1} · (1.73 m^2)$^{-1}$	52 (39–68)
Na^+, mmol/L	138 (135–141)
K^+, mmol/L	4.4 (4.0–4.7)
Hemoglobin, g/L	123 (107–137)
NT-proBNP, pmol/L	2,285 (1140–4036)
ADHERE group 3-5	16.7%
OPTIMIZE-HF score	35 (30–40)
GWTG-HF score	43 (38–48)
BNP 5-32	0.9 (0.4–1.6)
BNP 4-32	0.5 (0.2–0.7)
BNP 3-32	0.5 (0.3–0.8)
End points	
In-hospital death	60
Death at 1 year	227
Death/HF at 1 year	332
Death at 6 months	168
Death/HF at 6 months	267

[a] Data are reported as median (interquartile range) for continuous variables and as % for categorical.
[b] Molecular BNP forms are reported as a ratio of mass spectral peak signal intensity against an internal reference standard.
[c] BP, blood pressure; IHD, ischemic heart disease; HTN, hypertension; COPD, chronic obstructive pulmonary disease; JVP, jugular venous pressure.

Table 2. BNP molecular forms and associated clinical factors.

	BNP 5-32		BNP 4-32		BNP 3-32		NT-proBNP	
	r_s	*P* value	r_s	*P* value	r_s	*P* value	r_s	*P* value
Urea	0.246	<0.001	0.213	<0.001	0.187	<0.001	0.369	<0.001
eGFR	−0.211	<0.001	−0.183	<0.001	−0.158	<0.001	−0.284	<0.001
Age	0.132	<0.001	0.106	0.002	0.047	0.171	0.133	<0.001
Systolic BP[a]	−0.100	0.005	−0.089	0.013	−0.080	0.026	−0.164	<0.001
Respiratory rate	0.075	0.046	0.056	0.138	0.043	0.251	−0.045	0.232
Blood sodium	−0.076	0.034	−0.053	0.139	−0.016	0.664	−0.047	0.190
BNP 5-32			0.863	<0.001	0.809	<0.001	0.557	<0.001
BNP 4-32					0.813	<0.001	0.463	<0.001
BNP 3-32							0.438	<0.001

[a] BP, blood pressure; r_s, Spearman rho.

and for eGFR, $r_s = -0.158$ to -0.211 and -0.284 for the molecular BNP forms and NT-proBNP, respectively.

A linear regression model was used to investigate the independent predictors of log molecular BNP forms and log NT-proBNP. Independent predictors for each of the BNP molecular forms can be found in Table 3. The results showed that eGFR was independently predictive of all BNP forms ($P \leq 0.011$), and BNP 5-2 and NT-proBNP were independently predicted by blood urea concentrations ($P \leq 0.019$), thus illustrating that renal dysfunction is indicated in the presence of both molecular BNP forms and NT-proBNP. Interestingly, only BNP 5-32 and NT-proBNP were influenced by age (≤0.002) and BNP 4-32 and 3-32 the only biomarkers influenced by current severity of HF [as measured by New York Heart Association (NYHA) class, $P \leq 0.006$].

BNP MOLECULAR FORMS AS PREDICTORS OF DEATH

To investigate prognostic ability of BNP 5-32, BNP 4-32, and BNP 3-32 for death at 6 months and at 1 year and to compare these qualities to NT-proBNP, Cox survival analyses were conducted using a multivariable base model of cardiovascular disease risk factors. Models were adjusted for age, sex, past histories of cardiac risk markers (HF, hypertension, ischemia, renal failure, and diabetes), NYHA class, systolic blood pressure, respiratory rate, blood urea, eGFR and blood sodium. Independent abilities for the molecular BNP forms and NT-proBNP to predict outcome were tested by individually adding each biomarker to the base model. NT-proBNP was a univariate predictor of death at 6 months ($P \leq 0.001$) and 1 year ($P \leq 0.001$), and retained independent prediction for both end points (both $P = 0.014$) when adjusted for confounding variables. Similarly, BNP 5-32, 4-32, and 3-32 were all univariate predictors of death at 6 months ($P \leq 0.001$) and at 1 year ($P \leq 0.001$). When molecular BNP forms were added to the base model, all showed comparable predictive abilities to NT-proBNP and were able to independently predict death at 6 months ($P \leq 0.032$) and at 1 year ($P \leq 0.018$). Table 4 details the

Table 3. Linear regression model for independent predictors of BNP molecular forms and NT-proBNP.

BNP 5-32			BNP 4-32			BNP 3-32			NT-proBNP		
Variable	Std β	*P* value	Variable	Std β	*P* value	Variable	Std β	*P* value	Variable	Std β	*P* value
Age	0.128	0.002	eGFR	−0.181	0.001	NYHA Class	0.125	0.001	Urea	0.163	<0.001
PH of DM[a]	−0.110	0.004	PH DM	−0.114	0.003	eGFR	−0.153	0.005	Raised JVP	0.120	<0.001
DBP	0.125	0.008	NYHA Class	0.105	0.006	PH DM	−0.098	0.012	PH IHD	−0.134	<0.001
SBP	−0.120	0.011							Age	0.122	0.001
eGFR	−0.138	0.011							PH HF	0.096	0.007
Urea	0.123	0.019							PH DM	−0.070	0.045

[a] Std β, standardized β; PH, past history; DM, diabetes mellitus; JVP, jugular venous pressure; DBP, diastolic blood pressure; IHD, ischemic heart disease; SBP, systolic blood pressure.

Table 4. Independent prediction abilities of BNP molecular forms and NT-proBNP using multivariate Cox survival analyses for outcomes of all-cause mortality (death) and death or rehospitalization due to HF (death/HF) at 6 months and 1 year.[a]

Death at 6 months				Death at 1 year			
Biomarker	HR	95% CI	*P* value	Biomarker	HR	95% CI	*P* value
BNP 5-32	1.31	1.08–1.57	0.005	BNP 5-32	1.29	1.10–1.51	0.002
BNP 4-32	1.22	1.02–1.48	0.032	BNP 4-32	1.21	1.04–1.43	0.018
BNP 3-32	1.26	1.05–1.52	0.014	BNP 3-32	1.25	1.06–1.46	0.006
NT-proBNP	1.56	1.09–2.22	0.014	NT-proBNP	1.45	1.07–1.95	0.014
Death/HF at 6 months				**Death/HF at 1 year**			
Biomarker	HR	95% CI	*P* value	Biomarker	HR	95% CI	value
BNP 5-32	1.18	1.02–1.36	0.022	BNP 5-32	1.18	1.04–1.34	0.011
BNP 4-32	1.17	1.02–1.35	0.031	BNP 4-32	1.17	1.02–1.35	0.031
BNP 3-32	1.21	1.05–1.36	0.008	BNP 3-32	1.16	1.02–1.32	0.020
NT-proBNP	1.32	1.02–1.72	0.035	NT-proBNP	1.27	1.01–1.60	0.039

[a] Models adjusted for age, sex, past history (PH) of HF, PH of hypertension, PH of ischemic heart disease, PH of renal failure, PH of diabetes, NYHA class, heart rate, systolic blood pressure, respiratory rate, blood urea, eGFR, and blood sodium.

model statistics for all BNP measurements as predictors of death at 6 months and at 1 year, with additional independent predictors outlined in online Supplemental Tables 1 and 2. BNP 5-32 was the most superior of the forms overall with a strong independent predictive ability at both 6 months [HR (95% CI) 1.31 (1.08–1.57), $P = 0.005$] and 1 year [HR (95% CI) 1.29 (1.10–1.51), $P = 0.002$].

Kaplan–Meier survival analyses were performed to visualize the relationship of BNP molecular forms and death at 6 months and 1 year after stratification by tertiles. Mantel–Cox log-rank tests reported decreases in survival across tertiles for all forms at 6 months ($P \leq 0.004$) and 1 year (all $P < 0.001$). BNP 5-32 and NT-proBNP showed similar survival characteristics with increased event occurrence across higher concentrations, with differences observed beginning from point of admission. Alternatively, BNP 4-32 and 3-32 showed decreased survival rates across tertiles, but differences were only observed between the upper 2 tertiles after approximately 3 months postadmission (see online Supplemental Figs. 3 and 4). ROC curves were comparable between molecular BNP forms and NT-proBNP for death at 6 months [area under the curve (AUC) 0.592–0.643] and 1 year (AUC 0.595–0.641) and are detailed in online Supplemental Table 3.

BNP MOLECULAR FORMS AS PREDICTORS OF DEATH/HF

To investigate independent predictors of death/HF at 6 months and 1 year, Cox survival analyses were conducted employing the same base model as described for the analyses on the outcome of death. NT-proBNP was a univariate predictor of death/HF at 6 months ($P \leq 0.001$) and 1 year ($P \leq 0.001$), and retained independent prediction for both end points (6 months, $P = 0.035$; 1 year, $P = 0.039$) after adjustment. BNP 5-32, 4-32, and 3-32 were again univariate predictors of death/HF at 6 months ($P \leq 0.001$) and at 1 year ($P \leq 0.001$) and retained independent prediction death/HF at 6 months ($P \leq 0.035$) and at 1 year ($P \leq 0.039$) in a comparable manner to NT-proBNP. Table 4 details the model statistics for BNP measurements as predictors of death/HF at 6 months and 1 year, with additional independent predictors outlined in online Supplemental Tables 4 and 5. Kaplan–Meier survival analyses were performed to visualize the relationship of BNP molecular forms and death/HF at 6 months and 1 year after stratification by tertiles. Mantel–Cox log-rank tests reported decreases in survival across tertiles for all forms at 6 months ($P \leq 0.003$) and 1 year ($P \leq 0.004$). Similarly to survival curves for the end point of death, BNP 5-32 and NT-proBNP reported similar event incidence for death/HF with an increase across tertiles. However, BNP 4-32 and 3-32 reported increased but similar event occurrence for the upper 2 tertiles in comparison to the lowest tertile (see online Supplemental Figs. 5 and 6). ROC curves were comparable between molecular BNP forms and NT-proBNP for death/HF at 6 months (AUC 0.577–0.600) and 1 year (AUC 0.570–0.600) and are detailed in online Supplemental Table 3.

BNP MOLECULAR FORMS AS PREDICTORS OF IN-HOSPITAL MORTALITY

To investigate the usefulness of BNP 5-32, BNP 4-32, and BNP 3-32 as additive biomarkers for risk assessment of in-hospital mortality, analyses were performed alongside established clinical risk scores as either categorical (ADHERE) or continuous variables (ADHERE, OPTIMIZE-HF, and GWTG-HF). BNP 5-32 was a univariate predictor of in-hospital mortality [odds ratio (OR) (95% CI) 1.45 (1.10–1.91), $P = 0.009$] as was NT-proBNP [OR 2.82 (1.64–4.86), $P < 0.001$]. BNP 4-32 and 3-32, however, were not univariate predictors [BNP 4-32, OR 1.32 (0.99–1.75), $P = 0.059$; BNP 3-32, OR 1.26 (0.96–1.67, $P = 0.102$)]. After adjustment for clinical risk scores, BNP 5-32 was not able to independently predict in-hospital mortality ($P \geq 0.279$) nor was NT-proBNP when combined with ADHERE (categorical) [OR 1.74 (0.98–3.07), $P = 0.057$] or OPTIMIZE-HF scores [OR 1.67 (0.95–2.94), $P = 0.077$]. These results were not comparable to a previous investigation into NT-proBNP and in-hospital mortality in this cohort *(21)* as only patient samples with detectable molecular BNP forms were included in the present analyses.

Discussion

The results of this study demonstrate that specific molecular forms of BNP are associated with poor prognosis in patients hospitalized with acute HF. Molecular BNP forms 5-32, 4-32, and 3-32 were all independently able to predict all-cause mortality or a combination of all-cause mortality and rehospitalization due to HF at 6 months and 1 year after adjustment for clinical and physiological factors. These prognostic qualities were comparable to those for conventional clinical measurements of circulating BNP through analysis of plasma NT-proBNP concentrations. When stratified by tertiles, BNP molecular forms showed increased risk of adverse events with increased circulating concentrations. Previous reports have described the presence of molecular BNP forms in the circulation of patients with HF and shown that these forms correlate with conventional BNP concentrations *(12–14)*; however, the 3 common molecular forms investigated in this study represent only a fraction of those measured in commercially available BNP assays, with the inclusion of other BNP molecular forms and BNP 1-32 contributing to these results. Due to substantial levels of cross-reactivity in conventional BNP assays, proBNP may also contribute to the measured concentrations of BNP *(22)*. This study is the first to investigate prognostic implications of molecular BNP forms.

Proportional hazard analyses demonstrated BNP 5-32 as the superior form for the prediction of death and/or death/HF events. When categorized into tertiles for circulating concentrations, BNP 5-32 was observed to show the strongest association between increased concentrations and reduced survival. The reasons for the slight increase in association seen for BNP 5-32 over BNP 4-32 and BNP 3-32 are not known but may be linked to the kinetics and dynamics of BNP fragmentation/proteolytic characteristics. Molecular BNP forms were detectable in 838 (93%) of measured samples, with degradation of sample likely to be the major cause for this discrepancy. Mechanistic underpinnings are poorly understood and warrant further investigation. Prognostic implications of the BNP molecular forms were compared to NT-proBNP and were observed to possess comparable predictive qualities for the end points of death or death/HF at 6 months and 1 year. NT-proBNP has previously been reported to have short-term (<80 days) prognostic ability for mortality in acute HF *(22)* but has not been studied in isolation for the longer-term end points of 6 months and 1 year. These data indicate the utility of BNP molecular forms and NT-proBNP for these extended end points.

Interestingly, investigations into associations with markers of renal dysfunction showed that all molecular BNP forms correlated with blood urea and eGFR but at a level that was modestly reduced when compared with NT-proBNP. Effects of renal dysfunction augmenting concentrations of BNP in the circulation hampers wider clinical use of this biomarker in patients with renal dysfunction. NT-proBNP is particularly affected by renal dysfunction as approximately 55%–60% is known to be cleared from the circulating blood by the kidneys, whereas molecular BNP forms are removed through NP receptors or NEP *(23, 24)*. These physiological mechanisms may lead to impaired prognostic judgement when using circulating concentrations of NT-proBNP in patients with renal dysfunction, suggesting that molecular BNP forms may allow improved prognostic utility for patients with renal impairment.

The mechanistic pathways relating to the synthesis of these molecular forms are not fully understood, although reports suggest that numerous proteases are responsible. Each of the molecular forms investigated within this study have been associated with distinctive degradation pathways, including dipeptidyl peptidase-IV (BNP 3-32) *(26)* and a neutral endopeptidase (BNP 5-32) *(27)* processing of BNP 1-32 and a corin-mediated cleavage of proBNP (BNP 4-32) *(28)* (see online Supplemental Fig. 7).

In conclusion, this study is the first prognostic investigation of BNP molecular forms in a cohort of HF patients and demonstrates the prognostic usefulness of BNP forms in comparison to current clinical BNP measurement techniques. Further, this is the first report on the prognostic qualities of NT-proBNP in acute HF for events occurring at 6 months and 1 year after initial hospital admission. Circulating plasma concentrations of

BNP 5-32, BNP 4-32, and BNP 3-32 were associated with poor prognosis at 6 months and 1 year in patients with acute HF, and were comparable in prognostic ability when compared with NT-proBNP.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest.*

Employment or Leadership: None declared.

Consultant or Advisory Role: I.B. Squire, advisory boards for Novartis.

Stock Ownership: None declared.

Honoraria: I.B. Squire, honoraria for participation in educational events for Novartis.

Research Funding: T. Suzuki, (*a*) Sekisui Medical Co., (*b*) the Practical Research Project for Life-Style related Diseases including Cardiovascular Diseases and Diabetes Mellitus from Japan Agency for Medical Research and Development (AMED), (*c*) the University of Tokyo, and (*d*) the John and Lucille van Geest Foundation and the National Institute for Health Research Leicester Cardiovascular Biomedical Research Unit to the University of Leicester.

Expert Testimony: None declared.

Patents: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, and final approval of manuscript.

Acknowledgments: The authors are grateful to Sekisui Medical Co. for provision of antibodies.

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