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ORIGINAL RESEARCH

Plasma Neprilysin Displays No Relevant Association With Neurohumoral Activation in Chronic HFrEF

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BACKGROUND: Neprilysin is a transmembrane endopeptidase involved in the breakdown of a variety of vasoactive peptides and serves as a therapeutic target in heart failure with reduced ejection fraction (HFrEF). This study aimed to investigate the relationship of circulating neprilysin with neurohumoral activation and the impact of plasma neprilysin activity on prognosis in HFrEF.

METHODS AND RESULTS: A total of 369 chronic HFrEF patients were enrolled prospectively. Plasma neprilysin concentration and activity were determined by a specific ELISA and a fluorometric method. The association between plasma neprilysin and heart failure (HF) severity, neurohumoral activation, ie norepinephrine and absolute renin concentration, as well as all-cause mortality was assessed. Median plasma neprilysin concentrations and activity levels were 413 pg/mL (interquartile range 0–4111) and 2.36 nmol/mL per minute (interquartile range 1.16–4.59). No correlation could be shown between plasma neprilysin concentrations and activity (r_S=0.09, P=0.088). Plasma neprilysin activity correlated with HF severity reflected by New York Heart Association stage (P=0.003) and tertiles of N-terminal pro-B-type natriuretic peptide (P<0.001), whereas neprilysin concentrations did not (P=0.220; P=0.849). There was no relevant relationship between plasma neprilysin concentrations and activity, with neurohumoral activation reflected by absolute renin concentration (r_S=−0.02, P=0.648; r_S=0.03, P=0.574) or norepinephrine levels (r_S=−0.06, P=0.248; r_S=0.20, P<0.001). Neither circulating neprilysin concentrations nor activity were associated with outcome.

CONCLUSIONS: Plasma neprilysin concentrations and activity are not directly related to neurohumoral activation, indicating that neprilysin regulation is either more complex or not correctly mirrored by circulating neprilysin as a biomarker. Circulating neprilysin concentrations and activity were not associated with overall survival, implicating limited prognostic value of plasma neprilysin measurements in HFrEF patients.

Key Words: biomarker ■ heart failure ■ neprilysin ■ neprilysin activity

The pharmacological inhibition of the renin-angiotensin-system (RAS) and the sympathetic nervous system (SNS) has shown remarkable success in the treatment of heart failure with reduced ejection fraction (HFrEF) and thus been the mainstay of therapy in the past 30 years. Recently, the therapeutic strategy of neprilysin inhibition became the focus of interest because of the clinical benefits of the novel dual-acting angiotensin receptor-neprilysin inhibitor (ARNi) demonstrated in the PARADIGM-trial (Prospective Comparison of ARNi with Angiotensin-Converting Enzyme Inhibitor [ACEi] to Determine Impact on Global Mortality and Morbidity in Heart Failure).[1]

Neprilysin, also known as enkephalinase, cluster of differentiation 10 (CD10) or EC3.4.24.11, is a widely

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CLINICAL PERSPECTIVE

What Is New?

- Our data could not confirm any association of plasma neprilysin concentrations with heart failure severity or prognosis; similarly, plasma neprilysin activity was not related to outcomes, but based on a weak association with heart failure severity it may inhere some prognostic information.
- This report provides the first data on the relationship between neurohumoral activation and neprilysin regulation in heart failure with reduced ejection fraction.
- Circulating neprilysin shows no meaningful association with the renin-angiotensin-system and the sympathetic nervous system, suggesting either an independent regulation of neprilysin or that circulating neprilysin is not a good surrogate for true in vivo neprilysin actions.

What Are the Clinical Implications?

- The lack of correlation between neprilysin regulation and neurohumoral activation might be part of the explanation why neprilysin inhibition is effective on top of renin-angiotensin-system and sympathetic nervous system blockade in heart failure with reduced ejection fraction.
- At the same time the data add another piece of evidence that plasma neprilysin concentrations may not be useful as biomarkers in heart failure with reduced ejection fraction.
- Plasma neprilysin activity may inhere some prognostic information and might be tested in larger cohorts. A better understanding of neprilysin regulation in heart failure with reduced ejection fraction would provide further insights into HF disease mechanisms and might enable further risk stratification and therapy.

Nonstandard Abbreviations and Acronyms

ACEi	angiotensin-converting enzyme inhibitor
ALT	alanine aminotransferase
ANP	atrial natriuretic peptide
AP	alkaline phosphatase
ARB	angiotensin II receptor blocker
ARC	absolute renin concentration
ARNi	angiotensin receptor-neprilysin inhibitor
AST	aspartate aminotransferase
BMI	body mass index
BNP	B-type natriuretic peptide
CD10	cluster of differentiation 10
CNP	C-type natriuretic peptide
EDTA	ethylenediaminetetraacetic acid
GGT	gamma glutamyl transferase
HF	heart failure
HFrEF	heart failure with reduced ejection fraction
HR	hazard ratio
IQR	interquartile range
LC-MS/MS	liquid chromatography-tandem mass spectrometry
NT-proBNP	N-terminal pro-B-type natriuretic peptide
NYHA	New York Heart Association
PBS	phosphate-buffered saline
RAS	renin-angiotensin-system
SNS	sympathetic nervous system

expressed transmembrane zinc-dependent endopeptidase located on the cell surface of neutrophils, endothelial cells, and fibroblasts and in various tissues including the kidneys, brain, testes, lungs, heart, and gastrointestinal tract.[2] In addition to the tissue-based form, neprilysin is also detectable as a soluble nonmembrane-bound form in the blood, where it probably retains activity.[3] Neprilysin catalyzes the degradation of various vasodilators including adrenomedullin and the atrial (ANP), B-type (BNP), and C-type (CNP) natriuretic peptides and potent vasoconstrictors such as angiotensin (Ang) II and endothelin-1.[2] A net beneficial effect on the homeostasis of related hormones has been hypothesized as a mechanism of action of neprilysin inhibition in HFrEF. A just published study indeed indicated that treatment with sacubitril/valsartan results in strongly reduced plasma neprilysin activity accompanied by increased bioavailability of neprilysin substrates regulating vasoactivity.[4] Neurohumoral activation is strongly implicated in balancing the vasoactive state as both the RAS and the SNS regulate blood pressure and organ perfusion.[5,6] Renin is a primarily circulating enzyme catalyzing the rate-limiting step of the RAS cascade, which involves the formation of AngII, the primary effector of RAS with strong vasoconstrictive properties.[7] Norepinephrine is one of the main effector neurotransmitters of the SNS and equally plays a central role in vasoregulation. Elevated levels of renin and norepinephrine are characteristic for neurohumoral activation and are associated with worse prognosis in HFrEF.[8] Although neprilysin could be linked to neurohumoral activation by (1) reflecting heart failure (HF) disease severity, (2) regulating vasoactive state in

concert with the RAS and SNS, or because of (3) direct involvement in the metabolism of AngII, the main effector peptide of RAS, no data on the relationship between plasma neprilysin measures and neurohumoral dysregulation exist.

Regarding neprilysin as a potential biomarker, several studies have investigated the possible role of circulating neprilysin concentrations and neprilysin activity in HF. In chronic HF, elevated plasma neprilysin levels were associated with poor prognosis.[9] In contrast, no prognostic value of plasma neprilysin concentrations could be observed in a cohort of HF patients with preserved ejection fraction.[10] In a mixed cohort of stable and acutely decompensated patients, BNP levels inversely correlated with plasma neprilysin activity but not with plasma neprilysin concentrations.[11] Also, no correlation between plasma neprilysin concentrations and activity has been found, whereas a direct correlation could be shown elsewhere using a different assay for detecting circulating neprilysin.[3] In contrast to plasma neprilysin concentrations, the prognostic impact of plasma neprilysin activity in HFrEF has not been described yet.

This study aimed to investigate plasma neprilysin concentrations and activity in a large cohort of chronic stable HFrEF patients under optimal guideline-directed therapy in order to elucidate the relationship of circulating neprilysin with neurohumoral dysregulation (ie, activation of the RAS and the SNS), and to further characterize the prognostic utility of circulating neprilysin.

METHODS

The data supporting the findings of this study can be made available from the corresponding author upon reasonable request.

Study Population

Between February 2016 and November 2018, consecutive outpatients with chronic stable HFrEF were enrolled in a prospective registry at the Medical University of Vienna, a university-affiliated tertiary care center. Comorbidities, medical therapy including RAS-inhibitors, New York Heart Association (NYHA) functional class, HF cause, and follow-up data were recorded. All-cause mortality was defined as the primary study outcome measure. For validation of the neprilysin activity measurement, 20 additional patients were recruited from the same registry. Inclusion criteria were age >18 years and chronic stable HFrEF. The diagnosis of HFrEF was made according to the European Society of Cardiology guidelines. Written informed consent was obtained by all participating patients. The study was approved by the local Ethics Committee and performed according to the current revision of the Helsinki Declaration.

Sampling and Routine Local Laboratory Analysis

Blood samples were obtained from the cubital vein at study inclusion. Routine laboratory markers including NT-proBNP (N-terminal pro-B-type natriuretic peptide) were assessed according to the local laboratory standards. Additional blood samples were immediately centrifuged, transferred into cryotubes, and frozen at −80°C until use.

Measurement of Renin and Norepinephrine

Active renin concentration (ARC) was assessed according to the local laboratory standards. Norepinephrine was determined in 100 µL ethylenediaminetetraacetic acid (EDTA) samples by a specific ELISA (KA3836, Abnova, Taipei, Taiwan) according to the manual of the manufacturer. Intra-assay coefficient of variation of the same matrix is indicated between 8.4% and 15.6% for concentrations between 33 and 1377 pg/mL by the manufacturer.

Measurement of Plasma Neprilysin Concentrations and Plasma Neprilysin Activity

Plasma neprilysin concentrations were measured with a specific ELISA from EDTA plasma samples (DY1182, R&D Systems, Minneapolis MN, USA) according to the manufacturer's instructions. For the measurements, plasma samples were diluted 1:2 or 1:20 in phosphate-buffered saline (PBS) (D8537, Sigma-Aldrich, St. Louis, MO, USA) and final extinction was assessed using the Victor3 multilabel plate reader (PerkinElmer, Waltham, MA, USA) at 450 nm. The intra-assay and inter-assay coefficients of variation as well as linearity for the method were reported elsewhere.[12]

For plasma neprilysin activity measurements, a noncommercial fluorometric method was performed as previously described.[13] Heparin or serum samples were diluted with PBS in a 1:20 concentration. Forty microliters of the diluted sample was mixed with 20 µL of 0.1 mol/L Tris/HCl buffer (for active wells) or with 20 µL of 0.1 mmol/L phosphoramidon solution (for control wells) and the reaction was started by adding 100 µL of 1 mmol/L substrate solution. After 60 minutes of incubation at 37°C, 20 µL of 0.1 mmol/L phosphoramidon and 20 µL of AminopeptidaseM solution (4 units/well) were added. Fluorescence was read after another 60 minutes of incubation at 37°C at 460 nm with excitation at 355 nm on a Victor3 multilabel plate reader.

In 20 patients, measurement of plasma neprilysin activity was performed using both the above-described method and liquid chromatography-tandem mass spectrometry (LC-MS/MS) as validation. Here, the enzymatic activity of neprilysin was determined in heparin plasma by a LC-MS/MS-based Ang1-7 formation assay using the natural substrate Ang1-10. Alternative Ang1-10 metabolizing enzymes were blocked using a proprietary inhibitor cocktail (Attoquant Diagnostics, Vienna, Austria), assuring stability of the substrate and the product in the plasma sample, without affecting neprilysin activity. The linear range of the neprilysin activity assay was determined by adding different concentrations of recombinant human neprilysin (R&D Systems) to a pool of plasma samples, which was analyzed in the absence and presence of 100 μM of the neprilysin inhibitor DL-thiorphan (Sigma Aldrich). Following an Ang1-7 generation period of 60 minutes at 37°C, all samples were stabilized and concentrations of Ang1-7 were determined by LC-MS/MS-based angiotensin quantification as previously described.[14] The neprilysin specific Ang1-7 formation rate in the reaction mix was expressed in ([ng Ang1-7/mL]/h).

Statistical Analysis

All statistical analyses were performed using SPSS software (IBM, Armonk, NY, USA) version 24. Descriptive statistics were expressed as median and interquartile ranges (IQR) for continuous parameters and as percentages and counts for categorical variables, respectively. To assess the relation between plasma neprilysin activity measurements by the fluorometric and mass-spectrometry–based method, linear regression analysis was performed. The distributions of neprilysin concentrations and activity were tested for normality by the Shapiro–Wilk test after lg-transformation. The correlation between plasma neprilysin concentrations and activity and various clinical parameters was assessed by calculating the Spearman rank correlation coefficient. Plasma neprilysin concentrations and activity for different NYHA class, tertiles of NT-proBNP, as well as HF cause and modality of RAS-blockade were compared using the Kruskal-Wallis and Mann–Whitney *U* test. To assess the association between neurohumoral activation and neprilysin, the Spearman rank correlation coefficient was calculated for the relationship between plasma neprilysin concentrations and activity with ARC and norepinephrine. Cox proportional hazard regression analysis was used to evaluate the effect of plasma neprilysin concentrations and activity on all-cause mortality. Results are presented as hazard ratios (HR) per IQR. All tests were 2-sided and a $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Baseline Characteristics

A total of 369 patients were included in the study. Table shows the detailed baseline characteristics of the study population. Median age was 64 (IQR 53–73) years, 75% of patients were male. Forty-four percent (n=163) were in NYHA functional class II and 37% (n=136) in NYHA III. Approximately half of the study population with 173 (46.9%) patients had a nonischemic cause of HF. HF medications were well established with 95%, 93%, and 70% of patients receiving RAS-blockade, β-blockers, and mineralocorticoid receptor antagonist therapy, respectively. Two hundred twenty-four patients (61%) received ACEi, 100 (27%) angiotensin-receptor blocker (ARB), and 28 (8%) ARNi. Median plasma ARC and norepinephrine levels were 153 μIE/mL (IQR 28.7–558.2) and 1062 pg/mL (IQR 794–1399) for the total cohort, respectively.

Validation of the Fluorometric Method for the Determination of Plasma Neprilysin Activity

For the 20 HFrEF patients, neprilysin activity was 0.493 nmol/mL per minute (IQR 0.234–0.997) when measured by the noncommercial fluorometric assay. Plasma neprilysin activity performed by the mass spectrometry-based assay resulted in a turnover rate of a substrate of 38.65 ng (Ang1-7)/mL/h (IQR 20.95–149.25). Figure S1 shows a scatter plot and linear regression analysis for plasma neprilysin activity determined by both methods revealing an excellent correlation (r_s=0.85, P<0.001).

Plasma Neprilysin Concentrations and Plasma Neprilysin Activity in Stable HFrEF Patients

Median plasma neprilysin levels were 413 pg/mL ([IQR 0–4111], range [0–200000]). Plasma neprilysin concentrations showed a relatively wide non-normal distribution, whereas in 132 (36%) cases plasma neprilysin concentrations were under the detection limit and 18 (5%) patients had concentrations >200000 pg/mL. After exclusion of the 18 patients with highest values, plasma neprilysin concentrations showed a lg-normal distribution (P=0.427). Median plasma neprilysin activity was 2.36 nmol/mL per minute ([IQR 1.16–4.59], range [0.00–34.85]) for the total cohort. Like plasma neprilysin concentrations, plasma neprilysin activity showed a lg-normal distribution (P=0.065). There was no significant correlation between plasma neprilysin concentrations and plasma neprilysin activity (r_s=0.09, P=0.088).

Table. Baseline Characteristics for the HFrEF Cohort

Baseline Characteristics	Total Study Population (n=369)
Age (IQR)	64 (53–73)
Male sex, n (%)	275 (74.5)
BMI, kg/m^2 (IQR)	27 (24–31)
Systolic blood pressure, mmHg (IQR)	126 (110–141)
Diastolic blood pressure, mmHg (IQR)	80 (70–85)
Heart rate, beats/min (IQR)	70 (62–81)
NYHA functional class	
NYHA I, n (%)	70 (19.0)
NYHA II, n (%)	163 (44.2)
NYHA III, n (%)	136 (36.9)
Comorbidities	
Nonischemic cause of HF, n (%)	173 (46.9)
Hypertension, n (%)	148 (40.1)
Type 2 diabetes mellitus, n (%)	116 (31.4)
Atrial fibrillation, n (%)	86 (23.3)
Laboratory parameters	
Hemoglobin, g/dL (IQR)	13.5 (12.2–14.7)
Serum creatinine, mg/dL (IQR)	1.2 (0.9–1.6)
Blood urea nitrogen, mg/dL (IQR)	23.4 (17.5–33.0)
Total cholesterol, mg/dL (IQR)	169 (136–196)
C-reactive protein, mg/dL (IQR)	0.3 (0.1–0.8)
ALT, U/l (IQR)	24 (17–34)
AST, U/l (IQR)	25 (21–32)
Total bilirubin, mg/dL (IQR)	0.6 (0.4–0.9)
GGT, U/l (IQR)	27 (50–108)
AP, U/l (IQR)	74 (58–102)
BChE, U/l (IQR)	6.9 (5.6–8.3)
Neurohormones	
NT-proBNP, pg/mL (IQR)	1937 (850–4153)
ARC, µIE/mL (IQR)	153 (28.7–558.2)
NE, pg/mL (IQR)	1062 (794–1399)
Medication	
Beta-blocker, n (%)	342 (92.7)
Diuretics, n (%)	170 (46.1)
Mineralocorticoid antagonist, n (%)	259 (70.2)
I_f inhibitor, n (%)	23 (6.2)
ACEi/ARB/ARNi, n (%)	224/100/28 (60.7/27.1/7.6)
Dose equivalent, ≥50%	154/70/20 (68.8/70.0/71.4)

Continuous variables are given as medians and interquartile ranges (IQR), counts are given as numbers and percentages. ACEi indicates angiotensin-converting enzyme inhibitor; ALT, alanine aminotransferase; AP, alkaline phosphatase; ARB, angiotensin II receptor blocker; ARC, active renin concentration; ARNi, angiotensin receptor-neprilysin inhibitor; AST, aspartate aminotransferase; BChE, butyrylcholinesterase; BMI, body mass index; GGT, gamma glutamyl transferase; HF, heart failure; HFrEF, heart failure with reduced ejection fraction; IQR, interquartile range; NE, norepinephrine; NT-proBNP, N-terminal pro-B-type-natriuretic peptide; NYHA, New York Heart Association.

Correlation of Circulating Neprilysin With Clinical and Laboratory Parameters

Plasma neprilysin concentrations and activity were not associated with age, systolic blood pressure, or body mass index (BMI) (*P*=ns for all). Similarly, no correlation of plasma neprilysin concentrations and activity could be observed with hemoglobin, sodium, albumin, C-reactive protein, triglycerides, and total cholesterol levels (*P*=ns for all). Plasma neprilysin activity, but not concentrations, showed a modest correlation with kidney functional parameters as creatinine and urea (r_s=0.12, *P*=0.033 and r_s=0.15, *P*=0.005). Interestingly, plasma neprilysin activity correlated meaningfully and highly significantly with liver functional parameters (alkaline phosphatase: r_s=0.32, *P*<0.001; gamma glutamyl transferase [GGT]: r_s=0.72, *P*<0.001; aspartate aminotransferase [AST]: r_s=0.40, *P*<0.001; alanine aminotransferase [ALT]: r_s=0.41, *P*<0.001; bilirubin: r_s=0.15, *P*=0.004; butyrylcholinesterase: r_s=−0.12, *P*=0.031). Plasma neprilysin concentrations showed only weak relationship to some of the liver enzymes as AST, ALT, and GGT (r_s=0.17, *P*=0.001; r_s=0.12, *P*=0.033; r_s=0.11, *P*=0.044).

Association of Circulating Neprilysin With HF Severity, HF Cause, and Modality of RAS-Blockade

Figure 1 shows the association of circulating neprilysin concentrations and neprilysin activity with HF severity, HF cause, and RAS-inhibitor therapy. Plasma neprilysin activity increased with HF severity reflected by NYHA stage (*P*=0.003) and tertiles of NT-proBNP (*P*<0.001), whereas no difference could be shown for circulating neprilysin concentrations (*P*=0.220; *P*=0.849). There was no difference in neprilysin concentrations or activity between ischemic and nonischemic cause of HF. ARNi patients showed reduced levels of plasma neprilysin activity compared with patients receiving ACEi or ARB (ARNi: 0.80 nmol/mL per minute [IQR 0.38–1.84], ACEi: 2.55 nmol/mL per minute [IQR 1.44–5.10], ARB: 2.54 nmol/mL per minute [IQR 1.22–4.61]; *P*<0.001 for comparison between all groups). No differences could be observed for plasma neprilysin concentrations (*P*=0.351).

Relationship of Circulating Neprilysin With Neurohumoral Dysregulation

Figure 2 displays the relationship between plasma neprilysin concentrations and activity with neurohumoral dysregulation (ie, circulating ARC and norepinephrine concentrations). Neither plasma neprilysin concentrations nor neprilysin activity correlated with RAS-activation reflected by ARC (r_s=−0.02, *P*=0.648

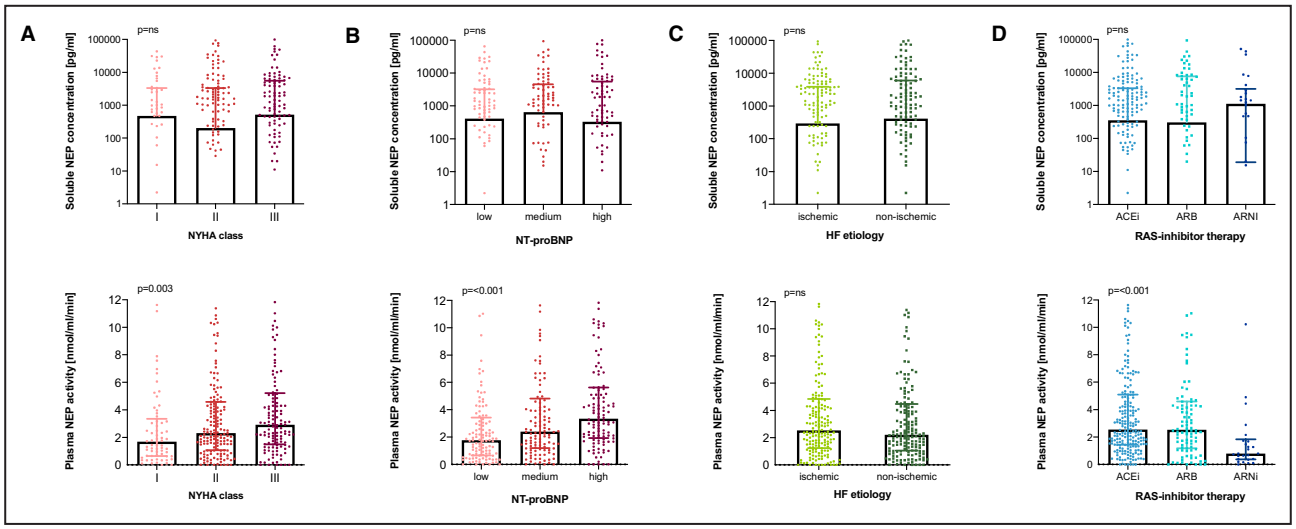

Figure 1. Relationship of plasma NEP concentrations and activity with HF severity, cause, and therapy mode.
Individual values as well as median and IQR are shown for (**A**) NYHA class, (**B**) tertiles of NT-proBNP, (**C**) HF cause (ischemic vs nonischemic), and (**D**) RAS-inhibitor therapy. Comparison between groups has been assessed by using the Kruskal–Wallis test, and levels of significance are indicated in the respective plots. Regarding soluble NEP concentrations, out of range individual values of 200 000 pg/mL and, because of logarithmic transformation, 125 values with 0 pg/mL are not displayed in the graphs. ACEi indicates angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker; ARNi, angiotensin-receptor neprilysin inhibitor; HF, heart failure; IQR, interquartile range; NEP, neprilysin; NT-proBNP, N-terminal pro-B-type natriuretic peptide; NYHA, New York Heart Association; and RAS, renin-angiotensin-system.

for plasma neprilysin concentrations and r_S=0.03, P=0.574 for plasma neprilysin activity). Similarly, there was no significant association between plasma neprilysin concentrations and the activation of the SNS reflected by plasma norepinephrine levels (r_S=–0.06, P=0.248); however, a weak association of plasma neprilysin activity with norepinephrine could be observed (r_S=0.20, P<0.001).

Association of Plasma Neprilysin Concentrations and Activity With Outcome

During the median follow-up period of 23 (IQR 12–31) months, 78 (21%) patients died. Univariate Cox-regression analysis did not reveal an association with the primary endpoint neither for plasma neprilysin concentrations nor plasma neprilysin activity in the total cohort (crude HR, 1.00 [95% CI, 0.99–1.02], P=0.692 for plasma neprilysin concentrations and crude HR, 1.08 [95% CI, 0.94–1.23], P=0.283 for plasma neprilysin activity).

DISCUSSION

This is the first study to specifically investigate the association of circulating neprilysin with neurohumoral activation in a reasonably large cohort of stable HFrEF outpatients. Additionally, this is the first description of plasma neprilysin concentrations in HFrEF using the R&D Systems kit in a larger HFrEF cohort and the impact of plasma neprilysin activity on survival. Interestingly, although neprilysin is known to be involved in the breakdown of the most potent vasoactive mediators, there was no relevant relationship with neurohumoral systems regulating vasoactivity (ie, activation of the RAS and the SNS), which represent the main targets of HF therapy. Although increased plasma neprilysin activity seems to be related to a more severe disease state, no clear association with outcome could be shown either for plasma neprilysin concentrations or for plasma neprilysin activity.

Biology of Neprilysin

Neprilysin is a zinc-dependent oligopeptidase with ubiquitous expression in various tissues including the kidney, liver, lung, fat, brain, heart, and bone marrow.[15,16] As an ectoenzyme, neprilysin is anchored to the plasma membrane with its catalytic site exposed to the extracellular surface.[17] Neprilysin plays a key role in the degradation of many potent biological mediators such as enkephalins, the B chain of insulin, chemotactic peptide, interleukin-l-beta, substance P, bradykinin, neurotensin, oxytocin, Ang I and II, bombesin-like peptides, amyloid-beta peptide, glucagon-like peptide-1, endothelin-1, and natriuretic peptides.[2] Functional relevance for the breakdown of these substrates in vivo, however, is difficult to predict. In malignant disease, tissue neprilysin expression seems to be involved in tumor development

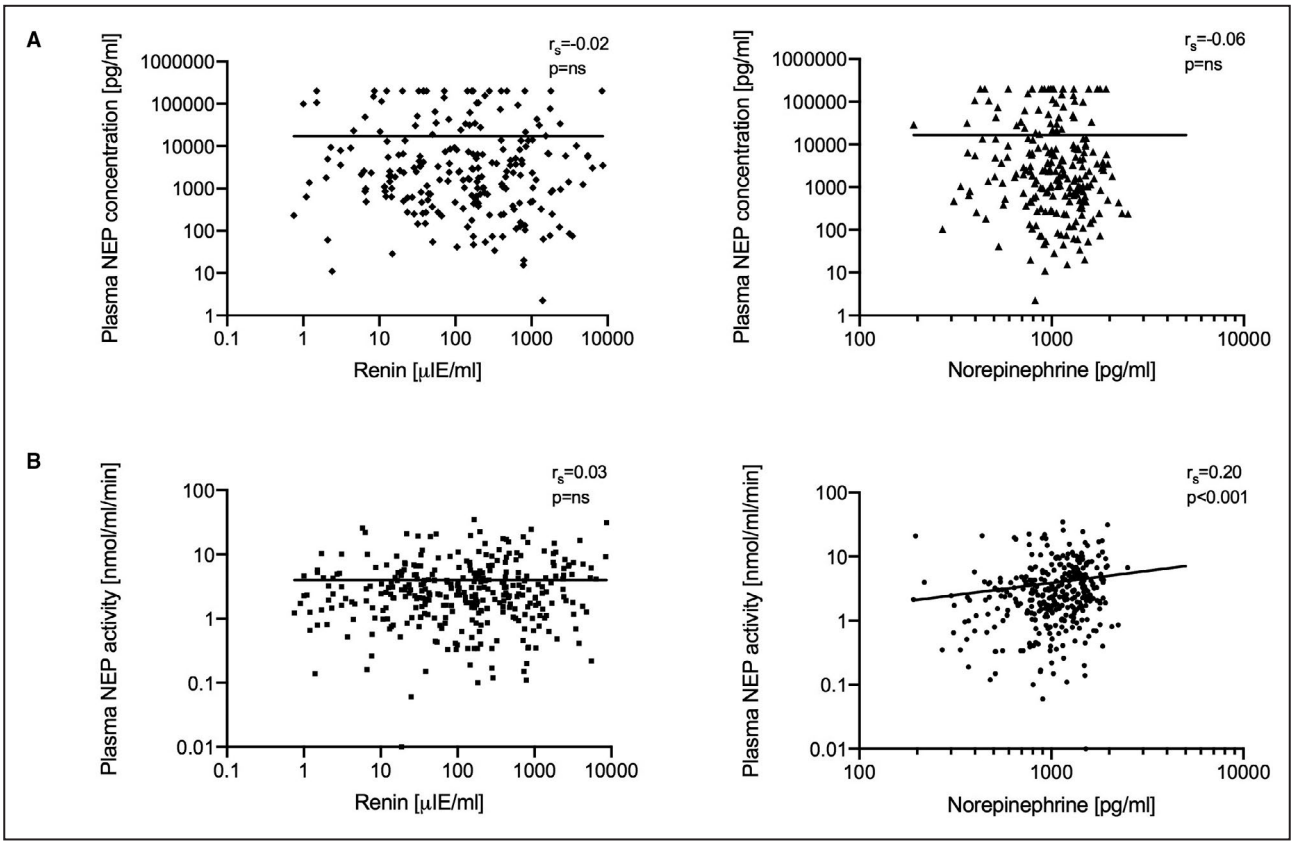

Figure 2. Relationship of circulating neprilysin (NEP) with neurohumoral dysregulation.
Scatter plots for (**A**) plasma NEP concentrations and (**B**) plasma NEP activity with absolute renin concentration and norepinephrine are shown. Linear regression analysis was performed and log-log-line is displayed. Spearman-Rhos correlation coefficient and level of significance are indicated in the respective plots.

and progression,[18] whereas CD10 expression on peripheral blood cells enables diagnosis and is linked to prognosis in acute lymphoblastic leukemia.[19] Neprilysin-deficient mice appear to be more susceptible to septic shock, hypoxic pulmonary vascular remodeling, and hypertension,[20,21] and are more likely to develop late-onset obesity and Alzheimer disease by accumulation of beta-amyloid peptides.[22,23] Besides the membrane-associated form, neprilysin, as other metallopeptidases, can be released into the extracellular space by ectodomain shedding and bound on exosomes.[24,25] Nonmembrane-associated plasma neprilysin concentrations have been described in multiple cohorts with various diseases, and neprilysin activity has indeed been detected in several body fluids such as plasma, urine, and cerebrospinal fluid.[26,27]

Association of Plasma Neprilysin With Neurohumoral Activation

In HF, the unfavorable overactivation of the neurohumoral axis is characterized by increased plasma concentrations of endogenous vasoconstrictors including AngII and norepinephrine.[28] Plasma renin concentration represents a good surrogate for RAS-activation directly correlating with circulating AngI and AngII metabolite levels.[14] Similarly, plasma norepinephrine concentrations have been shown to relate to the degree of HF severity and reflect SNS activity.[8] Besides its vasoconstrictive properties, AngII mediates numerous detrimental effects including inflammation and fibrosis via stimulation of AT_1 receptors.[29,30] Norepinephrine levels have been linked to direct cardiotoxic effects characterized by increased myocyte apoptosis, growth, and hypertrophy.[31,32] In fact, elevated levels of plasma renin and norepinephrine were found to predict worse outcome in HF patients.[8]

The development of the dual-acting ARNi and the marked clinical benefits attributable to neprilysin inhibition have prompted the interest in the role of neprilysin status in patients with HFrEF. Neprilysin is believed to be critically involved in vasoactive homeostasis cleaving a broad range of substrates including the strongest vasoactive mediators.[2] It has been hypothesized that neprilysin inhibition may oppose the maladaptive neurohumoral activation by

augmentation of ANP and BNP exerting beneficial effects. Neprilysin inhibition with sacubitril/valsartan has been shown to increase the bioavailability not only for ANP and BNP but also for adrenomedullin.[4] In contrast, infusion of the neprilysin inhibitor candoxatril resulted in enhanced RAS and SNS activity, reflected by increased plasma levels of AngII, aldosterone, and norepinephrine.[33] This is consistent with in vitro data showing that neprilysin is directly involved in the breakdown of AngII,[2] which is known to potentiate SNS activity supposedly acting through facilitation of norepinephrine release.[34]

Although it can be assumed that neprilysin is strongly related to the neurohumoral and vasoactive system because of one of the following: (1) neprilysin correlates with neurohumoral activation as a function of HF disease severity, (2) regulation of the neprilysin system is interrelated with RAS and SNS activity because neprilysin substrates involve potent vasoactive mediators, and⁄or because (3) neprilysin is directly involved in the metabolism of AngII, the main effector peptide of RAS, there are no data on the association of neprilysin with neurohumoral dysregulation.

Interestingly, the data from our study do not support a relevant association between neprilysin and neurohumoral activation. It is possible that plasma neprilysin measures are not appropriately reflecting systemic neprilysin actions. The results might also suggest that neprilysin regulation and its relationship to the RAS and the SNS is more complex than initially thought and that neprilysin regulation functions rather independently. This could be an explanation for the marked clinical benefit of neprilysin inhibition in addition to guideline-directed therapy including optimal blockade of the RAS and the SNS.

Plasma Neprilysin Concentrations

The predictive value of circulating neprilysin concentrations has been discussed controversially.[9,10,12,35–38] This might be partly explained by the alarmingly high lack of correlation between the currently available immunoassays for the detection of plasma neprilysin.[39] The commercially available ELISAs mostly use polyclonal antibodies and target different epitopes; thus, a variety of different neprilysin fragments might be recognized, resulting in obviously limited comparability. A recently published study compared the performance of the Aviscera Bioscience kit (Santa Clara, CA, USA) and the kit from R&D Systems used in this study.[38] The authors observed a good correlation (r=0.831, $P<0.0001$) between the 2 assays and described a better inter- and intra-assay variability for the assay by R&D Systems. Additionally, a greater sensitivity for higher concentrations has been described for the R&D Systems kit. Our data revealed a wide range of plasma neprilysin concentrations with values not detected in 36% of the samples. To our knowledge, there is only 1 report by Zaidi et al[40] using the same assay for the measurement of plasma neprilysin concentrations in HFrEF patients. In this pilot study, the concentration of circulating neprilysin was higher in patients with HFrEF than in controls, and neprilysin levels seemed to increase with HF severity. However, no information was given about median concentrations. On the contrary, Vodovar et al[11] reported higher levels in chronic HF patients compared with patients with acute HF (352 pg/mL [IQR 325–380] versus 314 pg/mL [IQR 257–377], $P=0.012$) measured by another assay (USCN Life Science, Wuhan, China). A study by Nougue et al[4] observed similar plasma neprilysin levels in HFrEF patients (241 pg/mL [IQR 205–303]) using the same detection assay. A further report, using the Aviscera Bioscience kit reported substantially higher concentrations of plasma neprilysin in chronic HFrEF patients (0.642 ng/mL [IQR 0.385–1.219]).[9] Here, values for plasma neprilysin were below the detection limit in ≈15% of patients. The data gathered so far report rather differing ranges of plasma neprilysin concentrations, thus not allowing the true estimation of plasma neprilysin levels in HFrEF. The median plasma neprilysin levels of 413 pg/mL (IQR 0–4111) of this study, however, seem to lie within the range of these values.

In our study, neprilysin concentrations were associated with neither HF severity or cause nor with the modality of RAS-inhibition. Moreover, plasma neprilysin concentrations were not related to survival. A lack of correlation between plasma neprilysin concentrations, BNP, and NT-proBNP has also been reported in previous studies.[9,11,37] Plasma neprilysin was similarly not related to NYHA class, but was reportedly higher in nonischemic versus ischemic cause of HF.[9] To date, 2 studies have described the predictive value of plasma neprilysin concentrations in a reasonably large cohort of chronic HFrEF patients.[9,35] Here, plasma neprilysin concentrations were a risk factor for cardiovascular mortality independent from NT-proBNP; however, no significant association could be proven with all-cause mortality. In concordance with our data, in an unselected community-based cohort, no relationship among BMI, age, and plasma neprilysin concentrations was observed.[38]

Plasma neprilysin Activity

Plasma neprilysin activity is, by its virtue, a probably superior biomarker for systemic neprilysin regulation than mere concentrations of neprilysin. The detection of plasma neprilysin activity is not biased by shed nonfunctional neprilysin fragments, and the quantification of neprilysin activity, although more elaborate

than plasma neprilysin concentrations, is not inherent for the difficulties of immunoassay measurements as discussed above. Thus, the measurement of plasma neprilysin activity might be a better candidate for risk stratification and guiding pharmacological therapy in HF in terms of personalized medicine. Plasma neprilysin activities have only been measured in a few studies.[3,4,11,37] In patients with chronic kidney disease, plasma neprilysin activity was a risk factor for HF hospitalizations.[37] To our knowledge, the prognostic value of plasma neprilysin activity in HFrEF patients has not been reported yet.

For this study, the measurement of plasma neprilysin activity was performed using a fluorometric assay, which has been developed and described first by Yandle et al[13] This method revealed an excellent correlation with the more sensitive and probably more reliable but costly mass spectrometry–based method.

In our data, plasma neprilysin activity was comparable between ischemic and nonischemic HF cause and reduced in patients receiving ARNi compared with ACEi/ARB. The findings regarding plasma neprilysin activity under different RAS-inhibitor therapies are in concordance with a recently published study by Nougue et al.[4] This study described a decrease of plasma neprilysin activity in 73 chronic HF patients undergoing conversion from ACEi/ARB-based to ARNi-based treatment, while plasma neprilysin concentration remained unaffected.

Moreover, neprilysin activity tended to increase with HF severity, reflected by NT-proBNP and NYHA class, but no association with overall survival could be observed. Since NT-proBNP and NYHA class are strong prognostic surrogate markers, it cannot be excluded that neprilysin activity may reveal an association with outcome in a larger cohort of HFrEF patients. Notably, Vodovar et al[11] reported an inverse relationship between plasma neprilysin activity and immunoreactive BNP (BNP and proBNP) in HF patients and subsequently suggested an in vitro inhibitory effect of BNP and proBNP on neprilysin activity. BNP is a substrate for neprilysin and the results imply that proBNP may similarly enter the catalytic site. However, there are no data in regard to NT-proBNP, which probably does not affect neprilysin activity directly, and similarly there are hints that changing neprilysin activity might alter the BNP/NT-proBNP ratio, at least following ARNi initiation.[4]

Circulating Neprilysin as a Biomarker?

The rationale for a possible association between plasma neprilysin levels and HF outcomes is based on the assumption that HF is the leading pathophysiologic condition with most prominent influence on neprilysin regulation and the fact that neprilysin inhibition has shown impressive clinical benefits in HF patients.[1] Additionally, plasma neprilysin measures need to be a good surrogate for tissue neprilysin actions. Most data revealing an association between neprilysin regulation and relevant non-HF pathologies have investigated tissue-based neprilysin by expression data or have looked at neprilysin knockdown, which inevitably prohibits tissue neprilysin expression.[19–23,41,42] In clinical HF, plasma neprilysin measures seem attractive as biomarkers: If their utility could be shown for stratification of disease states or outcomes, it would not only extend our understanding of the disease but also of the mechanism of action of ARNi and may eventually be used for directing personalized medicine. However, the determination of circulating neprilysin concentrations turned out to be an analytical challenge, and there is no evidence that plasma neprilysin activity reflects tissue-neprilysin actions. Prior research on the relationship between neprilysin concentration and activity has revealed conflicting results,[3,11,37,43] and it may not be surprising that plasma neprilysin concentrations are not associated with plasma neprilysin activity in our study, as suggested by others.[11,37] The neutral findings of this study and other data regarding the association of plasma neprilysin with HF severity and outcomes underscore the assumption that circulating neprilysin measures may either not reflect systemic neprilysin regulation or that neprilysin regulation is more complex in the condition of HF. Whether the determination of membrane-associated neprilysin measures perform better must be investigated in future studies.

Neprilysin and Liver Function

Our results revealed a strong association between plasma neprilysin and liver function, showing a worsening of liver parameters with increasing plasma neprilysin activity. The relationship between neprilysin and liver failure has been established by a few experimental studies in rodents. A study by Klein et al[44] reported reduced signs of liver fibrosis in neprilysin knockout-mice. Sansoè et al demonstrated an increased neprilysin content in both liver and renal tissue from animals with liver cirrhosis.[45,46] Moreover, intravenous administration of the neprilysin inhibitor candoxatrilat led to reduced portal pressure in rats with cirrhotic livers, suggesting that neprilysin might represent a therapeutic target for portal hypertension.[45] Although further data are required to clarify the pathophysiologic relationship, it seems likely that neprilysin might affect the intrahepatic vascular resistance and fibrosis, both phenomena that occur in advanced stages of HF. The highly significant positive correlation of plasma neprilysin activity and GGT, AST, and ALT found in our study support the idea of a significant role of neprilysin in progressive liver failure in HFrEF.

Limitations

We acknowledge several limitations in this study. First, it must be considered that 95% and 93% of the selected study population were treated with RAS inhibitors and beta-blockers, respectively, which thereby might alter the neurohumoral profile and may confound possible associations. In the meantime, the study investigates a well-treated real-world HFrEF cohort. Second, this study was set up in a single tertiary care center, which may limit generalization. However, the potential benefits from a single-center setting approach are the homogeneous nature of the patient cohort and the continuity in clinical routine. Third, we did not investigate secondary cardiovascular end points, which could have revealed additional information about the prognostic value of circulating neprilysin. Fourth, the quantification of catecholamines in biological systems is challenging. Since norepinephrine outflow varies among tissues and organs, blood samples taken from the forearm may not reflect overall sympathetic activity. However, enzymatic immunoassay measurement of norepinephrine represents a well-validated method that shows good correlation with highly sensitive and selective chromatographic analytical techniques.[47] Lastly, it has to be noted that multiple comparisons increase the probability of committing a type I error.

CONCLUSIONS

In conclusion, although there is evidence indicating that neprilysin interferes with the RAS and the SNS, we did not detect a direct relationship in HFrEF patients. Despite previous findings describing a prognostic value for circulating neprilysin, no such association could be confirmed in this study. Neprilysin activity may increase with HF failure severity, reflected by NYHA stage and NT-proBNP levels.

ARTICLE INFORMATION

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Disclosures

None.

Supplementary Material

Figure S1

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SUPPLEMENTAL MATERIAL

Figure S1. Comparison of different methods for the determination of plasma NEP activity (n=20).