Multimarker strategy for heart failure prognostication. Value of neurohormonal biomarkers: Neprilysin vs. NTproBNP

Estrategia multimarcador para estratificar el pronóstico en insuficiencia cardíaca. Valor de los marcadores neurohumorales: Neprilisina vs. NTproBNP

Antoni Bayes-Genis, MD, PhD ^{1,2*}, Jaume Barallat, MD^{2,3}, Amparo Galán, MD, PhD ^{2,3}, Marta de Antonio, MD, PhD ^{1,2} Mar Domingo, MD, PhD¹, Elisabet Zamora, MD, PhD ^{1,2}, Paloma Gastelurrutia, PhD⁴, Joan Vila, MSC^{5,6}, Judith Peñafiel, BSC^{5,6}, Carolina Galvez-Monton, DVM, PhD⁴, Josep Lupón, MD, PhD ^{1,2}

From: ¹Heart Failure Clinic, Cardiology Service. Hospital Universitari Germans Trias i Pujol. Badalona (Barcelona), Spain. ²Department of Medicine, Universitat Autònoma de Barcelona, Barcelona, Spain. ³Biochemistry Service, Hospital Universitari Germans Trias i Pujol Badalona (Barcelona), Spain; ⁴Germans Trias i Pujol Health Research Institute, Badalona, Spain. ⁵IMIM (Hospital del Mar Medical Research Institute), Barcelona. Spain. ⁶CIBER Epidemiology and Public Health, Barcelona. Spain.

Word Count: 4.907

***Address for correspondence:**

Antoni Bayes-Genis. MD, PhD
Chief of the Cardiology Service, Department of Medicine, UAB
Hospital Universitari Germans Trias i Pujol
Carretera del Canyet s/n 08916 Badalona, Spain
Cell phone: +34-679418491
Tel: +34 934978915 Fax: +34 934978939
Email: abayesgenis @gmail.com

ABSTRACT

Introduction and objectives: Neprilysin (NEP) breaks down numerous vasoactive peptides. The soluble form of NEP (sNEP), which was recently identified in heart failure (HF), is associated with cardiovascular (CV) outcomes. Within a multi-biomarker strategy, we directly compared sNEP and NTproBNP as risk stratifiers in a real-life cohort of HF patients.

Methods: sNEP, NTproBNP, ST2, and high sensitivity troponin T (hs-TnT) levels were measured in 797 consecutive ambulatory HF patients followed for 4.7 years. Comprehensive multivariable analyses and sNEP vs. NTproBNP head-to-head assessments of performance were performed. A primary composite endpoint included CV death or HF hospitalization. A secondary endpoint explored CV death alone.

Results: Median sNEP and NTproBNP concentrations were 0.64 ng/mL and 1187 ng/L, respectively. Both biomarkers significantly correlated with age ($p<0.001$) and ST2 ($p<0.001$), but only NTproBNP significantly correlated with estimated glomerular filtration rate ($p<0.001$), body mass index ($p<0.001$), left ventricular ejection fraction ($p=0.02$) and hs-TnT ($p<0.001$). In multivariable Cox regression analyses, sNEP remained independently associated with the composite endpoint (HR 1.14, 95% CI 1.02 to 1.27, $p=0.03$) and CV death (HR 1.15, 95% CI 1.01 to 1.31, $p=0.04$), but NTproBNP did not. The head-to-head sNEP vs. NTproBNP comparison showed good calibration and similar discrimination and reclassification for both neurohormonal biomarkers, but only sNEP improved global goodness of fit.

Conclusions: When added to a multimarker strategy sNEP remained an independent prognosticator while NTproBNP lost significance as risk stratifier in ambulatory patients with HF. Both biomarkers performed similarly in head-to-head analyses.

Key words: heart failure, Neprilysin, NTproBNP, prognosis, survival

RESUMEN

Introducción y objetivo: Neprilisina (NEP) degrada numerosos péptidos vasoactivos.

La forma soluble de NEP (sNEP), identificada recientemente en insuficiencia cardíaca (IC), se asocia con los acontecimientos cardiovasculares (CV). Se compararon de forma directa sNEP y NT-proBNP como estratificadores riesgo dentro de una estrategia multimarcador, en una cohorte de pacientes con IC de la vida real.

Métodos: se midieron sNEP, NTproBNP, ST2, y troponina T de alta sensibilidad niveles (hs-TnT) en 797 pacientes ambulatorios consecutivos con IC, seguidos durante 4,7 años. Se realizaron análisis multivariantes exhaustivos y se compararon directamente sNEP y NTproBNP mediante medidas estadísticas de rendimiento. El objetivo final primario fue el compuesto por muerte CV u hospitalización por IC. Un objetivo secundario exploró muerte CV aislada.

Resultados: Las concentraciones medianas de sNEP y NTproBNP fueron 0,64 ng/ml y 1.187 ng/L, respectivamente. Ambos biomarcadores correlacionaron con edad ($p < 0,001$) y ST2 ($p < 0,001$), pero sólo NTproBNP lo hizo con filtrado glomerular ($p < 0,001$), índice de masa corporal ($p < 0,001$), fracción de eyección ($p = 0,02$) y hs-TnT ($p < 0,001$). En los análisis multivariantes de regresión de Cox, sNEP mantuvo la asociación independiente con el objetivo compuesto (HR 1,14, IC95%: 1,02-1,27; $p = 0,03$) y con muerte CV (HR 1,15, IC95%: 1,01-1,31; $p = 0,04$), no así NTproBNP. Ambos biomarcadores mostraron discriminación y reclasificación similares, pero sólo sNEP mejoró la bondad global del ajuste.

Conclusiones: sNEP se mantuvo como pronosticador independiente al incluirlo en una estrategia multimarcador, mientras que NTproBNP perdió significación en la

estratificación del riesgo en pacientes ambulatorios con IC. Ambos biomarcadores obtuvieron medidas de rendimiento similares.

Palabras clave: insuficiencia cardíaca, Neprilisina, NTproBNP, pronóstico, supervivencia

ABBREVIATIONS

CV: cardiovascular

HF: heart failure

hs-TnT: High sensitivity troponin T

LVEF: Left ventricular ejection fraction

NTproBNP: N-terminal pro-brain natriuretic peptide

sNEP: Soluble Neprilysin

ABREVIACIONES

CV: cardiovascular

hs-TnT: Troponina T de alta sensibilidad

IC: Insuficiencia cardíaca

FEVI: fracción de eyección de ventrículo izquierdo

NTproBNP: Porción N-terminal del pro-péptido natriurético cerebral

sNEP: Neprilisina soluble

INTRODUCTION

Heart failure (HF) is a growing public epidemic, with increasing incidence and prevalence¹. Despite important progress in recent decades, mortality remains high for patients with HF. Prognostication may be refined by the use of biomarkers for different pathophysiological processes not reflected by established mortality risk factors. In 2008, Braunwald² classified circulating biomarkers into seven categories based on their pathophysiological effects in HF and hypothesized that multiple biomarkers in combination would provide a valuable means for risk stratification³. These pathways include myocardial stretch, myocyte injury, extracellular matrix, inflammation, renal dysfunction, neurohormonal activation and oxidative stress.

At present all multimarker approaches include natriuretic peptides as surrogates of the neurohormonal activation pathway⁴. However, soluble neprilysin (sNEP) has recently emerged as a potential alternative. NEP is a membrane-bound enzyme that cleaves numerous vasoactive peptides, including natriuretic peptides, adrenomedullin, angiotensin-I and -II, bradykinin, and substance P^{5,6}. NEP is quite ubiquitous and expressed mainly within cell membranes, but a circulating soluble form of NEP was recently reported in HF⁷. sNEP was found to be an independent predictor of cardiovascular (CV) death and HF hospitalizations in an ambulatory cohort of patients with HF⁷. By virtue of its central role in neurohormonal regulation, sNEP prognostic value reports on the status of several pathophysiological pathways involved in HF. Therefore, we directly compared sNEP, which is indicative of comprehensive neurohormonal activation, and N-terminal prohormone of brain natriuretic peptide (NTproBNP), a surrogate of natriuretic peptide release and current standard-of-care, in combination with high sensitivity troponin T (hs-TnT – myocyte injury), ST2

(myocardial fibrosis and inflammation) and estimated glomerular filtration rate (renal dysfunction) for HF prognostication.

METHODS

Study Population

The present is a multimarker subanalysis of the total studied cohort previously reported⁷ performed in those patients with availability of all the examined biomarkers (sNEP, NTproBNP, hs-TNT and ST2). From May 2006 to May 2013, ambulatory patients treated at a multidisciplinary HF clinic were consecutively included in the study. Referral inclusion criteria and blood sample collection were described elsewhere^{7,8}. All biomarkers were analysed in the same blood sample stored at -80° without previous freeze-thaw cycles. All samples were obtained between 09:00 am and 12:00 pm.

All participants provided written informed consent, and the local ethics committee approved the study. All study procedures were in accordance with the ethical standards outlined in the Helsinki Declaration of 1975, as revised in 1983.

Follow-up and Outcomes

All patients were followed at regular pre-defined intervals, with additional visits as required in the case of decompensation. The regular visitation schedule included a minimum of quarterly visits with nurses, biannual visits with physicians, and elective visits with geriatricians, psychiatrists, and rehabilitation physicians⁹. Patients who did not attend the regular visits were contacted by telephone.

The primary outcome was a composite of CV death or HF hospitalization. CV and all-cause deaths were also explored as secondary outcomes. A death was considered CV in origin if it was caused by HF (decompensated HF or treatment-resistant HF in the absence of another cause), sudden death (unexpected death, witnessed or not, of a

previously stable patient with no evidence of worsening HF or any other cause of death), acute myocardial infarction (directly related in time with acute myocardial infarction due to mechanic, hemodynamic, or arrhythmic complications), stroke (associated with recently appearing acute neurologic deficit), procedural (post-diagnostic or post-therapeutic procedure death), and other CV causes (e.g., rupture of an aneurysm, peripheral ischaemia, or aortic dissection). Hospitalizations were identified from the clinic records of patients with HF, hospital wards, and the electronic Catalan history record. Fatal events were identified from the clinical records of patients with HF, hospital wards, the emergency room, general practitioners, and by contacting the patient's relatives. Data were verified by the databases of the Catalan and Spanish Health Systems. Events were adjudicated by two of the authors (MD and JL). Follow-up was closed at March, 31, 2014.

Neprilysin Assay

Human NEP was measured using a modified sandwich immunoassay (HUMAN NEP/CD10 ELISA KIT, Aviscera Biosciences, Santa Clara, USA, Ref. SK00724-01, Lot No. 20111893). Several modifications were made to improve the analytical sensitivity of the method and obtain a lower limit of sample quantification, as reported elsewhere⁷. The modified protocol displayed analytical linearity for 0.250 to 4 ng/mL. Samples with concentrations higher than 4 ng/mL were diluted to a final concentration between 0.250 and 64 ng/mL. At a positive control value of 1.4 ng/mL, the intra- and inter-assay coefficients of variation were 3.7% and 8.9%, respectively.

NTproBNP Assay

NTproBNP levels were determined using an immuno-electrochemiluminescence method (Elecsys®, Roche Diagnostics, Switzerland). This assay has <0.001% cross-

reactivity with bioactive BNP, and in the constituent studies in this report, the assay had inter-run coefficients of variation ranging from 0.9% to 5.5%.

High-sensitivity cardiac troponin T (hs-TnT) Assay

Troponin levels were measured by electrochemiluminescence immunoassay using an hs-TnT assay on the Modular Analytics E 170 (Roche Diagnostics). The hs-TnT assay had an analytic range from 3 to 10,000 ng/L. At the 99th percentile value of 13 ng/L, the coefficient of variation was 9%. The assays were run with reagents from lot 157123, not affected by the analytical issues emerged with Roche hs-TnT assays.

ST2 Assay

ST2 was measured from plasma samples using a high-sensitivity sandwich monoclonal immunoassay (Presage[®] ST2 assay, Critical Diagnostics, San Diego, CA, USA). The ST2 assay had a within-run coefficient of <2.5% and total coefficient of variation of 4%.

Statistical Analysis

Categorical variables were expressed as percentages. Continuous variables were expressed as means (standard deviation [SD]) or medians (quartile Q1-Q3) according to normal or non-normal distributions. Normal distribution was assessed with normal Q-Q plots. The correlation between sNEP and NTproBNP concentrations with age, left ventricular ejection fraction (LVEF), estimated glomerular filtration rate, assessed by CKD-EPI equation, blood urea and body mass index were analysed using the rho Spearman coefficient due to skewed distribution. Statistical differences (p-value for trend) in NTproBNP and sNEP concentrations among eGFR subgroups (≥ 60 , 30 to <60, and <30 mL/min/1.73 m²) and body mass index subgroups (<20.5, 20.5 to < 25.5, 25.5 to <30, and ≥ 30 Kg/m²) were determined using the Spearman test.

Multivariable Cox regression analyses were performed using the backward step method. To fulfill the assumption of linearity of the co-variables sNEP, NT-proBNP, hs-TnT, and ST2, the logarithmic functions of sNEP, NT-proBNP and hs-TnT, and the quadratic term of log(hs-TnT) and of ST2 were used in the Cox models. For HR calculation in the three logarithm-transformed variables 1SD increase was used, and ST2 analyses were performed per every 10 ng/mL change. In patients with SNEP levels below the lower range of detection (0.250 ng/mL), a concentration of 0.249 ng/mL was introduced as a continuous variable. The following variables were incorporated into the model: age, sex, ischaemic aetiology of HF, LVEF, NYHA functional class, presence of diabetes mellitus, heart rate, systolic blood pressure, haemoglobin, serum sodium, estimated glomerular filtration rate, NTproBNP, hs-TnT, ST2, β -blocker treatment, angiotensin-converting enzyme inhibitor or angiotensin II receptor blocker treatment, and sNEP. In order to compare the prognostic values of both sNEP and NTproBNP, different measurements of performance were used (discrimination, calibration, and reclassification) as described previously^{9,10}. For calculation of NRI, we used risk categories based on tertiles of risk for the composite end-point (<21%, 21-39%, and >39%) and CV death (<11%, 11-23%, and >23%).

Statistical analyses were performed using SPSS 15 (SPSS Inc., Chicago, IL) and the R (version 2.11.1) statistical package (Foundation for Statistical Computing, Vienna, Austria). A two-sided $p < 0.05$ was considered significant.

RESULTS

Circulating sNEP, NTproBNP, hs-TnT and ST2 were measured in 797 patients with HF who were consecutively enrolled in the study between May 2006 and July 2010. Table 1 provides the baseline characteristics and biomarker metrics of the studied cohort.

During a mean follow-up period of 4.7 ± 2.4 years, 393 patients died, 216 from CV causes (54.9%), 147 from non-CV causes (37.4%), and 30 from unknown causes (7.6%). Among the known CV causes of death, refractory HF was the cause in 110 (50.9%) patients, sudden death in 47 (21.8%) patients, acute myocardial infarction in 19 (8.8%), stroke in 9 (4.2%), CV procedure in 5 (2.3%) and other CV cause in 26 (12%) patients. During the follow-up period, 193 patients were admitted to the hospital for HF and 300 patients fulfilled the primary endpoint of CV death or HF hospitalization. Five patients were lost to follow-up and adequately censored.

sNEP and NTproBNP Concentrations

The median soluble sNEP concentration was 0.64 ng/mL (Q1-Q3 0.42-1.23) and the median NTproBNP concentration was 1187 ng/L (Q1-Q3 476–278). sNEP levels were below the analytical measurement range in 101 patients (12.7%). Both biomarkers significantly correlated with age (ρ 0.41, $p < 0.001$ and ρ 0.19, $p < 0.001$), and NTproBNP significantly correlated with estimated glomerular filtration rate (ρ -0.37 , $p < 0.001$), blood urea (ρ 0.31, $p < 0.001$), body mass index (ρ -0.29 , $p < 0.001$), and weakly with LVEF (ρ -0.08 , $p = 0.02$); but sNEP did not (ρ -0.03 , $p = 0.44$; ρ 0.002, $p = 0.95$; ρ 0.04, $p = 0.23$; and ρ 0.008, $p = 0.88$, respectively). According to pre-defined estimated glomerular filtration rate and body mass index strata, the serum concentration of NTproBNP increased as renal function worsened (Figure 1A) and decreased at higher body mass index (Figure 1B), whereas sNEP remained unaffected by these comorbidities (Figures 1C and 1D).

Moreover, NTproBNP highly correlated with hs-TnT (ρ 0.57, $p < 0.001$), and also significantly with ST2 (ρ 0.33, $p < 0.001$), whereas sNEP did not correlate with hs-TnT (ρ < 0.01 , $p = 0.83$), and correlated modestly with ST2 (ρ 0.15, $p < 0.001$).

sNEP and NTproBNP did not correlate in the whole sample as previously reported ($\rho=0.01$; $p=0.68$)⁷; and in the present multimarker substudy the results remained non-significant ($\rho=0.04$, $p=0.28$).

Prognostic Role in Outcomes

In separate Cox regression multivariate analyses that included clinical parameters and the well-recognized HF biomarkers hs-TnT and ST2, sNEP remained independently associated with both the composite end-point (HR 1.14, 95% CI 1.02 to 1.27, $p=0.03$, Table 2) and CV death (HR 1.15, 95% CI 1.01 to 1.31, $p=0.04$, Table 3), but NPproBNP did not (HR 1.09, 95% CI 0.93 to 1.28, $p=0.27$, Table 2; and CV death (HR 1.18, 95% CI 0.97 to 1.43, $p=0.1$, Table 3, respectively).

Neither sNEP (HR 1.02, 95% CI 0.92 to 1.14, $p=0.68$) nor NTproBNP (HR 1.09, 95% CI 0.95 to 1.26, $p=0.23$) were independently associated to all-cause death in the multivariate analysis. By contrast, in the analysis focused only on HF endpoints sNEP remained independently associated with both HF-related death (HR 1.31, 95% CI 1.11 to 1.55, $p=0.001$) and with the composite endpoint HF-related death or HF hospitalization (HR 1.20, 95% CI 1.06 to 1.35, $p=0.005$), whereas NTproBNP did not (HR 1.18, 95% CI 0.97 to 1.43, $p=0.1$ and HR 1.07, 95% CI 0.89 to 1.29, $p=0.48$, respectively).

Direct comparison of sNEP vs. NTproBNP in a predictive model containing 11 clinical variables plus hs-TnT and ST2, did not show differences in discrimination (all differences in AUC $p>0.05$, Figure 2) and reclassification (all IDI and NRI $p>0.05$) for both end-points added to the reference model (Table 4). The calibration was good, with a non-significant Hosmer-Lemeshow test in all models (all $p>0.05$), although the models containing sNEP showed slightly lower AIC and BIC values (better calibration) (data not shown). The addition of sNEP improved global goodness of fit assessed by the

likelihood ratio test for both the composite primary end-point ($p=0.02$) and CV death ($p=0.03$), but NTproBNP did not ($p=0.22$ and $p=0.11$ respectively). A significant P value in this test means that adding a new variable to the model significantly improves the accuracy of the model of reference.

Figure 3 shows Cox regression event-free survival curve for the primary composite endpoint of CV death or HF hospitalization (A) and survival curve for CV death (B) according to the serum concentrations below or above the median of sNEP, hs-TnT and ST2.

DISCUSSION

Natriuretic peptides have become standard-of-care biomarkers in HF and they are currently the only biomarkers that have crossed the research boundary to become routinely used in every day practice. Used as surrogates of myocardial stretch, natriuretic peptides are just one of the multiple counter-regulatory mechanisms activated in HF. In contrast, NEP is an essential enzyme that cleaves a majority of HF-activated neurohormones, including, but not limited to, natriuretic peptides. Therefore, the recent identification of a soluble form of NEP with strong independent prognostic value⁷ raised the potential of sNEP as a truly comprehensive neurohormonal biomarker in HF. Here, we performed a head-to-head comparison of both biomarkers within a multimarker strategy that also included ST2 and hs-TnT for HF prognostication. Three conclusions emerged from this study. First, both sNEP and NTproBNP performed similarly in risk stratification in a large cohort with long-term follow-up of real-life patients with HF. Second, sNEP was unaffected by renal dysfunction and body mass index. Third, in the context of multimarker analyses, particularly with the incorporation of ST2 and hs-TnT, both of which have been shown consistently to be strong prognosticators in chronic HF, only sNEP retained its prognostic value.

Natriuretic peptides were recognized as class IA biomarkers of prognosis in the 2013 ACC/AHA HF guidelines¹¹. A multitude of prospective and retrospective studies have consistently confirmed the usefulness of both BNP and NTproBNP in predicting risk in HF^{12,13}. Nevertheless, in the post-PARADIGM era, NTproBNP may emerge as a stand-alone natriuretic peptide biomarker. Indeed, with the advent of angiotensin receptor and neprilysin inhibitors, particularly after the ground-breaking results of the Prospective comparison of neprilysin inhibitors with ACEI to Determine Impact on Global Mortality and morbidity in Heart Failure trial (PARADIGM-HF) with LCZ696¹⁴, circulating levels of BNP will almost certainly not be suitable for prognosis, monitoring, and therapeutic guidance. Packer *et al.*¹⁵ elegantly demonstrated that treatment with LCZ696 exerts its beneficial effects by inhibiting NEP, which subsequently inhibits BNP degradation, persistently maintaining high BNP levels among treated patients. LCZ696 dissociates the molecular balance between BNP and NTproBNP such that circulating BNP may no longer reflect the true myocardial stretch, but rather sustained NEP inhibition¹⁵. In contrast, NTproBNP, not a NEP substrate, exhibited a progressive decline in LCZ696-treated patients as HF improved.

In a recent study⁷, our group demonstrated for the first time that high levels of sNEP are found in the circulation of patients with HF and that sNEP concentrations are indicators of adverse outcomes for both CV mortality and morbidity. These data were important for better understanding sNEP pathobiology in HF and to put the results obtained in the PARADIGM-HF Trial into context. Nevertheless, the results reported here show that sNEP also provides independent information to other biomarkers commonly used for HF risk stratification and may be an alternative to natriuretic peptides. A current limitation is that the assay used for sNEP determination is not approved for clinical use and requires *ad hoc* fine-tuning, but it has good intra- and

inter-assay coefficients of variation. This assay displays 0% cross-reactivity with the two metallopeptidases most similar to this sequence, namely endothelin-converting enzymes (ECE) 1 and 2, and does not display cross-reactivity with erythrocyte cell-surface antigen (KELL), another protein with strong homology with NEP¹⁶.

Renal dysfunction and high BMI are becoming epidemic in HF patients. A recent report from the ESC HF long-term registry indicates that 26.4% of patients hospitalized with HF and 18.2% of chronic HF patients suffer from renal dysfunction and have a median BMI of 28 kg/m².¹⁷ Moreover, data from our group showed that, depending on the equation used for the calculation of eGFR, the prevalence of renal failure (defined as eGFR <60 mL/min/1.73 m²) may be as high as 64% in ambulatory patients with chronic HF¹⁸. The use of a biomarker significantly affected by these comorbidities may hamper widespread clinical use for prognostication. Given that plasma NTproBNP is excreted by the kidney we found, as expected, that decreased renal function is independently associated with higher plasma NTproBNP concentrations. Previous studies have suggested that plasma NTproBNP concentrations increase in patients with kidney dysfunction due to impaired clearance¹⁹; however, other studies have suggested that increased cardiac secretion due to coexistent CV disease may explain the finding²⁰. In this study, sNEP was unaffected by renal function, but many unsettled issues remain and the exact excretion mechanism of sNEP is currently uncertain. In regards to BMI, an important clinical factor influencing outcomes in HF patients^{21,22}, sNEP concentration remained unaffected across BMI strata, whereas NTproBNP showed a well-recognized reduction at higher BMIs²³. Taken together, our data indicate that sNEP is a novel independent prognostic biomarker that does not require adjustments for most common HF comorbidities.

Interest is increasing in multimarker strategies to examine panels of biomarkers that assess different pathophysiological pathways²⁴. Several recently reported scores for risk prediction assessment showed that multiple biomarker scoring is superior to a conventional risk score including clinical parameters and NTproBNP^{25,26}. Additional predictive information from different biological pathways reflects the multisystemic character of HF. In this study, we examined the value of ST2, which is indicative of fibrosis²⁷, and hs-TnT, which is reflective of myocyte injury²⁸ in combination with sNEP or NTproBNP, which are both indicative of neurohormonal activation, using a comprehensive clinical model including 11 prognostically meaningful variables (age, sex, ischaemic aetiology of HF, LVEF, NYHA functional class, diabetes mellitus, haemoglobin, sodium, eGFR, β -blocker, and ACEI/ARB treatment). Remarkably, sNEP retained its predictive value in combination with ST2 + hs-TnT for CV- and HF-related outcomes, whereas NTproBNP was no longer relevant. A number of biomarker panels may perform equivalently; choosing which one to employ in clinical practice will depend on factors such as cost, ease of assay, and potential therapeutic implications. Our present multimarker substudy, beyond the description of sNEP as a valuable biomarker in HF recently described⁷, suggests that the triad ST2, hs-TnT, and sNEP may eventually become a panel of choice once the sNEP assay is refined.

Neither sNEP nor NTproBNP were independently associated to all-cause death in the multivariate analysis. It should be taken into account that non-CV death was not negligible (37.4% of patients) and this might have influenced the results. We chose the same endpoints of PARADIGM-HF Trial, which are the most accepted in recent HF trials. Remarkably, sNEP also remained independently associated with HF-related death and HF hospitalization whereas NTproBNP did not.

Limitations

The experimental assay for sNEP measurement described here has long incubation times, making it ill-suited for daily clinical use. We have no data on stability of sNEP while frozen so we can not discard that the sNEP concentrations found would have been different in fresh samples. Samples were obtained during routine visits and no data of clinical stability of patients (i.e. possible decompensation during the 3 previous months) have been collected. However, the sample is representative of ambulatory chronic HF patients in real life. Although the study population was a real-life HF population with different HF aetiologies, it was treated at a specific multidisciplinary HF unit in a tertiary care hospital; most patients were referred from the cardiology department and, thus, were relatively young men with HF of ischaemic aetiology and reduced LVEF. As such, these results cannot necessarily be extrapolated to a more global HF population. In the near future, with the likely widespread use of neprilysin inhibitors in patients with HF and reduced ejection fraction, the prognostic value and use of sNEP and other circulating biomarkers may change.

Prospective studies are needed to assess tailored strategies for pharmacological NEP inhibition based on measurements of sNEP levels in patients with HF. The appropriate use of biomarkers supporting management of patients with HF should aid in reducing costs of a very costly disease in developed countries²⁹.

CONCLUSIONS

When added to a multimarker strategy that also incorporates ST2 and hs-TnT, sNEP remained an independent prognosticator while NTproBNP lost significance as risk stratifier in ambulatory patients with HF. sNEP performed similarly to NTproBNP in head-to-head analyses, but it was less influenced by comorbidities (renal function and BMI).

ACKNOWLEDGEMENTS

We thank Beatriz González, Roser Cabanes, Margarita Rodríguez, Nuria Benito and Alba Ros for data collection and invaluable work in the HF Clinic.

We also wish to acknowledge Redes Temáticas de Investigación Cooperativa en Salud (RETICS), Red Cardiovascular (RD12/0042/0047; Postdoctoral RIC fellowship), Red de Terapia Celular–TerCel [RD12/0019/0029], and Ministerio de Economía y Competitividad (Juan de la Cierva, JCI-2012-14025).

REFERENCES

- 1- McMurray JJ, Adamopoulos S, Anker SD, Auricchio A, Böhm M, Dickstein K, et al; ESC Committee for Practice Guidelines. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. *Eur Heart J*. 2012;33:1787-847
- 2- Braunwald E. Biomarkers in heart failure. *N Engl J Med* 2008;358: 2148–59.
- 3- Lupón J, de Antonio M, Vila J, Peñafiel J, Galán A, Zamora E, et al. Development of a novel heart failure risk tool: the barcelona bio-heart failure risk calculator (BCN bio-HF calculator). *PLoS One*. 2014;9:e85466.
- 4- Pascual-Figal DA, Manzano-Fernandez S, Boronat M, Casas T, Garrido IP, Bonaque JC, et al. Soluble ST2, high sensitivity troponin T and N-terminal pro-Btype natriuretic peptide: complementary role for risk stratification in acutely decompensated heart failure. *Eur J Heart Fail* 2011;13:718–25.
- 5- Kenny AJ, Bourne A, Ingram J. Hydrolysis of human and pig brain natriuretic peptides, urodilatin, C-type natriuretic peptide and some C-receptor ligands by endopeptidase-24.11. *Biochem J* 1993;291(Pt 1):83–8.
- 6- Vijayaraghavan J, Scicli AG, Carretero OA, Slaughter C, Moomaw C, Hersh LB.. The hydrolysis of endothelins by neutral endopeptidase 24.11 (enkephalinase). *J Biol Chem* 1990;265:14150–5.
- 7- Bayes-Genis A, Barallat J, Galan A, de Antonio M, Domingo M, Zamora E, et al. Soluble Neprilysin is Predictive of Cardiovascular Death and Heart Failure Hospitalization in Heart Failure Patients. *J Am Coll Cardiol* 2014;65:657-65.

- 8- Bayes-Genis A, de Antonio M, Galan A, Sanz H, Urrutia A, Cabanes R, et al.
Combined Use of High Sensitivity ST2 and NTproBNP to Improve the Prediction of Death in Heart Failure. *Eur J Heart Fail* 2012;14:32-8.
- 9- Bayes-Genis A, de Antonio M, Vila J, Peñafiel J, Galán A, Barallat J, Zamora E, et al. Head-to-head comparison of 2 fibrosis biomarkers for long-term heart failure risk stratification. ST-2 versus Galectin-3. *J Am Coll Cardiol*. 2014;63:158–166.
- 10- Pencina MJ, D'Agostino RB, Steyerberg EW. Extensions of net reclassification improvement calculations to measure usefulness of new biomarkers. *Stat Med* 2011;30:11–21.
- 11- Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE Jr, Drazner MH, et al;
American College of Cardiology Foundation; American Heart Association Task Force on Practice Guidelines.. 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *J Am Coll Cardiol* 2013;62:e147-239.
- 12- Oremus M, McKelvie R, Don-Wauchope A, Santaguida PL, Ali U, Balion C, et al.
A systematic review of BNP and NT-proBNP in the management of heart failure: overview and methods. *Heart Fail Rev*. 2014;19:413-9
- 13- Ahmad T, Fiuzat M, Felker GM, O'Connor C. Novel biomarkers in chronic heart failure. *Nat Rev Cardiol*. 2012;9:347-59.
- 14- McMurray JJ, Packer M, Desai AS, Gong J, Lefkowitz MP, Rizkala AR, et al;
PARADIGM-HF Investigators and Committees. Angiotensin-neprilysin inhibition versus enalapril in heart failure. *N Engl J Med*. 2014;371:993-1004.
- 15- 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*. 2014 [Epub ahead of print].
- 16- Oefner C, D'Arcy A, Hennig M, Winkler FK, Dale GE. Structure of human neutral endopeptidase (Neprilysin) complexed with phosphoramidon. *J Mol Biol*. 2000;296:341–9.
 - 17- Maggioni AP, Anker SD, Dahlström U, Filippatos G, Ponikowski P, Zannad F, et al; Heart Failure Association of the ESC. Are hospitalized or ambulatory patients with heart failure treated in accordance with European Society of Cardiology guidelines? Evidence from 12,440 patients of the ESC Heart Failure Long-Term Registry. *Eur J Heart Fail*. 2013;10:1173-84.
 - 18- Zamora E, Lupón J, de Antonio M, Vila J, Peñafiel J, Galán A, et al. Long-term prognostic value for patients with chronic heart failure of estimated glomerular filtration rate calculated with the new CKD-EPI equations containing cystatin C. *Clin Chem*. 2014;60:481-9.
 - 19- Anwaruddin S, Lloyd-Jones DM, Baggish A, Chen A, Krauser D, Tung R, et al. Renal function, congestive heart failure, and amino-terminal pro-brain natriuretic peptide measurement: results from the ProBNP Investigation of Dyspnea in the Emergency Department (PRIDE) Study. *J Am Coll Cardiol*. 2006;47:91–97.
 - 20- van Kimmenade RR, Januzzi JL Jr, Bakker JA, Houben AJ, Rennenberg R, Kroon AA, et al. Renal clearance of B-type natriuretic peptide and amino terminal pro-B-type natriuretic peptide a mechanistic study in hypertensive subjects. *J Am Coll Cardiol*. 2009;53:884–890.
 - 21- Zamora E, Lupón J, Urrutia A, González B, Mas D, Pascual T, et al. Does body mass index influence mortality in patients with heart failure? *Rev Esp Cardiol* 2007;60:1127-1134.

- 22- Puig T, Ferrero-Gregori A, Roig E, Vazquez R, Gonzalez-Juanatey JR, Pascual-Figal D, et al. Value of body mass index and waist circumference in patients with chronic heart failure (Spanish REDINSCOR Registry). *Rev Esp Cardiol* 2014;67:101-6.
- 23- Madamanchi C, Alhosaini H, Sumida A, Runge MS. Obesity and natriuretic peptides, BNP and NT-proBNP: mechanisms and diagnostic implications for heart failure. *Int J Cardiol*. 2014;176:611-7.
- 24- Bayes-Genis A, Ordonez-Llanos J. Multiple biomarker strategies for risk stratification in heart failure. *Clin Chim Acta*. 2014 [Epub ahead of print].
- 25- Ky B, French B, Levy WC, Sweitzer NK, Fang JC, Wu AH, et al. Multiple biomarkers for risk prediction in chronic heart failure. *Circ Heart Fail*. 2012;5:183-90
- 26- Lupón J, de Antonio M, Vila J, Peñafiel J, Galán A, Zamora E, et al. Development of a novel heart failure risk tool: the barcelona bio-heart failure risk calculator (BCN bio-HF calculator). *PLoS One*. 2014;9:e85466.
- 27- Daniels LB, Bayes-Genis A. Using ST2 in cardiovascular patients: a review. *Future Cardiol*. 2014;10:525-39.
- 28- de Antonio M, Lupón J, Galan A, Vila J, Urrutia A, Bayes-Genis A. Combined use of high-sensitivity cardiac troponin T and N-terminal pro-B type natriuretic peptide improves measurements of performance over established mortality risk factors in chronic heart failure. *Am Heart J*. 2012;163:821-8.
- 29- Delgado JF, Oliva J, Llano M, Pascual-Figal D, Grillo JJ, Comín-Colet J, et al. Health care and nonhealth care costs in the treatment of patients with symptomatic chronic heart failure in Spain. *Rev Esp Cardiol*. 2014;67:643-50.

FIGURE LEGENDS

Figure 1. Boxplots for NTproBNP and sNEP according to estimated glomerular

filtration rate and body mass index subgroups.

A) NTproBNP concentration according to grouped estimated glomerular filtration rate. B) NTproBNP serum concentration according to grouped body mass index values. C) sNEP serum concentration according to grouped estimated glomerular filtration rate. D) sNEP concentration according to grouped body mass index values. The central box represents the values from the lower to the upper quartile and the middle line represents the median. The whiskers extend from the minimum to the maximum value, excluding outside values, which are not displayed.

Figure 2. AUC for the predictive models. Core model (black line): age, sex, ischaemic

aetiology of HF, LVEF, NYHA functional class, presence of diabetes mellitus, haemoglobin, serum sodium, estimated glomerular filtration rate, hs-TnT, ST2, β -blocker treatment, and angiotensin-converting enzyme inhibitor or angiotensin II receptor blocker treatment. Model containing as well sNEP is depicted as red line and model containing also NTproBNP is depicted as blue line. A) Composite end-point of CV death and HF hospitalization. $P=0.83$ for the comparison between the core model and the model containing also NTproBNP; $P=0.24$ for the comparison between the core model and the model containing also sNEP; and $P=0.22$ for the direct comparison between the two models containing neurohormonal biomarkers.

B) CV death. $P=0.87$, $P=0.26$ and $P=0.40$ respectively for the same comparisons.

Figure 3. Cox regression survival curves according to the number of elevated biomarkers (sNEP, hsTnT and ST2; $N=797$).

A) Event-free survival curve for the primary composite endpoint of CV death or HF hospitalization. B) Survival curve for CV death.

A biomarker was considered elevated if equal or above the median values:

sNEP= 0.64 ng/mL; hsTnT= 22.3 ng/L; ST2= 38.1 ng/mL.

Table 1. Demographic and clinical characteristics at baseline and treatments during follow-up

	Total Cohort
	N= 797
Age, yr [*]	66.5 ± 12.3
Male, n (%)	579 (71.5)
White, n (%)	791 (99.2)
Aetiology, n (%)	
Ischaemic heart disease	416 (52.2)
Dilated cardiomyopathy	74 (9.3)
Hypertensive	74 (9.3)
Valvular	101 (12.7)
Other	132 (16.5)
HF duration, months [†]	27.2 (5–72.6)
LVEF, in % ^a	33.4 ± 13.3
NYHA functional class, n (%)	
I	52 (6.5)
II	535 (67.1)
III	204 (25.6)
IV	6 (0.8)
Hypertension, n (%)	494 (62)
Diabetes mellitus, n (%)	275 (34.5)
Sodium, mmol/L [*]	139.3 ± 3.4
Haemoglobin, g/dL [*]	12.9 ± 1.9
eGFR, mL/min/1.73 m ² [*]	53.7 ± 26.4
NTproBNP, ng/L [†]	1187 (476–278)
Neprilysin, ng/mL [†]	0.64 (0.42–1.23)
Hs-TnT, ng/L [†]	22.3 (10.2–39.2)
ST2, ng/mL [†]	38.1 (30.7–50.4)
Treatment (follow-up), n (%)	
ACEI or ARB	729 (91.5)
β-blocker	717 (90)

MRA	464 (58.2)
Loop diuretic	738 (92.6)
Digoxin	338 (42.4)
CRT	60 (7.5)
ICD	99 (12.4)

*Mean $\pm$ standard deviation; †Median (Q1-Q3);

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker;

CRT, cardiac resynchronization therapy; eGFR, estimated glomerular filtration rate

(CKD-EPI); HF, heart failure; ICD, implantable cardiac defibrillator; LVEF, left

ventricular ejection fraction; MRA, mineralocorticoid receptor antagonist; NTproBNP,

N-terminal pro-brain natriuretic peptide; NYHA, New York Heart Association.

Table 2. Multivariable Cox regression analysis for risk of the composite primary endpoint (cardiovascular death or HF hospitalization)

	Model with sNEP			Model with NTproBNP		
	HR	95% CI	P-value	HR	95% CI	P-value
Age	1.03	1.01 to 1.04	<0.001	1.02	1.01 to 1.03	<0.001
Female	0.86	0.66 to 1.13	0.28	0.86	0.65 to 1.13	0.28
Ischaemic aetiology of HF	1.01	0.78 to 1.30	0.96	1.00	0.77 to 1.29	0.97
LVEF	1.01	1.00 to 1.02	0.19	1.01	1.00 to 1.02	0.17
NYHA functional class	1.48	1.18 to 1.85	0.001	1.47	1.17 to 1.84	0.001
eGFR, mL/min/1.73 m ²	1.00	0.99 to 1.00	0.19	1.00	0.99 to 1.00	0.11
Heart rate	1.00	0.99 to 1.01	0.48	1.00	1.00 to 1.01	0.54
Systolic blood pressure	1.00	1.00 to 1.01	0.32	1.00	1.00 to 1.01	0.33
Diabetes mellitus	1.34	1.06 to 1.69	0.01	1.35	1.07 to 1.70	0.01
ACEI or ARB treatment	0.68	0.47 to 1.0	<0.05	0.64	0.44 to 0.94	0.02
β-blocker treatment	0.63	0.45 to 0.88	0.007	0.61	0.44 to 0.86	0.005
Sodium	0.96	0.94 to 1.01	0.14	0.98	0.95 to 1.01	0.18
Haemoglobin	0.90	0.84 to 0.96	0.002	0.91	0.85 to 0.97	0.006
hs-TnT*,†	1.69	1.43-2.01	<0.001	1.70	1.44-2.02	<0.001
ST2‡,§	1.12	1.02-1.22	0.02	1.12	1.02-1.24	0.02
NTproBNP*	–	–	–	1.09	0.93 to 1.28	0.27

sNEP*	1.14	1.02 to 1.27	0.03		–	–	–
--------------	-------------	---------------------	-------------	--	---	---	---

*hs-TnT as log(hs-TnT); NTproBNP as log(NTproBNP); Neprilysin as log(Neprilysin). For HR calculation in the three logarithm-transformed variables, a 1SD increase was used.

†For $\log(\text{hs-TnT})^2$, $p=0.001$ in the model containing NEP and $p=0.001$ in the model containing NTproBNP.

‡ST2 as ST2/10 ng/mL.

§For ST2^2 , $p=0.05$ in the model containing NEP and $p=0.04$ in the model containing NTproBNP.

HR, hazard ratio; HF, heart failure; hs-TnT, high sensitivity cardiac troponin T; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association; eGFR, estimated glomerular filtration rate; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; NTproBNP, N-terminal prohormone of brain natriuretic peptide; ST2, high-sensitivity soluble ST2; NEP, neprilysin.

Table 3. Multivariable Cox regression analysis for risk of cardiovascular death

	Model with sNEP			Model with NTproBNP		
	HR	95% CI	P-value	HR	95% CI	P-value
Age	1.03	1.02 to 1.05	<0.001	1.03	1.02 to 1.05	<0.001
Female	0.78	0.56 to 1.08	0.13	0.76	0.54 to 1.07	0.11
Ischaemic aetiology of HF	0.97	0.72 to 1.31	0.84	0.96	0.71 to 1.29	0.76
LVEF	1.01	1.00 to 1.02	0.13	1.01	1.00 to 1.02	0.13
NYHA functional class	1.56	1.21 to 2.05	0.001	1.57	1.21 to 2.05	0.001
eGFR, mL/min/1.73 m²	1.00	0.99 to 1.00	0.11	1.00	0.99 to 1.00	0.12
Heart rate	1.00	0.99 to 1.01	0.82	1.00	0.99 to 1.01	0.30
Systolic blood pressure	1.00	0.99 to 1.01	0.83	1.00	0.94 to 1.01	0.81
Diabetes mellitus	1.31	1.00 to 1.72	0.05	1.32	1.01 to 1.74	<0.05
ACEI or ARB treatment	0.63	0.41 to 0.98	0.04	0.59	0.38 to 0.90	0.02
β-blocker treatment	0.54	0.37 to 0.79	0.002	0.54	0.37 to 0.79	0.001
Sodium	0.96	0.92 to 1.00	0.03	0.96	0.92 to 1.00	<0.05
Haemoglobin	0.96	0.88 to 1.04	0.27	0.99	0.98 to 1.01	0.29
hs-TnT*,†	1.78	1.45-2.18	<0.001	1.74	1.42-2.14	<0.001
ST2‡,§	1.13	1.00-1.26	0.04	1.14	1.02-1.28	0.02

NTproBNP*	–	–	–	1.18	0.97 to 1.43	0.10
sNEP*	1.15	1.01 to 1.31	0.04	–	–	–

Food notes as table 2.

[†]For $\log(\text{hs-TnT})^2$, $p = 0.007$ in the model containing NEP and $p=0.01$ in the model containing NTproBNP.

[§]For ST2^2 , $p = 0.09$ in the model containing NEP and $p=0.05$ in the model containing NTproBNP.

Table 4. Reclassification for the composite end-point of CV death and HF hospitalization and for CV according to the addition of sNEP or NTproBNP to the core model

Composite Primary end-point			Cardiovascular death	
	Model with NTproBNP [*]	Model with sNEP [†]	Model with NTproBNP [*]	Model with sNEP [†]
IDI	0 (−0.3 to 0.3); ‡P= 0.94	0.5 (0 to 1); ‡P= 0.056	0 (−0.4 to 0.4); ‡P= 0.89	0.4 (−0.1 to 1); ‡P= 0.12
NRI	0.3 (−3.1 to 3.6); ‡P= 0.86	−2.6 (−6.9 to 1.6); ‡P= 0.23	−0.6 (−3.9 to 2.7); ‡P= 0.73	−0.7 (−5 to 3.7); ‡P= 0.76
IDI	Reference	0.5 (−0.1 to 1.1); §P= 0.08	Reference	0.4 (−0.2 to 1); §P= 0.22
NRI	Reference	−1.5 (−5.7 to 2.7); §P= 0.48	Reference	−0.7 (−5.8 to 4.3); §P= 0.78

Core model: age, sex, NYHA functional class, LVEF, Ischemic etiology, diabetes, eGFR, Hb, Na, Beta-blocker treatment, and ACEI or ARB treatment.

^{*}Model with NTproBNP: core model + NTproBNP

[†]Model with NEP: core model + sNEP

[‡]Versus core model.

[§] Model with sNEP versus model with NTproBNP

IDI = integrated discrimination improvement; NRI = net reclassification improvement.

Fig 1

Fig 2

Fig 3