Journal of Cardiac Failure Vol. 00 No. 00 2021

Active B-Type Natriuretic Peptide Measured by Mass Spectrometry and Response to Sacubitril/Valsartan

ERICA M. DILLON, MD, MSCI,[1,2] SHOUZUO D. WEI,[1] DEEPAK K. GUPTA, MD, MSCI,[3,4] HUI NIAN, PhD, MS,[5] BROOKS S. RODIBAUGH, BS,[1] KATHERINE N. BACHMANN, MD, MSCI,[3,6,7] ALLEN J. NAFTILAN, MD, PhD,[4] LYNNE W. STEVENSON, MD,[4] AND NANCY J. BROWN, MD[1,2,8]

Nashville, Tennessee; and New Haven, Connecticut

ABSTRACT

Background: B-type natriuretic peptide (BNP) immunoassays (BNP_{ia}) do not differentiate active and inactive forms. Inactive NT-proBNP is used to track heart failure (HF) during treatment with sacubitril/valsartan, which inhibits BNP degradation. Mass spectrometry (MS) may better assess effects of HF treatment on biologically active BNP1-32.

Methods and Results: We developed a MS assay with immediate protease inhibition to quantify BNP1-32 over a linear range, using labeled recombinant BNP standard. In 4 healthy volunteers, BNP1-32 by MS (BNP_{MS}) increased from below the 5 pg/mL detection limit to 228 pg/mL after nesiritide. In patients with HF, BNP_{MS} was measured in parallel with BNP and NT-proBNP immunoassays before and during sacubitril/valsartan treatment. BNP_{MS} was 4.4-fold lower than BNP_{ia} in patients with HF. Among patients not taking sacubitril/valsartan and without end-stage renal disease, BNP_{MS} correlated with BNP_{ia} ($r_s = 0.77$, $P < .001$) and NT-proBNP ($r_s = 0.74$, $P < .001$). After a median of 8 weeks on sacubitril/valsartan, active BNP_{MS} levels decreased by 50% (interquartile range −98.3% to 41.7%, $n = 22$, $P = .048$) and correlated with NT-proBNP ($r_s = 0.64$, $P < .001$), but not with BNP_{ia} ($r_s = 0.46$, $P = .057$).

Conclusions: Active BNP measured by MS accounts for only a small amount of BNP measured by immunoassays. Although decreased BNP production was anticipated to be masked by inhibition of degradation, levels of active BNP decreased during chronic sacubitril/valsartan treatment. (*J Cardiac Fail 2021;00:1−9*)

Key Words: B-type natriuretic peptide (BNP), BNP1-32, NT-proBNP, natriuretic peptides, sacubitril/valsartan, neprilysin, mass spectrometry, heart failure.

B-Type natriuretic peptide (BNP), a hormone released from cardiomyocytes in response to myocardial wall stress, exerts compensatory effects including natriuresis, vasodilation, inhibition of neurohormonal activation, and decrease in hypertrophy and fibrosis.[1,2] Given its central role in cardiovascular physiology BNP has proven to be not only a robust biomarker for heart failure (HF) diagnosis and prognosis, but also a therapeutic target. Therefore, understanding BNP-related physiology is of biologic and clinical importance. Like many hormones, BNP undergoes a series of post-translational processing steps resulting in several biologically inactive fragments in addition to the physiologically active form. Current clinically approved immunoassays, however, lack specificity for differentiating between active and inactive forms thereby confounding the study of BNP kinetics.[2−7]

The gene for BNP is transcribed and translated into a preprohormone, which undergoes intracellular processing to proBNP1-108, which can then be cleaved by furin or corin yielding biologically active BNP1-32 and the inactive N-terminal fragment, NT-proBNP1-76.[8] BNP1-32 is degraded into truncated peptides in the circulation by enzymes

From the [1]Division of Clinical Pharmacology, Vanderbilt University Medical Center, Nashville, Tennessee; [2]Department of Internal Medicine, Vanderbilt University Medical Center, Nashville, Tennessee; [3]Vanderbilt Translational and Clinical Cardiovascular Research Center, Vanderbilt University School of Medicine, Nashville, Tennessee; [4]Division of Cardiovascular Medicine, Vanderbilt University Medical Center, Nashville, Tennessee; [5]Division of Biostatistics, Vanderbilt University Medical Center, Nashville, Tennessee; [6]Veterans Administration Tennessee Valley Healthcare System, Nashville, Tennessee; [7]Division of Diabetes, Endocrinology, and Metabolism, Vanderbilt University Medical Center, Nashville, Tennessee and [8]Department of Internal Medicine, Yale School of Medicine, New Haven, Connecticut.

Manuscript received February 1, 2021; revised manuscript received May 14, 2021; revised manuscript accepted May 21, 2021.

Reprint requests: Erica M. Dillon, MD, MSCI, Vanderbilt University School of Medicine, Division of Clinical Pharmacology, 2200 Pierce Ave, 566 Robinson Research Building, Nashville, TN 37232. Tel: (916) 835-8510; Fax: (214) 387-1205. E-mails: erica.m.dillon@vumc.org, ericadillon10@gmail.com

Supported by HL143619, HL145293, HL128928, HL133860, HL148661, TR002243 and T32GM007569 from the National Institutes of Health (NIH) and 1IK2CX001678 from the U.S. Department of Veterans Affairs Clinical Sciences Research and Development (CSR&D) Program.

1071-9164/$ - see front matter

© *2021 Elsevier Inc. All rights reserved.*

https://doi.org/10.1016/j.cardfail.2021.05.026

including neprilysin, dipeptidyl peptidase IV, insulin degrading enzyme, and in rodents, meprin.[4,5,9] The half-life of BNP has been estimated to be approximately 22 minutes using conventional methods.[10] The rapid enzymatic processing of BNP also complicates measurement accuracy under standard blood collection techniques.

Clinically available immunoassays for BNP have large interassay variation and overestimate bioactive BNP1-32 owing to cross-reactivity with proBNP and the truncated peptide degradation products.[3,4,11,12] Establishing a sensitive method for the specific quantification of BNP1-32 can help to elucidate the mechanisms of HF therapies and guide the use of circulating BNP as a surrogate endpoint for decreased myocardial stress.[13,14] Richards and colleagues developed a Luminex-based BNP1-32 immunoassay that shows 0.1% or less cross-reactivity to proBNP but still cross-reacts with BNP2-32; this assay is not clinically approved.[15] Several groups have measured BNP1-32 using mass spectrometry (MS), typically finding levels lower than those measured by immunoassay.[16–18] The prior methodology, however, did not include immediate inhibition of enzymes at specimen collection to ensure full yield of the peptide before its rapid degradation.

Sacubitril/valsartan is a combination neprilysin inhibitor and angiotensin receptor blocker demonstrated to improve multiple outcomes in HF.[19,20] Benefits have been attributed in part to decreased degradation and thus increased circulating levels of natriuretic peptides including BNP1-32.[13] In the PARADIGM-HF cohort, treatment with sacubitril/valsartan was associated with increased concentrations of BNP as measured by immunoassay at 4 weeks and sustained after 8 months, and measured decreases of the inactive NT-proBNP indicated the clinical benefit to decrease myocardial stress.[20] A switch to the clinical immunoassay for NT-proBNP rather than for BNP is in fact recommended during sacubitril/valsartan treatment, based on the expectation that inhibited degradation of circulating BNP will mask the decrease in BNP production that indicates clinical improvement.[21]

To overcome the limitations of approved clinical immunoassays, we developed an assay to measure the active peptide BNP1-32 using MS (BNP_{MS}) in blood specimens collected with immediate inhibition of degradative enzymes. We applied this methodology to (1) measure BNP1-32 in normal volunteers during placebo or steady-state infusion of BNP (Nesiritide, Scios LLC, Titusville, NJ), (2) compare BNP_{MS} with clinical immunoassays for BNP (BNP_{ia}) or NT-proBNP in patients with HF, and (3) evaluate the effect of chronic treatment with sacubitril/valsartan on BNP_{MS} and clinical BNP_{ia} and NT-proBNP levels.

Methods

Study Participants and Procedures

Study procedures were approved by the Vanderbilt Institutional Review Board, and all patients gave written informed consent. We collected blood from 4 healthy volunteers and 38 patients with HF from June 2018 to February 2020 at Vanderbilt University Medical Center. The healthy volunteers were randomized in a blinded crossover fashion to treatment with placebo or intravenous recombinant BNP (nesiritide) given as a 100 ng/kg loading dose followed by 10 ng/kg/min over 240 minutes before BNP1-32 measurement. BNP_{ia} and NT-proBNP were measured in The Vanderbilt Clinical Chemistry Lab using the Abbott Architect (Abbott Laboratories, Abbott Park, IL) and proBNP II (Roche Diagnostics, Indianapolis, IN) assays, respectively.

BNP_{MS} measurements were obtained in 34 patients with HF who were not taking sacubitril/valsartan, of whom 25 and 18 also had same-day clinical immunoassay measurements of BNP and NT-proBNP, respectively. In 23 of the 25 patients in whom blood was collected for BNP_{MS} and BNP_{ia}, samples were obtained from the same blood draw. In all 18 of the patients in whom blood was collected for BNP_{MS} and NT-proBNP, samples were obtained from the same blood draw. A total of 26 patients had blood collected for paired BNP_{MS}, BNP_{ia}, and/or NT-proBNP before and during chronic treatment with sacubitril/valsartan. Of these 26 patients, 22, 19, and 13 patients had paired measurements for BNP_{MS}, BNP_{ia}, and NT-proBNP, respectively. Among the paired blood samples drawn, the median duration of time between baseline and post-treatment collection was 8 weeks (interquartile range 5−23). Blood was collected from a total of 28 patients with HF who were treated with sacubitril/valsartan for correlation analyses between BNP_{MS} and BNP_{ia} ($n = 26$) or NT-proBNP ($n = 20$). In all 28 patients treated with sacubitril/valsartan, blood samples were obtained from the same blood draw. Correlation analyses were performed between BNP_{MS} and immunoassay measurements from samples drawn on the same day.

BNP Collection and Measurement by MS

Blood for measurement of BNP and NT-proBNP by immunoassays was collected in lavender top EDTA tubes according to clinical protocol and immediately processed by the clinical laboratory. Blood (2 mL) for BNP_{MS} was collected into BD P100 tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) containing a proprietary protease inhibitor cocktail specifically formulated for stabilization of human plasma proteins at the point of collection. Tubes were inverted and placed on ice immediately and centrifuged at $3250\times g$, at 4 ˚C for 2 minutes. Next, 0.5 mL of supernatant (plasma) was added to a 2 mL low protein retention tube containing 1.5 mL of 80% CH_3CN/H_20, 0.5% formic acid and 2 μL L-methionine (45 mg/mL) solution, and 10 ng of internal standard ($^{13}C_{12}$,$^{15}N_8$-labeled BNP, CPC Scientific) was added to the last 34 samples assayed. In the prior samples, internal standard was added at sample extraction and purification step, as described below. Pellets were centrifuged at $16{,}000\times g$, at 4 ˚C for 3 minutes and removed. The supernatant was flash frozen with liquid nitrogen and stored at −80 ˚C until analysis.

For sample extraction and purification, 1.3 mL H_2O, 0.4 mL NH_4HCO_3 (1 M), 2 μL L-methionine (45 mg/mL)

was added and solution was well-mixed. The peptide was then reduced and alkylated by adding 0.12 mL DTT (50 mM at 50˚C for 20 minutes), and 0.13 mL Iodoacetamide (100 mM in dark at 37˚C for 40 minutes). With 2 mL of 10 mM KPi (pH 7.4) added in, the mixture was centrifuged for any pellets, and the supernatant was passed through a 30,000 Da Amicon Ultra-4 filter (4500×*g* at 4 ˚C for 70 minutes) and purified (in a 4 ˚C cold room) using a 60 mg Nexus cartridge (Agilent), preconditioned with 3 mL MeOH, 5 mL H2O, and 3 mL KPi (10 mM, PH 7.4). After the alkylated peptide was applied, the cartridge was washed with 3 mL H_2O containing 45 μg/mL L-methionine, dried under a vacuum and the peptide eluded with 1.5 mL MeOH containing 0.1% HCl (1 N) and 45 μg/mL L-methionine. The eluate was kept at −80 ˚C until analysis.

For liquid chromatography/MS/MS analysis, the eluate was evaporated in N_2 at room temperature and the residue dissolved in 30 μL of 20% CH_2CN, 0.2% formic acid/H_2O. Samples were centrifuged at 22,000×*g* for 2 minutes to remove any pellets before being resolved by UPLC (Waters) at 40˚C with an Acquity BEH (C8) column (1.7 μm, 1.0 × 50 mm) using a CH_3CN/H_2O/FA (5/94.8/0.2 to 30/69.8/0.2, v/v/v, over 5 minutes at flow rate 0.12 mL/min) linear gradient. The alkylated peptide was quantified by positive electrospray ionization/MS/MS-selected reaction monitoring on a TSQ Vantage triple quadrupole mass spectrometer equipped with a heated electrospray ionization probe monitoring product ions: m/z 728.2 originating from m/z 597.5 (BNP) and m/z 734.2 originating from m/z 602.5 (internal standard). Quantification was done by the ratio of BNP to internal standard.

In the preanalytical stages of BNP MS assay development, measurements of MS BNP1-32 in samples collected (1) in the absence of inhibitors and (2) with an in-house inhibitor cocktail yielded inconsistent results. The key step of protease inhibition at time of blood collection with BD P100 tubes proved to increase the sensitivity needed for BNP1-32 detection.

To assess linearity, we added varying amounts of unlabeled BNP to a fixed amount of labeled BNP internal standard (250 pg) and measured the ratio of the isotopes to compare with the expected ratio. The standard curve is linear for unlabeled/labeled BNP ranging from 5 to 800 pg/mL in plasma (Supplemental Fig. 1). The intra and interassay coefficients of variation were calculated by mixing charcoal-stripped plasma with BNP (Sigma-Aldrich) at a concentration of 50 pg/mL (low control) or a concentration of 500 pg/mL (high control). Aliquots were frozen, and later thawed, processed, and analyzed for BNP on 3 separate days over 1 month. On each day, the sample was assayed 5 times. The lower limit of detection in plasma was determined by assaying serial dilutions of charcoal-stripped plasma until the coefficient of variation for the measurement of a concentration exceeded 16%. The lower limit of detection was determined as the next lowest concentration with a coefficient of variation less than 16%. Clearance of nesiritide was calculated in the healthy volunteers using the formula Clearance = $F \times R_0/C_{SS}$ where F is the bioavailability factor (which is 1 in the case of intravenous administration), R_0 is the constant dosing rate, and C_{SS} is the steady-state concentration.

Statistical Analysis

Data are presented as median (25th, 75th percentile), mean ± standard deviation, or counts (percentages). Comparisons between groups were made using the Wilcoxon rank-sum test for continuous variables or Pearson χ^2 test for categorical variables. Comparisons before and during chronic treatment with sacubitril/valsartan were made using the Wilcoxon signed rank test. Agreement between BNP_{ia} and BNP_{MS} was assessed by Spearman's correlation coefficient, as well as by a linear regression model using BNP_{ia} as the dependent variable. For all analyses, BNP_{MS} levels below the limit of detection were assigned a value of 0 pg/mL. All analyses were made using IBM SPSS version 26 or R (3.6.3). (R Core Team [2020], R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria; available from: www.R-project.org/.)

Results

MS BNP Assay Characteristics

The assay was linear over a concentration range from 5 to 800 pg/mL in plasma ($r^2 = 1.000$). The lower limit of quantification in plasma was 5 pg/mL. The intra-assay precision (coefficient of variation) was 12.0% and 6.8% for the 50 pg/mL and 500 pg/mL concentration levels, respectively. The interassay precision was 5.2% and 2.2% for the same concentrations.

MS BNP Concentrations in Normal Volunteers During Placebo and Nesiritide Infusions. BNP_{MS} concentrations were below the limit of detection in 3 of 4 normal volunteers at baseline or during infusion of vehicle. During steady-state infusion of nesiritide, plasma concentrations of BNP increased to 228 pg/mL (Fig. 1). Using circulating BNP1-32 levels measured by MS, the calculated clearance of BNP1-32 was 49.0 ± 19.5 mL/min/kg or 4.2 ± 1.9 L/min.

Baseline Characteristics of Patients With HF and BNP Before Sacubitril/Valsartan. Table 1 provides baseline characteristics of all patients with HF in whom BNP_{MS} was measured. BNP levels were statistically similar in men and women and in black and non-black patients regardless of assay method. BMI was inversely correlated with BNP_{ia} levels ($n = 29$, $r_s = -0.44$, $P = .016$) as has previously been demonstrated,[6,22–24] but was not related to BNP as measured by MS ($n = 34$, $r_s = -0.19$, $P = .29$).

Relationship Between BNP or NT-proBNP Measured by Clinical Immunoassay and BNP Measured by MS. Among patients with HF not taking sacubitril/valsartan, the relationship between BNP_{MS} and BNP_{ia} or NT-proBNP is displayed in Fig. 2. Although BNP_{MS} correlated with BNP_{ia} ($n = 25$, $r_s = 0.73$, $P < .001$), BNP levels measured by immunoassay

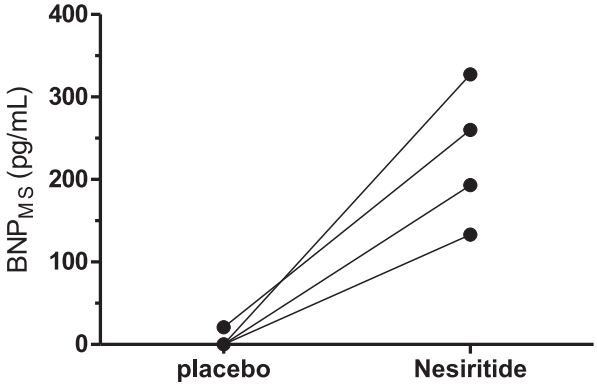


Fig. 1. B-type natriuretic peptide (BNP)1-32 by mass spectrometry (MS) (BNP_{MS}) in healthy volunteers during nesiritide vs vehicle. Plasma BNP1-32 concentrations (pg/mL) in 4 healthy volunteers measured by mass spectrometry during vehicle or during infusion of recombinant BNP (Nesiritide, Scios LLC, Titusville, NJ) given at a loading dose of 100 ng/kg followed by steady-state infusion of 10 ng/kg/min.

were 4.4-fold higher (95% CI 2.7−6.1) than levels measured by MS. BNP_{MS} also correlated with NT-proBNP ($n = 18$, $r_s = 0.72$, $P < .001$). Exclusion of the 2 patients with end-stage renal disease slightly increased the correlations of BNP_{MS} with BNP_{ia} ($n = 23$, $r_s = 0.77$, $P < .001$) and NT-proBNP ($n = 17$, $r_s = 0.74$, $P < .001$).

Effect of Sacubitril/Valsartan Treatment on BNP or NT-proBNP Measured by Immunoassay and on BNP Measured by MS. Blood was collected in 26 patients before and during sacubitril/valsartan with 19 (73%), 5 (19%), and 2 (8%) taking doses of 50 mg, 100 mg, and 200 mg, respectively. Systolic and diastolic blood pressures and heart rate decreased after treatment with sacubitril/valsartan (Table 2). Weight and creatinine did not significantly change after treatment. Neither BNP nor NT-proBNP concentrations measured by conventional immunoassays changed following treatment with sacubitril/valsartan (Fig. 3). BNP_{MS} decreased significantly (median percent change −50%, interquartile range −98.3% to 41.7%) during chronic treatment with sacubitril/valsartan ($n = 22$, $P = .048$, Fig. 3). BNP_{MS} levels decreased in 14 patients, increased in 6 patients, and were below the detection limit before and during treatment in 2 patients. In patients whose BNP_{MS} decreased and immunoassays were available for comparison, NT-proBNP also decreased in 4 of 7 patients, and BNP_{ia} decreased in 7 of 10 patients. Of the patients in whom BNP_{MS} increased, NT-proBNP also increased in 4 of 4 patients in whom before and after NT-proBNP immunoassay measurements were available, and BNP_{ia} increased in 4 of 5 patients in whom BNP_{ia} measurements were available. One patient did not have immunoassay measurements available for comparison. There was no relationship between the within-patient change in BNP_{MS} and the duration of time between baseline and post-treatment blood collection. There was no relationship between BNP_{MS} and sacubitril/valsartan dose at post-treatment collection.

During chronic treatment with sacubitril/valsartan, BNP_{MS} and BNP_{ia} were not significantly correlated ($n = 20$, $r_s = 0.36$, $P = .12$), even after the exclusion of 2 patients with end-stage renal disease ($n = 18$, $r_s = 0.46$, $P = .057$). The correlation of BNP_{MS} with NT-proBNP was modest ($n = 26$, $r_s = 0.45$, $P = .023$) and increased after exclusion of 2 patients with end-stage renal disease ($n = 24$, $r_s = 0.64$, $P < .001$).

Table 1. Characteristics of Patients With Heart Failure Not Receiving Sacubitril/Valsartan in Whom BNP_{MS} Was Measured ($n = 34$)

Characteristic	Median (25th, 75th Percentile) or % (Count)
Age, years	59 (52, 64)
Female sex	24 (8)
Race	
White	68 (23)
Black	29 (10)
Hispanic	3 (1)
LV ejection fraction, %	25 (19, 33)
Atrial fibrillation	29 (10)
Diabetes mellitus	41 (14)
Coronary artery disease	35 (12)
Hypertension	85 (29)
Prior RAAS blockade	
ACEi	50 (17)
ARB	38 (13)
β-Blocker	97 (33)
Loop diuretic	71 (24)
Mineralocorticoid antagonist	56 (19)
Weight, kg	88.0 (75.8, 106.2)
BMIç kg/m^2	29.3 (26.1, 33.1)
Heart rate, bpm	81 (70, 85)
Systolic blood pressure, mm Hg	118 (108, 128)
Diastolic blood pressure, mm Hg	78 (68, 84)
Creatinine, mg/dL	1.17 (0.94, 1.63)

ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; BNP_{MS}, BNP1-32 by mass spectrometry; LV, left ventricular; RAAS, renin−angiotensin−aldosterone system.

Discussion

BNP plays a pivotal role in cardiovascular physiology and serves as an important biomarker and therapeutic target in clinical medicine. The inability of clinically approved immunoassays to distinguish between biologically active and inactive BNP forms presents a barrier to understanding kinetics and changes in BNP after targeted therapy. Therefore, we developed a MS assay to investigate active BNP1-32 concentrations in patients with HF, including during sacubitril/valsartan therapy. Our principal findings are that circulating levels of BNP1-32 (1) account for only approximately 20%−25% of BNP detected by conventional clinical immunoassays, (2) correlate modestly with immunoassay measurements of BNP and NT-proBNP, and (3) decrease after approximately 8 weeks of treatment with sacubitril/valsartan. Collectively, these findings support utilization of MS-based measurement of active BNP1-32 to better understand the natriuretic peptide processing and kinetics and the

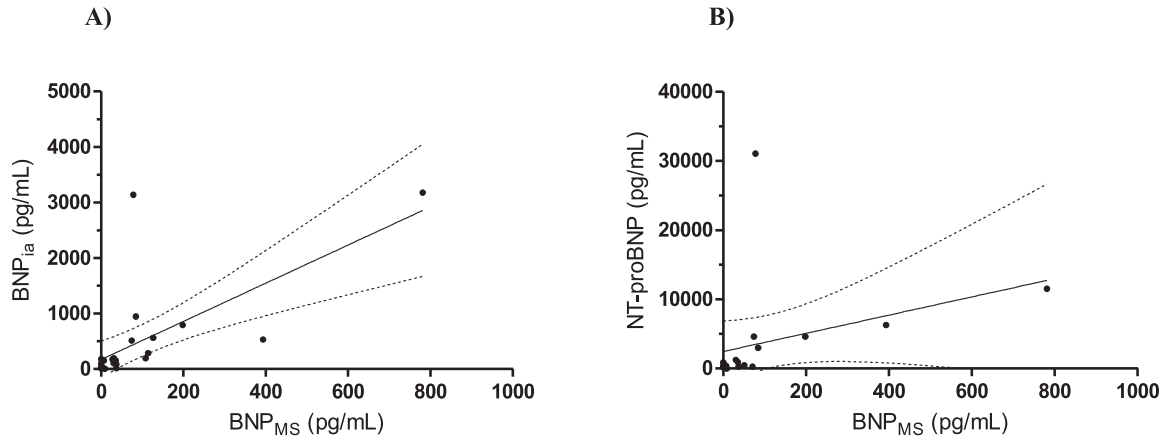


Fig. 2. Relationships of BNP_{MS} with BNP_{ia} and NT-proBNP. Correlations between baseline BNP_{MS} and (**A**) BNP_{ia} ($n = 23$, $r_s = 0.77$, $P < .001$) or (**B**) NT-proBNP ($n = 17$, $r_s = 0.74$, $P < .001$). Samples for correlation analyses between BNP_{MS} and immunoassays were collected within 24 hours of each other. Patients with end-stage renal disease were excluded from the analyses. Concentrations are in picograms per milliliter. Abbreviations as in Fig. 1.

mechanisms of action and integrated impact for drugs such as sacubitril/valsartan.

Our MS assay provides specific and precise measurement of BNP1-32. The lower limit of detection is 5 pg/mL with good interassay and intra-assay coefficient of variations, comparable with those of clinically available immunoassays. Reassuringly, as negative and positive controls, we found BNP_{MS} concentrations to be below the limit of detection in 3 of the 4 healthy volunteers and increased in these same individuals after recombinant BNP infusion. As an additional positive control of clinical relevance, we found BNP1-32 levels by MS to be detectable in the majority of patients with HF. Although other groups have quantified natriuretic peptides using MS, our group was the first to our knowledge to use MS with a collection protocol that includes immediate protease inhibition upon obtaining specimens, which we believe enhances the sensitivity of detection and more likely captures in vivo BNP1-32 levels.[16–18]

Table 2. Characteristics for 26 Patients in Whom BNP_{ia}, NT-proBNP, or BNP_{MS} Was Measured Before and During Chronic Sacubitril/Valsartan Treatment

Characteristic	Baseline	After treatment	*P* Value
Age, years	59 (54, 66)		
Female	19 (5)		
Race			
White	85 (22)		
Black	11 (3)		
Hispanic	4 (1)		
LV ejection fraction, %	22 (18, 32)		
Atrial fibrillation	35 (9)		
Diabetes mellitus	42 (11)		
Coronary artery disease	39 (10)		
Hypertension	81 (21)		
RAAS blockade			
ACEi	58 (15)	0 (0)	
ARB	23 (6)	100 (26)	
β-Blocker	92 (24)	100 (26)	.50
Loop diuretic	73 (19)	65 (17)	.50
Mineralocorticoid antagonist	54 (14)	62 (16)	.50
Weight, kg	87.2 (79.0, 106.2)	87.5 (79.1, 110.2)	.14
BMI, kg/m^2	28.2 (26.3, 33.3)	30.9 (26.1, 33.9)	.084
Heart rate, bpm	82 (71, 86)	70 (66, 80)	.024
Systolic blood pressure, mm Hg	119 (109, 126)	106 (98, 120)	.008
Diastolic blood pressure, mm Hg	77 (69, 84)	67 (61, 74)	.002
Creatinine, mg/dL	1.24 (0.93, 1.56)	1.31 (1.06, 1.49)	.35

Data presented as median (25th, 75th percentile) or % (count).

P values from Wilcoxon signed-rank test or McNemar test for continuous and categorical variables, respectively.

BNP_{ia}, clinical immunoassays for BNP; NP, natriuretic peptide. Other abbreviations as in Table 1.

The measurement of active BNP1-32 by MS during nesiritide infusion enabled accurate estimation of clearance in healthy individuals and comparison with clearance calculated in prior studies using immunoassay to measure BNP. The calculated BNP clearance 49.0 ± 19.5 mL/min/kg or 4.2 ± 1.9 L/min is higher than that reported in the nesiritide label (9.2 mL/min/kg),[25] but consistent with previously published studies (range 4.2 to 5.8 L/min).[10,26–28] The rapid clearance of active BNP1-32 may influence the development of pharmacologic strategies aiming to increase BNP effects. Measurements that detect biologically inactive fragments of BNP may not accurately reflect the enzymatic degradation of BNP1-32.

Among patients with HF, clinically approved BNP immunoassays detect primarily inactive degradation fragments; the concentrations of BNP measured by immunoassay were approximately 4 to 5 times higher than the active moiety detected by MS. Our findings agree with prior reports by Miller et al,[17] Hawkridge et al,[16] and Niederkofler et al,[18] who also reported lower BNP1-32 levels detected by MS than clinical immunoassay in patients with HF. Our results differ, however, from these prior studies with regard to the absolute levels of BNP1-32 detected. For example, Miller et al reported a mean BNP_{MS} of 19 ± 33 pg/mL in 39 patients with HF,[17] whereas our group mean was 61 ± 122 pg/mL, which may reflect different patient characteristics or an increased yield from our protocol of immediate protease inhibition.

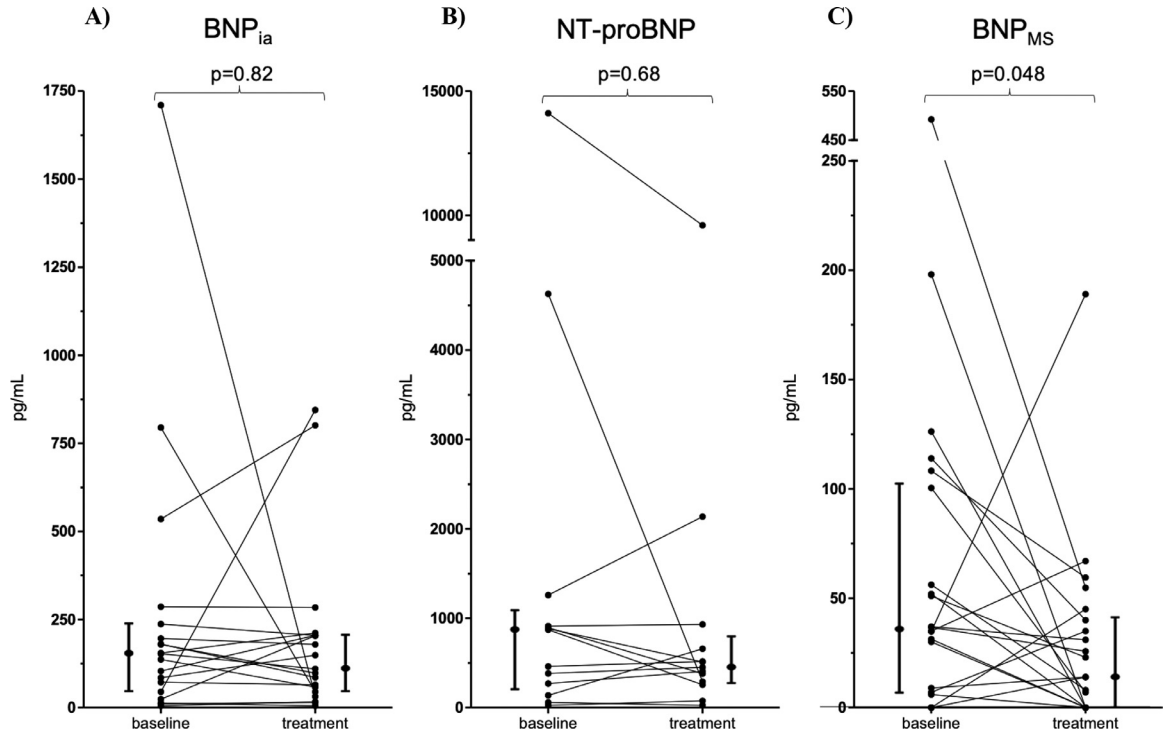


Fig. 3. Comparisons of natriuretic peptide concentrations at baseline and during chronic sacubitril/valsartan treatment. (**A**) BNP_{ia}, (**B**) NT-proBNP, and (**C**) BNP_{MS}, at baseline and after a median of 8 and mean of 16 weeks of treatment with sacubitril/valsartan. Vertical bars range from first to third quartiles with medians indicated by solid circles. Concentrations are in picograms per milliliter. *P* values are from Wilcoxon signed-rank test. Abbreviations as in Fig. 1.

Consistent with prior studies, we found BNP_{MS} concentrations in patients with HF correlated modestly with BNP_{ia} and NT-proBNP.[17] The modest correlation suggests that natriuretic peptides measured by various methods may offer somewhat different information. Conventional immunoassays are robust diagnostic and prognostic markers in clinical settings, whereas BNP measured by the MS assay may provide more insights into HF pathophysiology and responses to therapy. For example, in studies using immunoassays, inverse associations between BNP and NT-proBNP and BMI have been reported in both HF and healthy patients.[6,22–24] BNP concentrations have also been reported to be lower in non-white individuals and are generally higher in women compared with men.[29–32] We found an inverse correlation between BNP_{ia} and BMI, but not between BNP_{MS} and BMI. It is possible that obesity affects the clearance of inactive peptides detected by immunoassays to a greater extent than the clearance of the active BNP1-32, perhaps owing to differential affinities of BNP fragments vs mature peptide for the clearance receptor.[33,34] The potential differential association of BMI with BNP measured by MS and by immunoassay is intriguing and merits future study in larger cohorts, as do race and sex-based differences in BNP_{MS} levels.

BNP measured by the MS assay may be more informative than approved immunoassays for understanding treatment strategies, such as natriuretic peptide supplementation or inhibition of enzymatic degradation. The effects of sacubitril/valsartan on natriuretic peptide concentrations early and late after initiation have been studied using conventional immunoassays. A study performed on the PARADIGM-HF cohort demonstrated a modest increase (20%) in BNP_{ia} that peaked at 8–10 weeks of treatment.[35] Interestingly, the prognostic value of BNP_{ia} during treatment was robust and comparable with NT-proBNP; consequently the authors speculated that increases in BNP_{ia} may not be explained by drug effects alone.[35] Ibrahim et al[36] demonstrated a similar general increase in BNP_{ia} over subsequent outpatient dose titration visits; however, the effect on BNP was inconsistent when measured with different immunoassays in the same patients. In their study, of the patients whose BNP_{ia} did increase during treatment, BNP_{ia} tended to be higher at baseline, and the most dramatic increases occurred after titration to a dose of 100 mg twice daily.[36] Nougue et al[37] found no significant change in BNP_{ia} before or during treatment. The cross-reactivity between active and inactive fragments in BNP immunoassays limits this approach to understand how sacubitril/valsartan specifically affects active BNP1-32. NT-proBNP decreases with more consistency after 4–12 weeks of treatment with sacubitril/valsartan (28%–47%).[35–38]

Using our MS assay, we found that circulating levels of the active BNP moiety decreased after a median of 8 weeks of treatment with sacubitril/valsartan. BNP1-32 is believed to be a substrate of sacubitril/valsartan; thus, 2 explanations are possible for our findings of reduced BNP_{MS} during chronic treatment. As speculated by other investigators, a decrease in myocardial stress by sacubitril/valsartan may diminish the stimulus for BNP production to a greater

extent than it increases circulating BNP levels by inhibiting degradation. Another potential explanation is that neprilysin inhibition of BNP1-32 clearance could be offset by enhanced clearance activity from other enzymes such as dipeptidyl peptidase IV or insulin degrading enzyme.

The observed lack of increase in circulating BNP1-32 also suggests the chronic benefits of sacubitril/valsartan in patients with HF with reduced EF are unlikely to be attributable primarily to decreased BNP1-32 degradation by neprilysin inhibition. We propose that the effect of neprilysin inhibition on BNP1-32 may vary over time, with a possible initial increase owing to decreased degradation, followed by progressive decrease in myocardial wall stress leading to decreased production.

The effect of 8 weeks of treatment with sacubitril/valsartan likely is a combination of decreased BNP production and degradation, with decreased production exerting the predominant effect on blood concentrations, resulting in a net decrease in BNP1-32. This hypothesis is supported by the findings from the PARADIGM-HF cohort and others who reported an increase in BNP_{ia} in the acute period after initiation of sacubitril/valsartan followed by a decrease weeks to months later.[35,36] Additional support and a potential contributory mechanism for a biphasic BNP response to sacubitril/valsartan relates to the glycosylation state of proBNP. In acute HF, proBNP released from cardiomyocytes is predominantly nonglycosylated, leaving the cleavage site open to corin and furin, resulting in more active BNP1-32. In chronic HF, glycosylated proBNP predominates, which is less amenable to cleavage and subsequent release of active BNP1-32.[39] Based on differential glycosylation patterns, Chen and Burnett[40] proposed that neprilysin inhibition would be more effective in increasing circulating BNP1-32 in acute HF compared with chronic HF. The decrease in BNP_{MS} we observed after 8 weeks of treatment is consistent with this theory.

Our findings not only support the use of the BNP_{MS} assay to understanding therapeutic mechanisms for medications such as sacubitril/valsartan, but also suggest that measuring BNP1-32 by MS may be informative for assessing response to therapy because of the significant reduction during treatment in our HF cohort. However, the therapeutic effect of sacubitril/valsartan may relate more to decreased degradation of other vasoactive peptides such as bradykinin, substance P, or atrial natriuretic peptide (ANP). Additional studies are needed to isolate the effects of these other known substrates of neprilysin, with consideration of how the effects of this medication on other natriuretic peptides may vary over time and between individuals. As shown in the literature, sacubitril/valsartan may increase ANP.[41] We did not measure ANP in this study, but we have begun to measure ANP by MS in our ongoing studies.

Limitations of our study include a relatively small sample of patients in whom we have concentrations of BNP_{MS} before and during sacubitril/valsartan treatment, and the variability of time between measurements. Although we did not find a relationship between within-patient changes in BNP_{MS} and the duration of time between baseline and post-treatment blood collection, additional studies with larger cohorts are needed to assess the relationship between BNP_{MS} and treatment duration. Detection and quantitative measurement of other circulating natriuretic peptide degradation products that could also contribute to some of the clinical effects of neprilysin inhibition have been reported; however, we did not measure these products in the current study.[17,18] The focus of our current study was to evaluate our MS assay as an effective method for quantifying BNP1-32 in patients with HF. We were able explore the pharmacologic responses of natriuretic peptides that were previously difficult to interpret based on conventional immunoassays.

A more precise measurement of biologically active components of the natriuretic peptide system will enhance our understanding of HF and its response to therapies. However, it is not clear whether the MS assays would ever become practical for routine clinical use. Greater precision and resources are required to obtain and process samples for this assay than are needed for the commercial assays that continue to provide such useful diagnostic and prognostic information in HF. The commercial assays may be even more effectively used when we better understand how they reflect the various peptides of this important regulatory system.

In conclusion, we developed a MS assay to measure accurately active BNP1-32 concentrations. Among patients with HF BNP1-32 accounts for only 20%−25% of BNP fragments detected by conventional clinical immunoassays, correlates modestly with immunoassay measured BNP and NT-proBNP, and decreases after approximately 8 weeks of treatment with sacubitril/valsartan.

Acknowledgments

The authors appreciate the assistance of C. Anthony DeMatteo Jr. from the Division of Clinical Pharmacology at Vanderbilt University Medical Center, Nashville, Tennessee, for assistance with obtaining supplies and specimen processing, and Daniel W. Byrne, MS, from the Department of Biostatistics at Vanderbilt University Medical Center, Nashville, Tennessee, for offering writing and statistical analysis feedback.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.cardfail.2021.05.026.

References

1. Semenov AG, Katrukha AG. Different susceptibility of B-type natriuretic peptide (BNP) and BNP precursor (proBNP) to cleavage by neprilysin: the N-terminal part does matter. Clin Chem 2016;62:617–22.
2. Vasile VC, Jaffe AS. Natriuretic peptides and analytical barriers. Clin Chem 2017;63:50–8.

3. Fu S, Ping P, Zhu Q, Ye P, Luo L. Brain natriuretic peptide and its biochemical, analytical, and clinical issues in heart failure: a narrative review. Front Physiol 2018;9:692.
4. Xiao P, Li H, Li X, Song D. Analytical barriers in clinical B-type natriuretic peptide measurement and the promising analytical methods based on mass spectrometry technology. Clin Chem Lab Med 2018;57:268–75.
5. Yandle TG, Richards AM. B-type natriuretic peptide circulating forms: analytical and bioactivity issues. Clin Chim Acta 2015;448:195–205.
6. Maisel AS, Krishnaswamy P, Nowak RM, McCord J, Hollander JE, Duc P, et al. Rapid measurement of B-type natriuretic peptide in the emergency diagnosis of heart failure. N Engl J Med 2002;347:161–7.
7. McCullough PA, Nowak RM, McCord J, Hollander JE, Herrmann HC, Steg PG, et al. B-type natriuretic peptide and clinical judgment in emergency diagnosis of heart failure: analysis from Breathing Not Properly (BNP) Multinational Study. Circulation 2002;106:416–22.
8. Semenov AG, Tamm NN, Seferian KR, Postnikov AB, Karpova NS, Serebryanaya DV, et al. Processing of pro-B-type natriuretic peptide: furin and corin as candidate convertases. Clin Chem 2010;56:1166–76.
9. Dickey DM, Potter LR. Human B-type natriuretic peptide is not degraded by meprin A. Biochem Pharmacol 2010;80:1007–11.
10. Holmes SJ, Espiner EA, Richards AM, Yandle TG, Frampton C. Renal, endocrine, and hemodynamic effects of human brain natriuretic peptide in normal man. J Clin Endocrinol Metab 1993;76:91–6.
11. Luckenbill KN, Christenson RH, Jaffe AS, Mair J, Ordonez-Llanos J, Pagani F, et al. Cross-reactivity of BNP, NT-proBNP, and proBNP in commercial BNP and NT-proBNP assays: preliminary observations from the IFCC Committee for Standardization of Markers of Cardiac Damage. Clin Chem 2008;54:619–21.
12. Saenger AK, Rodriguez-Fraga O, Ler R, Ordonez-Llanos J, Jaffe AS, Goetze JP, et al. Specificity of B-type natriuretic peptide assays: cross-reactivity with different BNP, NT-proBNP, and proBNP peptides. Clin Chem 2017;63:351–8.
13. Hubers SA, Brown NJ. Combined angiotensin receptor antagonism and neprilysin inhibition. Circulation 2016;133:1115–24.
14. Solomon SD, Zile M, Pieske B, Voors A, Shah A, Kraigher-Krainer E, et al. The angiotensin receptor neprilysin inhibitor LCZ696 in heart failure with preserved ejection fraction: a phase 2 double-blind randomised controlled trial. Lancet 2012;380:1387–95.
15. Lewis LK, Raudsepp SD, Yandle TG, Prickett TC, Richards AM. Development of a BNP1-32 immunoassay that does not cross-react with proBNP. Clin Chem 2017;63:1110–7.
16. Hawkridge AM, Heublein DM, Bergen H.R. 3rd, Cataliotti A, Burnett J.C. Jr., Muddiman DC. Quantitative mass spectral evidence for the absence of circulating brain natriuretic peptide (BNP-32) in severe human heart failure. Proc Natl Acad Sci U S 2005;102:17442–7.
17. Miller WL, Phelps MA, Wood CM, Schellenberger U, Van Le A, Perichon R, et al. Comparison of mass spectrometry and clinical assay measurements of circulating fragments of B-type natriuretic peptide in patients with chronic heart failure. Circ Heart Fail 2011;4:355–60.
18. Niederkofler EE, Kiernan UA, O'Rear J, Menon S, Saghir S, Protter AA, et al. Detection of endogenous B-type natriuretic peptide at very low concentrations in patients with heart failure. Circ Heart Fail 2008;1:258–64.
19. McMurray JJ, Packer M, Desai AS, Gong J, Lefkowitz MP, Rizkala AR, et al. Angiotensin-neprilysin inhibition versus enalapril in heart failure. N Engl J Med 2014;371:993–1004.
20. Packer M, McMurray JJ, Desai AS, Gong J, Lefkowitz MP, Rizkala AR, et al. Angiotensin receptor neprilysin inhibition compared with enalapril on the risk of clinical progression in surviving patients with heart failure. Circulation 2015;131:54–61.
21. Yancy CW, Januzzi J.L. Jr., Allen LA, Butler J, Davis LL, Fonarow GC, et al. 2017 ACC Expert Consensus Decision Pathway for Optimization of Heart Failure Treatment: answers to 10 pivotal issues about heart failure with reduced ejection fraction: a report of the American College of Cardiology Task Force on Expert Consensus Decision Pathways. J Am Coll Cardiol 2018;71:201–30.
22. Das SR, Drazner MH, Dries DL, Vega GL, Stanek HG, Abdullah SM, et al. Impact of body mass and body composition on circulating levels of natriuretic peptides: results from the Dallas Heart Study. Circulation 2005;112:2163–8.
23. McCord J, Mundy BJ, Hudson MP, Maisel AS, Hollander JE, Abraham WT, et al. Relationship between obesity and B-type natriuretic peptide levels. Arch Intern Med 2004;164:2247–52.
24. Wang TJ, Larson MG, Levy D, Benjamin EJ, Leip EP, Wilson PW, et al. Impact of obesity on plasma natriuretic peptide levels. Circulation 2004;109:594–600.
25. NATRECOR® (nesiritide) [package insert] 2007 Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2009/020920s023lbl.pdf.
26. Florkowski CM, Richards AM, Espiner EA, Yandle TG, Frampton C. Renal, endocrine, and hemodynamic interactions of atrial and brain natriuretic peptides in normal men. Am J Physiol 1994;266:R1244–50.
27. Hunt PJ, Espiner EA, Richards AM, Yandle TG, Frampton C, Nicholls MG. Interactions of atrial and brain natriuretic peptides at pathophysiological levels in normal men. Am J Physiol 1995;269:R1397–403.
28. Richards AM, Crozier IG, Holmes SJ, Espiner EA, Yandle TG, Frampton C. Brain natriuretic peptide: natriuretic and endocrine effects in essential hypertension. J Hypertens 1993;11:163–70.
29. Maisel AS, Clopton P, Krishnaswamy P, Nowak RM, McCord J, Hollander JE, et al. Impact of age, race, and sex on the ability of B-type natriuretic peptide to aid in the emergency diagnosis of heart failure: results from the Breathing Not Properly (BNP) multinational study. Am Heart J 2004;147:1078–84.
30. Redfield MM, Rodeheffer RJ, Jacobsen SJ, Mahoney DW, Bailey KR, Burnett J.C. Jr.. Plasma brain natriuretic peptide concentration: impact of age and gender. J Am Coll Cardiol 2002;40:976–82.
31. Wang TJ, Larson MG, Levy D, Leip EP, Benjamin EJ, Wilson PW, et al. Impact of age and sex on plasma natriuretic peptide levels in healthy adults. Am J Cardiol 2002;90:254–8.
32. Krim SR, Vivo RP, Krim NR, Qian F, Cox M, Ventura H, et al. Racial/ethnic differences in B-type natriuretic peptide levels and their association with care and outcomes among patients hospitalized with heart failure: findings from Get With The Guidelines-Heart Failure. JACC Heart Fail 2013;1:345–52.
33. Charles CJ, Espiner EA, Nicholls MG, Richards AM, Yandle TG, Protter A, et al. Clearance receptors and endopeptidase 24.11: equal role in natriuretic peptide metabolism in conscious sheep. Am J Physiol 1996;271:R373–80.
34. Dickey DM, Potter LR. ProBNP(1-108) is resistant to degradation and activates guanylyl cyclase-A with reduced potency. Clin Chem 2011;57:1272–8.
35. Myhre PL, Vaduganathan M, Claggett B, Packer M, Desai AS, Rouleau JL, et al. B-type natriuretic peptide during treatment with sacubitril/valsartan: the PARADIGM-HF trial. J Am Coll Cardiol 2019;73:1264–72.
36. Ibrahim NE, McCarthy CP, Shrestha S, Gaggin HK, Mukai R, Szymonifka J, et al. Effect of neprilysin inhibition on various natriuretic peptide assays. J Am Coll Cardiol 2019;73:1273–84.

ARTICLE IN PRESS

37. Nougue H, Pezel T, Picard F, Sadoune M, Arrigo M, Beauvais F, et al. Effects of sacubitril/valsartan on neprilysin targets and the metabolism of natriuretic peptides in chronic heart failure: a mechanistic clinical study. Eur J Heart Fail 2019;21:598–605.
38. Velazquez EJ, Morrow DA, DeVore AD, Duffy CI, Ambrosy AP, McCague K, et al. Angiotensin-neprilysin inhibition in acute decompensated heart failure. N Engl J Med 2019;380:539–48.
39. Vodovar N, Seronde MF, Laribi S, Gayat E, Lassus J, Boukef R, et al. Post-translational modifications enhance NT-proBNP and BNP production in acute decompensated heart failure. Eur Heart J 2014;35:3434–41.
40. Chen Y, Burnett J.C. Jr.. Biochemistry, therapeutics, and biomarker implications of neprilysin in cardiorenal disease. Clin Chem 2017;63:108–15.
41. Murphy SP, Prescott MF, Camacho A, Iyer SR, Maisel AS, Felker GM, et al. Atrial natriuretic peptide and treatment with sacubitril/valsartan in heart failure with reduced ejection fraction. JACC Heart Fail 2020;9:127–36.