THE PRESENT AND FUTURE

REVIEW TOPIC OF THE WEEK

A Test in Context: Neprilysin

Function, Inhibition, and Biomarker

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ABSTRACT

Neprilysin is a zinc-dependent endopeptidase. It is ubiquitous in distribution and promiscuous in function, with >50 putative peptide substrates with varying levels of in vitro and/or in vivo evidence of functional relevance. In the first part of this review, we discuss the genetic, structural, substrate, and pathophysiological aspects of neprilysin. We incorporate information provided by genetically modified models, as well as pre-clinical and clinical data from investigations of synthetic neprilysin inhibitors. We next highlight the value of neprilysin as a biotarget and weigh the clinical benefits of synthetic neprilysin inhibitors, either alone or in combination with antagonists of the renin–angiotensin system. Finally, we provide evidence about soluble neprilysin as a biomarker surrogate in patients with heart failure and identify important gaps that require further research before soluble neprilysin is used clinically. In sum, neprilysin is a versatile, veteran player returning yet again to center stage after an eventful career spanning >40 years. (J Am Coll Cardiol 2016;68:639–53) © 2016 by the American College of Cardiology Foundation.

Neprilysin (NEP) is currently a focus of interest in cardiovascular medicine because of the impressive benefits of combining NEP inhibition and angiotensin 2 type 1 receptor blockade, demonstrated in the PARADIGM-HF (Prospective Comparison of ARNI with ACEI to Determine Impact on Global Mortality and Morbidity in Heart Failure) of LCZ696 (sacubitril/valsartan) for systolic heart failure (HF) (1). However, NEP EC 3.4.24.11 (also known as neutral endopeptidase 24.11, endoprotease 24.11, NEP, common acute lymphoblastic leukemia antigen [CALLA], neutrophil antigen cluster differentiation antigen 10 [CD10], membrane metalloendopeptidase EC 3.4.24.11, and enkephalinase) is actually a

versatile player returning yet again to the spotlight after an eventful career spanning >40 years.

First discovered in 1973, NEP was identified as the only endopeptidase among 7 hydrolases found in the brush border of rabbit proximal renal tubule microvilli (2), a location facilitating metabolism of substrate peptides appearing in renal filtrate. Its activity was first quantified in vitro through investigation of its cleavage of insulin B-chain, which proved to be a good substrate. Within 1 year, Kerr and Kenny (3,4) had demonstrated that the rabbit brush border enzyme was maximally active at neutral pH, could cleave glucagon, had an approximate molecular weight of 93 kDa, and was the first reported

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mammalian example of a zinc-activated endopeptidase. The renal enzyme was soon found to be identical to a brain endopeptidase, enkephalinase, which metabolizes endogenous opioids and for which inhibition has antinociceptive effects (5,6). NEP was also shown to be identical to a lymphocyte marker designated CALLA (CD10) (7,8). Subsequently, discoveries of the wide anatomic distribution of NEP and its bewilderingly extensive array of candidate substrates have pointed to potentially important roles for the endopeptidase in cardiovascular, renal, pulmonary, gastrointestinal, and neurological functions.

NEP SYNTHESIS, STRUCTURE, AND PHYSIOLOGY

The gene encoding human NEP is on chromosome 3 (3q21-27); it spans >80 kb and contains 24 exons (9-11). Exons 1 and 2 encode 5′ untranslated sequences; exon 3 contains the initiation codon, and it encodes the membrane and cytoplasmic domains. Twenty short exons (exons 4 to 23) encode the extracellular portion of the enzyme, and exon 24 encodes the carboxy-terminal 32 amino acids of the protein and the 3′ untranslated sequences. The pentapeptide sequence (His-Glu-Ile-Thr-His) is associated with metalloprotease zinc binding and substrate catalysis, and is encoded within exon 19. Three types of NEP complementary deoxyribonucleic acid types have been identified; they contain 5′ untranslated sequences differing from one another upstream of exon 3. The 3 human NEP complementary deoxyribonucleic acid types are the results of alternative splicing of exons 1, 2a, or 2b to the common exon 3. Moreover, exons 2a and 2b share the same 5′ sequence but have 2 distinct donor splice-sites 171 bp apart. The interspecies conservation of its 5′ untranslated sequences and the existence of 5′ alternative splicing are consistent with differential control of NEP expression during development and tissue-specific regulation (11).

NEP is an integral type II, membrane-bound, zinc-dependent endopeptidase composed of 749 amino-acid residues (12-14). Its ectodomain, a member of the M13 family of peptidases, comprises 2 alpha-helical structures forming a spherical, water-filled cleft presenting the catalytic site. The larger of the 2 ectodomain portions is structurally related to the bacterial protease thermolysin. It contains a single zinc atom, essential for catalytic activity, which is coordinated by His and Glu residues. The enzyme also has a transmembrane domain and a short intracellular domain. NEP cleaves peptides with a molecular weight generally at or below 3,000 Da and cannot attack large proteins. This degree of size-related specificity is conferred by size-restricted access to the catalytic crypt (**Figure 1**) (14). NEP hydrolyzes peptides to the amino side of hydrophobic amino acids, with a predilection for Phe or Leu at the P1 position and preferential release of di- or tripeptides from the carboxy-terminus of substrates.

NEP is found in epithelia, fibroblasts, and neutrophils and in soluble form in the circulation, urine, and cerebrospinal fluid (CSF) (15-19). After its original isolation from renal brush-border membranes, studies in several species, using NEP antibodies or labeled NEP inhibitors, have located NEP in the brain, heart, peripheral vasculature, adrenal gland, lungs, gastrointestinal secretory epithelial brush borders, thyroid, placental syncytiotrophoblasts, arthritic synovium, and the male genital tract (15,16,18,20-22). The renal brush border has long been recognized as rich in membrane-bound NEP, and a soluble form of the enzyme also appears in urine (15,16,19). In the circulation, NEP is a soluble circulating enzyme, and it is also present on the plasma membranes of neutrophils, where it may cleave the chemotactic peptide fMet-Leu-Phe and modulate the chemotactic response of neutrophils (16,23). Soluble neprilysin (sNEP) in blood, urine, and CSF retains catalytic activity, as demonstrated by assays of plasma NEP activity (17,18). The close similarities between membrane-bound NEP and sNEP with respect to affinity constants for various inhibitors, and optimal pH and Michaelis constant for test substrates, further suggest that sNEP plays a role in cleaving circulating substrates. However, measured maximal velocity values are much lower in CSF, plasma, and amniotic fluid, suggesting enzymatic activity is predominantly tissue based. The processes whereby NEP is solubilized to appear in these body fluids are unknown.

NEP is expressed in cardiac fibroblasts and cardiomyocytes, and it appears to be more important than angiotensin-converting enzyme (ACE) in clearance of cardiac bradykinin (24). In contrast to ACE, which is enriched in endothelial cells, vascular NEP is more of a fibroblast product (15,16). In lung, membrane-bound NEP appears within airways in cells that are associated with tachykinin receptors. NEP is present in the basal cells of airway epithelium, nerves, smooth muscle, glands, and blood vessels. It is also enriched in the pulmonary interstitium and alveolar cells (16,25-27). In the brain, the distribution of NEP maps with opioid receptors in structures related to pain

FIGURE 1 Interaction of Natriuretic Peptide With Neprilysin
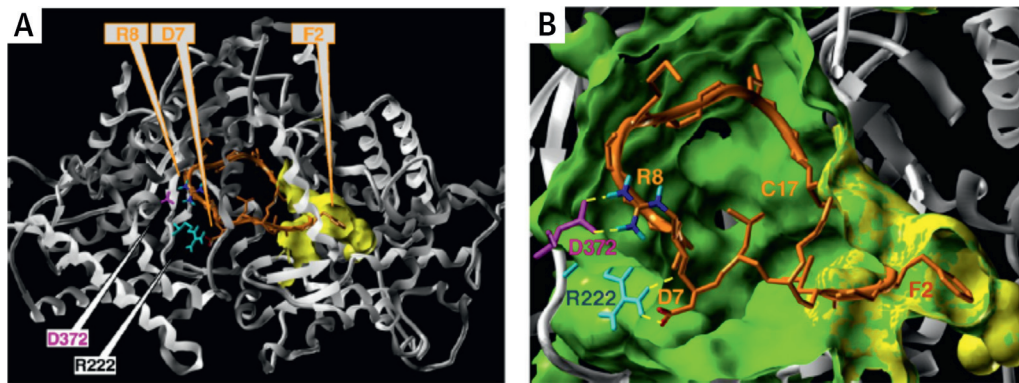
(A) Neprilysin molecule **(white)** entirely enclosing C-type natriuretic peptide (CNP) **(orange)**. **(B)** An expanded view of neprilysin's interior cavity **(green)** and catalytic site **(yellow)**. Key neprilysin amino acid residues include the recognition sites located opposite the catalytic site, arginine (R222) and aspartic acid (D372), which adjust orientation of the natriuretic peptide within the cleft by interacting with conserved natriuretic peptide residues D7 and R8, respectively. The common primary cleavage site for atrial natriuretic peptide (ANP) and CNP is the cysteine-phenylalanine bond ([F2] adjacent to the CNP ring structure cysteine-cysteine disulfide bridge [C17]). Reprinted with permission from Pankow et al. (14).

control and in the caudate-putamen, globus pallidus, and substantia nigra. In the latter 2 structures, NEP is associated with presynaptic axonal terminals well placed to metabolize neurotransmitters. It is abundant on epithelial surfaces in contact with the CSF, including choroid plexus, pial membranes, and ependymal cells (15,16,28). NEP is also present in the Schwann cells of many peripheral nerves (15).

In the gut, the jejunal brush-border epithelium is particularly enriched in NEP (29,30). The enzyme is also present in the male genital tract, including prostate, epididymis, and seminal plasma, where it may contribute to sperm motility (15,31,32).

The distribution of NEP, coupled with the many established and candidate substrates of the enzyme, point to potential roles in multiple organ systems in health and disease. On reviewing neutral endopeptidase 24.11 in 1989, Erdös and Skidgel tabled 26 peptides with in vitro evidence of cleavage by NEP and >7 with in vivo or in situ evidence (15). By 2016, more than 50 putative peptide substrates of NEP had been proposed (33), with varying levels of in vitro and/or in vivo evidence of functional relevance; **Table 1** lists those best studied. A comprehensive discussion of all possible NEP substrates and their putative roles in assorted organ systems is not possible within the bounds of the present review. However, in the context of HF, key substrates to consider include the natriuretic peptides (NPs), angiotensin 2, the endothelins, adrenomedullin, and bradykinin. With respect to potential unwanted consequences of NEP

inhibition, our focus is on its role in the central nervous, respiratory, and gastrointestinal systems.

The NPs atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) are of predominantly cardiac origin, whereas C-type natriuretic peptide (CNP) is largely sourced from endothelial cells throughout the systemic vasculature. All 3 are derived from propeptides cleaved to generate mature, bioactive carboxy-terminal peptides and associated amino-terminal fragments. Bioactive NPs

TABLE 1 Substrates for Human Neprilysin

Cardio-renal
ANP, BNP, and CNP; angiotensins 1, 2, 3, 1-9; endothelin-1, -2, and -3; adrenomedullin; bradykinin

Nervous system
Amyloid-beta (1-40), (1-42); enkephalins (met and leu); alpha-endorphin, gamma-endorphin; alpha-neoendorphin; beta-neoendorphin; nociceptin; corticotrophin-releasing factor; luteinizing hormone-releasing hormone; oxytocin; arginine vasopressin; neurotensin; neuropeptide Y; neurokinin A

Gastrointestinal
Gastrin-releasing peptide; gastric inhibitory peptide; vasoactive intestinal peptide; cholecystokinin

Respiratory
Substance P, other tachykinins

Metabolic
Glucagon; glucagon-like peptide; beta-lipotropin; insulin B-chain; secretin; CGRP; somatostatin

Inflammation
Chemotactic peptide; interleukin 1-beta

Multisystem/other
Substance P

ANP = atrial natriuretic peptide; BNP = B-type natriuretic peptide; CGRP = calcitonin gene-related peptide; CNP = C-type natriuretic peptide.

act via G protein-coupled transmembrane receptors activating cyclic guanosine monophosphate (cGMP) as a second messenger (34). ANP and BNP act via natriuretic peptide receptor (NPR)-A to exert natriuretic, diuretic, hemoconcentrating, and vasodilating effects in association with suppression of the renin-angiotensin-aldosterone and sympathetic nervous systems, and trophic effects that oppose cardiac hypertrophy and fibrosis. CNP, operating via NPR-B, is not natriuretic but is central to vasomotion, opposes vascular cell hyperplasia, and has a unique role in long bone development.

All 3 NPs are cleared via the NPR-C receptor in concert with proteolysis (35). NEP is responsible for the initial proteolytic cleavage of ANP and CNP. In humans, BNP is relatively resistant to degradation by NEP (36–39). Much of the impact of inhibiting NEP in preclinical and clinical settings has been presumed to be due to enhancement of NP bioactivity. The ranking of avidity of the enzyme for the NPs is CNP >ANP >BNP (34,39). The plasma half-lives of ANP and CNP in humans are 2 to 4 min but, unique to humans, the half-life of human BNP is considerably longer, at >20 min (34,40). The readiness with which NEP cleaves the different NPs in part reflects the ease with which the NPs 17-residue circular core gain access into NEP's catalytic cleft and the ability of the NP peptide to orient optimally once in the active site (**Figure 2**) (14). The NPs' amino- and carboxy-terminals alter their access to and orientation within the catalytic cleft and, together with the residue sequence of the 17-member circular motif, determine the efficacy of peptide catalysis. Occupation of NEP's catalytic cleft by human BNP without catalysis probably underlies the finding that increasing concentrations of human BNP act as an inhibitor of NEP (14,41). Ex vivo degradation of BNP in human blood samples is not inhibited by NEP inhibition, whereas the kallikrein-specific D-Phe-Phe-Arg-chloromethylketone (PPACK II) and a broad spectrum of serine protease inhibitors stabilize human BNP (42).

FIGURE 2   Interaction of Individual NPs With Neprilysin

The natriuretic peptides (NPs) enter the interior cavity of neprilysin **(green)**. The shorter amino and carboxy terminal tails of atrial natriuretic peptide (ANP) and C-type natriuretic peptide (CNP) **(upper panel)** allow optimal positioning and interaction with the catalytic site for primary cleavage of the NP ring structure at the cysteine-phenylalanine bond. The longer tails of B-type natriuretic peptide (BNP) **(lower panel)** cause spatial clashes at the entry to the cleft and hinder orientation for catalysis resulting in initial cleavage outside the ring structure at residues Met-Val. Modified with permission from Pankow et al. (14).

The exact relationship of these findings to in vivo clearance of human BNP is unclear (34).
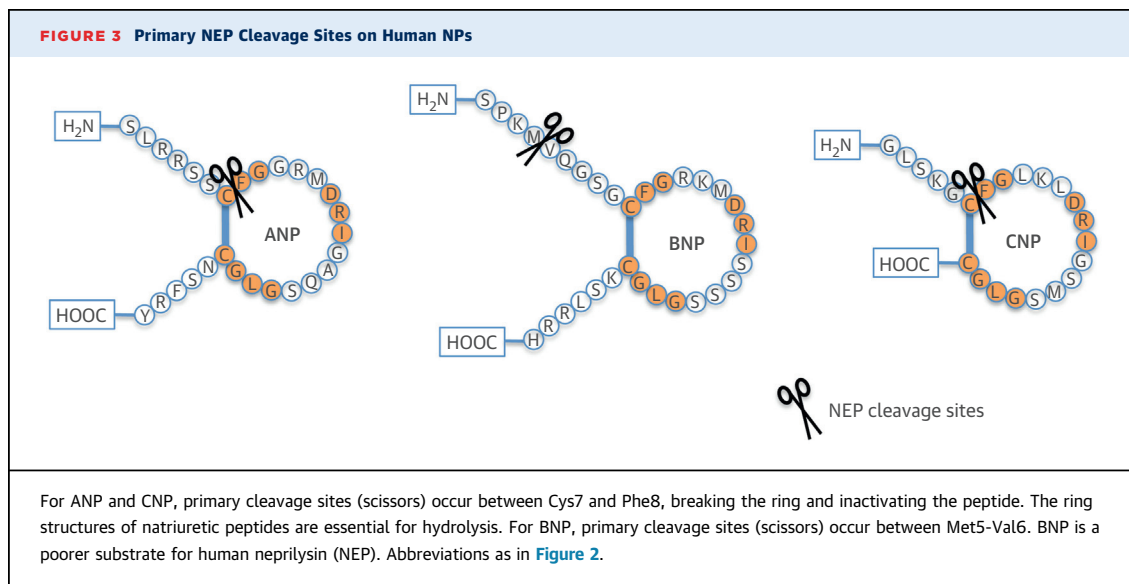
Initial and secondary NEP cleavage sites on NP are shown in **Figure 3**. In health, proteolytic cleavage and removal by the "clearance" NP receptor, NPR-C, play equal roles in metabolism of the NPs, but in high NP states, such as HF, it seems likely that NEP plays an increasingly important role (34). Elevation of BNP and concurrent decline in N-terminal pro-B-type natriuretic peptide (NT-proBNP) with angiotensin receptor neprilysin inhibitor (ARNi) therapy are commonly attributed to delayed BNP metabolism and decreased cardiac release of B-type peptides (presumed to reflect beneficially reduced intracardiac pressures), respectively. However, a recently proposed alternative explanation pivots around the idea that ARNis may promote peptide glycosylation, thus not only impeding cleavage of proBNP into BNP and NT-proBNP, but also rendering NT-proBNP invisible to current immunoassays. BNP assays cross-react to proBNP regardless of glycosylation; thus, an accumulation of the uncleaved precursor will generate a rise in BNP readings (43). This interesting hypothesis awaits further investigation.
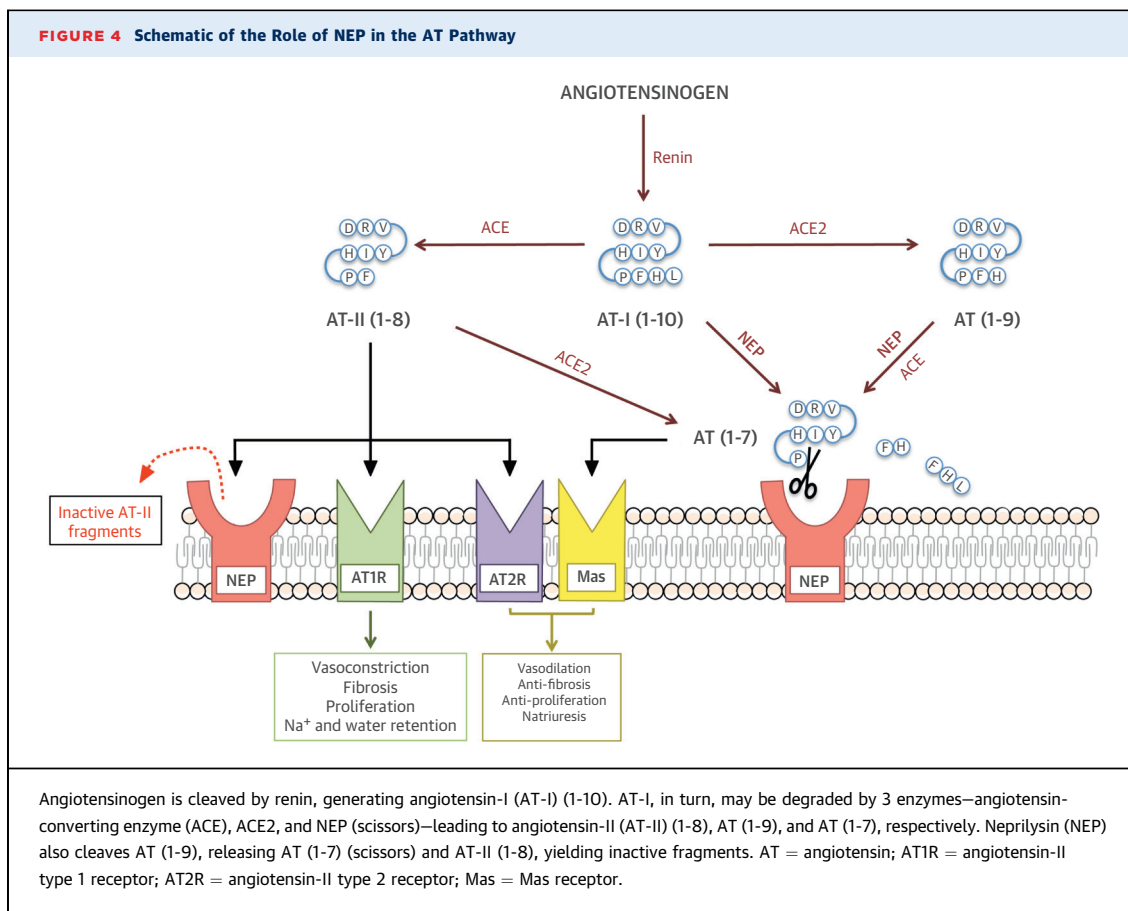
NEP AS A BIOTARGET

NEP INHIBITION AND THE HEART. The development of multiple neprilysin inhibitors (NEPis) has enabled clarification of the role of the enzyme in regulating levels of NPs and other substrates. The specificity and bioavailability of these agents has been well reviewed and cannot be detailed here (16). Clearly, relative affinity for NEP, achieved circulating concentrations, and the spectrum of tissue penetration by specific inhibitors (to variably access the wide array of NEP substrates), will influence the net impact of any single NEP inhibitor. Thiorphan, the first synthetic NEPi, was followed by many other agents suitable for both oral and parenteral administration; these agents have been tested in animals (42,44,45) and humans in health and disease (16,46-56). In healthy humans, and during hypertension and HF, NEPis consistently induce elevations in endogenous plasma concentrations of ANP and BNP, in association with increased circulating and urine cGMP, and at least brief beneficial hemodynamic responses (46-54).

NEPis also delay clearance of infused exogenous NPs (49-51). NP-related effects of NEPis, including natriuresis, increased renal filtration, and vasodilation, have been observed (46-51). However, the hypotensive effects of NEPis alone are modest and transient (47-49), and in severe HF, NEPis had no significant diuretic effect (54). After several days of NEPi administration, plasma ANP and cGMP remain elevated, but activation of both the renin-angiotensin-aldosterone and sympathetic nervous systems becomes evident, with increased plasma renin activity, angiotensin-II, aldosterone, and norepinephrine (49). In humans pretreated with NEPi, clearance of angiotensin-II during stepped infusions of exogenous angiotensin-II is clearly reduced (56). These findings in humans are consistent with in vitro data demonstrating the role of NEP in metabolizing angiotensin-II. In addition, NEP converts angiotensin-I to angiotensin 1-7, and inhibition of this process will enhance substrate for alternative conversion to angiotensin-II (**Figure 4**) (57). Bolus intravenous single

FIGURE 3 Primary NEP Cleavage Sites on Human NPs

For ANP and CNP, primary cleavage sites (scissors) occur between Cys7 and Phe8, breaking the ring and inactivating the peptide. The ring structures of natriuretic peptides are essential for hydrolysis. For BNP, primary cleavage sites (scissors) occur between Met5-Val6. BNP is a poorer substrate for human neprilysin (NEP). Abbreviations as in **Figure 2**.

FIGURE 4 Schematic of the Role of NEP in the AT Pathway

Angiotensinogen is cleaved by renin, generating angiotensin-I (AT-I) (1-10). AT-I, in turn, may be degraded by 3 enzymes—angiotensin-converting enzyme (ACE), ACE2, and NEP (scissors)—leading to angiotensin-II (AT-II) (1-8), AT (1-9), and AT (1-7), respectively. Neprilysin (NEP) also cleaves AT (1-9), releasing AT (1-7) (scissors) and AT-II (1-8), yielding inactive fragments. AT = angiotensin; AT1R = angiotensin-II type 1 receptor; AT2R = angiotensin-II type 2 receptor; Mas = Mas receptor.

doses of the NEPi SCH32615 administered to patients undergoing cardiac catheterization induced clear elevations in ANP and BNP throughout the circulation. Absolute and proportional increments in ANP were far more pronounced than those in BNP, presumably reflecting the lesser role of NEP in the clearance of human BNP compared with ANP (58). This scenario raises the question of which NP is exerting the greatest bioactivity during NEPi administration and which might be the preferred NP for monitoring the NEPi effect. Data about the response of CNP (the NP with the highest affinity for NEP) to NEPi are not available.

Bradykinin is a substrate of both ACE and NEP, and it could theoretically contribute to the clinical renal and vasodilator effects of NEPi. In practice, the relative contributions of bradykinin and NP are unclear. Preclinical studies have yielded inconsistent results, with either ANP antisera or bradykinin antagonists altering NEPi-induced renal and hemodynamic differently in various models (16). In patients with HF on background treatment with ACE inhibition, the NEPi thiorphan augmented the vasodilator response to bradykinin (59). Whether similar effects

(in part or in full) would be observed with background angiotensin receptor blockade rather than ACE inhibition is uncertain. The possible contribution to the cardiorenal effects of NEPi effects during health and/or HF by other vasoactive substrates of NEP is unclear. In vitro data demonstrate weak conversion of big endothelin to endothelin-1, and more active cleavage of endothelin-1 and endothelin-3 (and, to a lesser extent, endothelin-2) by NEP (60-63). In rats, NEPi elevates plasma endothelin-1 by ~40% (63). However, there is no certainty on the relevance of altered circulating or tissue-based endothelin concentrations in modulating the overall renal and hemodynamic responses to NEPi in human health or disease. NEPi reduces clearance of infused adrenomedullin and enhances renal responses to adrenomedullin in canine and ovine models (64,65). Whether this outcome is a direct effect of the potent vasodilator on proteolysis or reflects an indirect mechanism remains uncertain, and the contribution of this effect to the net renal and cardiovascular impact of NEPi is also uncertain. Recently, an in vitro study on isolated small human resistance arteries found that the prototype NEPi, thiorphan, enhanced the vasodilator response to

bradykinin but not that of adrenomedullin, CNP, calcitonin gene-related peptide, vasoactive intestinal polypeptide, or substance P (66).

Evidence of concurrent activation of the renin-angiotensin-aldosterone system, together with augmentation of NP bioactivity by NEPi monotherapy, inspired development and testing of agents that combine NEPi and ACE-inhibiting activity: the so-called vasopeptidase inhibitors. Several vasopeptidase inhibitors have been developed, including omapatrilat, fasidotril, sampatrilat, and mixanpril (67). Encouraging preclinical data and early-phase human trials resulted in the leading agent, omapatrilat, progressing to Phase II and III trials in HF (68,69). In the IMPRESS (Inhibition of Metallo Protease by Omapatrilat in a Randomized Exercise and Symptoms Study of Heart Failure) trial of 573 patients with New York Heart Association functional class II to III HF, omapatrilat decreased rates of a composite endpoint (death, hospitalization, or discontinuation of treatment for worsening HF) to 6%, compared with 10% on lisinopril (p = 0.035) (70). However, in the Phase III OVERTURE (Omapatrilat Versus Enalapril Randomized Trial of Utility in Reducing Events) trial

of 4,400 patients, omapatrilat was not superior to enalapril with respect to the primary endpoint of death from any cause or admission for HF (71). Notably, the main secondary endpoint (death from any cause or any cardiovascular admission) was significantly reduced by 9% and, in a post hoc analysis using the same diagnostic criteria as applied to the original placebo-controlled trials (SOLVD [Studies of Left Ventricular Dysfunction]) of enalapril in chronic HF, the modified composite primary endpoint was reduced by 11% (p = 0.012). These indications of benefit from vasopeptidase inhibition were overshadowed by an excess of severe angioedema, which led to cessation of vasopeptidase development (72). The increased incidence of angioedema is possibly due to excess bradykinin produced by the combined vasopeptidase inhibitor effects of inhibiting not only ACE and NEP but also aminopeptidase P (73) (Central Illustration). This issue could be avoided by the combination of NEPi with an angiotensin-receptor blocker (ARB) instead of an ACE inhibitor.

The combination of NEPi and ARB, also known as ARNi (angiotensin receptor-neprilysin inhibitor), is under evaluation in an extensive clinical trial

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CENTRAL ILLUSTRATION Neprilysin in Heart Failure: Valuable as Biotarget and Biomarker
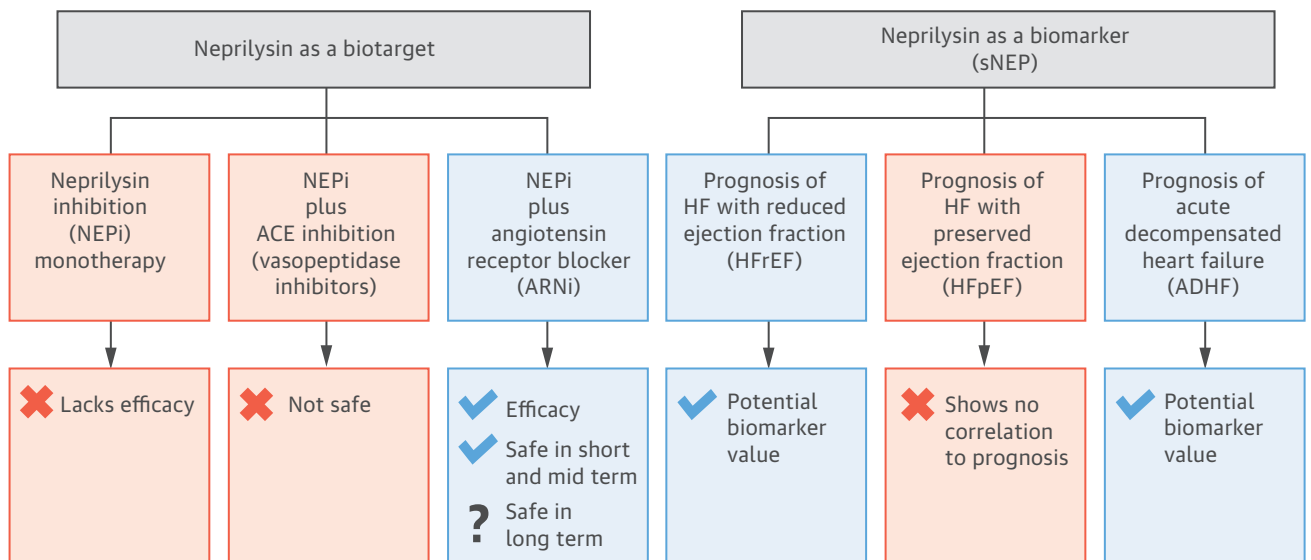
Bayes-Genis, A. et al. J Am Coll Cardiol. 2016;68(6):639-53.

Neprilysin is a highly versatile enzyme, again in the spotlight, after an eventful career of >40 years. For decades, neprilysin has been an important biotarget. Neprilysin inhibitor (NEPi) monotherapy failed due to lack of efficacy, and NEPi + angiotensin-converting enzyme (ACE) inhibition was abandoned due to lack of safety. Recent development of angiotensin receptor–neprilysin inhibitor (ARNi) has shown robust evidence of safety and short-term to mid-term efficacy in patients with heart failure with reduced ejection fraction (HFrEF). The value of neprilysin as a biomarker (soluble neprilysin [sNEP]) is more recent, and it has been explored in 3 settings: HFrEF, HF with preserved ejection fraction (HFpEF), and acutely decompensated heart failure (ADHF). Preanalytical and analytical gaps must be addressed before its translation from research-only to the clinic.

platform, ranging from hypertension to HF with both reduced and preserved ejection fraction. The PARADIGM-HF trial, the largest (n = 8,442) and most geographically diverse (1,043 clinical centers) trial in HF performed to date, evaluated the clinical benefit of sacubitril/valsartan compared with enalapril in New York Heart Association functional class II to IV HF and left ventricular ejection fraction <40% (protocol subsequently amended to left ventricular ejection fraction <35%) (1). The inclusion criteria were pioneering in clinical trial research for HF; they included the requirement of raised biomarker levels (BNP ≥150 pg/ml or NT-proBNP ≥600 pg/ml). Subjects were randomized to receive 200 mg of sacubitril/valsartan twice daily (n = 4,187) or 10 mg of enalapril twice daily (n = 4,212), in combination with conventional systolic HF therapy, including beta-blockers (93% of patients), diuretic agents (80%), mineralocorticoid receptor antagonists (56%), and digoxin (30%), as well as implantable cardioverter-defibrillators (15%) or cardiac resynchronization therapy (7%).

Abrupt trial termination occurred after a third interim efficacy analysis demonstrated overwhelming reductions in death from cardiovascular causes and the primary endpoint (cardiovascular death or hospitalization secondary to HF) (Central Illustration). The PARADIGM-HF trial is also referred to as the 20% trial because of the homogeneous relative reduction of approximately 20% for all studied endpoints, including the composite primary endpoint, cardiovascular death, sudden cardiac death, and hospitalization for HF. The run-in procedures and recruitment criteria (very few African-American subjects) may have biased the PARADIGM-HF population against inclusion of patients prone to angioedema. Indeed, angioedema was not significantly higher in patients receiving sacubitril/valsartan and, despite a higher incidence of hypotension in the sacubitril/valsartan arm, discontinuation of therapy was significantly less common overall in the sacubitril/valsartan group. No increased incidence of cognition- or dementia-related adverse events was reported in the PARADIGM-HF trial. However, these effects may not have been detected to date because dementia may take longer to develop than the current observation period for trial participants. Also, subjects with mild dementia were not expected to participate, and no specific test on cognitive function was performed in PARADIGM-HF.

The biomarker substudy of the PARADIGM-HF trial revealed that NEPi with sacubitril/valsartan (74) increased levels of both urinary cGMP and plasma BNP, and was associated with consistently lower levels of NT-proBNP (reflecting reduced cardiac wall stress) and troponin (reflecting reduced cardiac injury) throughout the trial. The contrasting effect of sacubitril/valsartan on the 2 NPs is an important finding. Despite the stoichiometric release of BNP and NT-proBNP during the course of HF, BNP (but not NT-proBNP) is a substrate for NEP. Hence, levels of BNP reflect the action of the drug, whereas levels of NT-proBNP reflect sacubitril/valsartan cardioprotective effects. Indeed, only NT-proBNP should be used to monitor the cardiac status of patients treated with sacubitril/valsartan, as needed.

The evidence from PARADIGM-HF supports the combination of NEPi with ARB as an effective strategy for further optimization of outcomes in patients with heart failure with reduced ejection fraction (HFrEF). Indeed, the absolute reductions in the primary endpoint, cardiovascular death, and all-cause mortality translate to numbers needed-to-treat of 21, 32, and 36 patients, respectively, over a 27-month period. The next step is to examine the value of ARNi in patients with heart failure with preserved ejection fraction (HFpEF). The PARAMOUNT (Prospective Comparison of ARNi With ARB on Management of Heart Failure With Preserved Ejection Fraction) study is a Phase II clinical trial in patients with HFpEF with a primary endpoint of change from baseline in NT-proBNP levels at 12 weeks (75). NT-proBNP was significantly reduced in the sacubitril/valsartan group compared with the valsartan group (p = 0.005), which prompted the development of the ongoing PARAGON-HF (Efficacy and Safety of LCZ696 Compared to Valsartan, on Morbidity and Mortality in Heart Failure Patients With Preserved Ejection Fraction) study, which is investigating whether sacubitril/valsartan significantly reduces morbidity and mortality in HFpEF.

NEP INHIBITION AND THE BRAIN. NEP participates with other peptidases in the degradation of endogenous opioids and amyloid-beta peptide (5,6,15,16). Early synthetic NEPis were developed as potential analgesic agents operating through enhancement of endogenous opioids (16). Genetic disruption of NEP induces relatively subtle phenotypic changes in enkephalin clearance and nociceptive function. Indeed, NEPi alone is not powerfully analgesic and requires coinhibition by other peptidases to match the analgesic effects of morphine (16). However, the role of NEP as the leading enzyme for clearance of amyloid-beta peptide is more central and, in this context, deletion or inhibition of brain NEP may have more important consequences (76-78). The accumulation of amyloid-beta peptide is a key pathophysiological component of Alzheimer's disease, in which NEP activity is reduced, the soluble and insoluble

amyloid-beta content of the brain is increased, and amyloid plaques appear along with neurofibrillary tangles. Introduction of an NEPi into mouse brain or a genetic deletion of NEP in mice that are usually also transgenic for an amyloid precursor protein variant generally replicates these findings, in association with indicators of impaired cognitive function (77-79). Introduction of the NEP gene using a lentivirus vector has been reported to rescue this progression (80). However, reports are not unanimous, and Meilandt et al. (81) have demonstrated reduction in plaque accumulation with no reduction in amyloid-beta oligomers and absence of improvement in deficits in learning and memory in amyloid precursor transgenic mice with overexpression of NEP.

Controversially, polymorphisms in the NEP gene may confer an increased risk for Alzheimer's disease, with susceptibility to this disease further enhanced when insulin-degrading enzyme, another amyloid-beta-degrading enzyme, also has a polymorphism (76). Recently, a further single-nucleotide variant of the NEP gene has been associated with increased risk of Alzheimer's disease in a Han Chinese population (82). Conversely, other studies do not report any association between NEP polymorphisms and risk of Alzheimer's disease, and a recent large meta-analysis found no association between variations in the NEP gene and Alzheimer's disease (83). Overall, these findings raise the possibility that chronic use of NEPis capable of crossing the blood-brain barrier might accelerate amyloid-beta accumulation and initiate or exacerbate dementia. In sharp contradistinction to the development of NEPis as a therapeutic avenue in heart disease, efforts have been made to develop NEP variants with enhanced affinity for amyloid-beta 1-40 and 1-42 for selective augmentation of brain NEP activity as a potential therapy for Alzheimer's disease (84). In view of the likely prolonged use of NEPIs in the context of combination ARB/NEPi agents in HF, clarification of the clinical relevance of this well-founded theoretical concern is mandatory.

NEP INHIBITION AND OTHER ORGAN SYSTEMS. The gastrointestinal and respiratory tracts are sites rich in NEP, together with key relevant substrates, including substance P and a clutch of gut regulatory hormones (**Table 1**). This raises the possibility of NEPis triggering unintended and potentially adverse effects in these systems.

In the lung, NEP's location near neurokinin receptors allows cleavage of tachykinins (exemplified by substance P) (25,26). NEP inactivates tachykinins released from pulmonary sensory nerves, thus limiting neurogenic inflammation and diminishing

smooth muscle contraction, bronchiolar secretion, neutrophil adhesion, and vascular permeability in experimental models of lung injury and inflammation (25,27). Epithelial injury from respiratory viral infections and exposure to irritants (e.g., cigarette smoke, toluene diisocyanate) reduces NEP levels (25). Accordingly, recombinant NEP aerosol suppresses cough responses during experimental neurogenic inflammation (27). Therefore, it is possible that NEP inhibition may lower thresholds for neurogenic lung and pulmonary airway inflammation.

In the gut, substance P and other tachykinins, operating via neurokinin receptors, may potentially exaggerate plasma extravasation and neutrophil infiltration in the case of inflammatory insults. In a mouse model of pancreatitis, genetic deletion of NEP significantly worsened survival (0 vs. 90% at 120 h; p < 0.001) and increased pancreatic myeloperoxidase release and pancreatitis-associated lung injury (85). In experimental enteritis triggered by *Clostridium difficile* toxin, both genetic deletion of NEP and its inhibition with phosphoramidon reduced the threshold secretory and inflammatory dose of toxin, and exacerbated the inflammatory responses, with 2-fold increases in stimulated granulocyte transmigration, myeloperoxidase activity, and histological damage, including depletion of enterocytes, edema, and neutrophil accumulation (86). In a NEP knockout animal, the enhanced inflammatory response was prevented by pretreatment with recombinant NEP. These experimental findings raise the possibility that NEP inhibition may exacerbate inflammatory gut pathology.

At present, these theoretical problems are supported by pre-clinical experimental data only. The data available in humans with respect to adverse side effect profiles from administration of NEPis, vasopeptidase inhibitors, or ARNis have not triggered alarm signals with respect to accelerated dementia or exacerbation of lung or gastrointestinal inflammatory pathologies. In total, exposure to NEPis must now amount to thousands of patient-years, and it seems likely that associations with specific classes of acute adverse events would have been recognized. Notably, the NEPi acetorphan is even marketed as an effective antidiarrheal agent (87). However, trial recruitment criteria inevitably lead to preferential participation by patients with, on average, a lesser burden of comorbidities than observed in "real-life" clinical heart failure populations, and continued monitoring is necessary. With respect to more chronic pathologies, such as progressive Alzheimer's disease, it is clear that a longer period of systematic monitoring of a sufficient population of older patients is needed before vigilance can be relaxed.

NEP AS A BIOMARKER IN HF

NEP, similar to other membrane-bound metalloproteases, may be released from the cell surface, producing a nonmembrane-associated form that retains catalytic activity (15-19,23,88). The mechanism by which NEP is released in the bloodstream may occur as a consequence of ectodomain shedding, or due to release of nonmembrane-associated sNEP via exosomes, a process dependent on ADAM metallopeptidase domain 17. Values for NEP activity in serum (assayed with 2-step hydrolysis) were reported 30 years ago in patients with acute respiratory distress syndrome and cardiogenic pulmonary edema (19). Nevertheless, evidence on the circulating enzyme as a pathobiological biomarker surrogate in patients with HF has only recently been reported (89).

In chronic HF, sNEP was evaluated in 1,069 patients, mainly with HFrEF (90). In comprehensive multivariable analyses that included conventional clinical risk stratifiers and NT-proBNP, sNEP remained significantly associated with both the composite endpoint of cardiovascular death and HF hospitalizations (hazard ratio: 1.18; p = 0.001) and cardiovascular death (hazard ratio: 1.18; p = 0.006) (90) (**Central Illustration**). By virtue of its central role in neurohormonal regulation, direct comparison of sNEP (reflective of multiple neurohormonal cleavage) and NT-proBNP (surrogate of NP release and current standard of care) was conducted in the same cohort. The lack of association between sNEP and NT-proBNP may be explained by the direct role of sNEP in cleaving neurohormones activated in HF (i.e., BNP, angiotensin-II) and its null effect on inactive circulating peptides, such as NT-proBNP. The data revealed that, when added to a multimarker strategy, sNEP remained an independent prognosticator, whereas NT-proBNP lost significance as a risk

stratifier in ambulatory HF. Moreover, sNEP was substantially less affected by comorbidities, in particular renal dysfunction and obesity (91). These data provide the rationale for further understanding the value of measuring sNEP in patients with HFrEF receiving sacubitril/valsartan, and they underscore the potential for precision medicine in HF using sacubitril/valsartan relative to circulating sNEP levels. In the setting of chronic HFpEF, Goliasch et al. (92) recently reported on a well-characterized cohort of 144 HFpEF patients. The investigators found unusually high levels of sNEP, 3-fold higher than those found in HFrEF, yet without correlation with prognosis. Issues regarding pre-analytic and analytic considerations will be discussed later.

More recently, the value of sNEP was also examined in acute HF in 2 studies. Bayés-Genís et al. (93), in a multicenter cohort of 350 patients admitted with acutely decompensated HF, found that circulating sNEP levels also have biomarker value by offering independent prognostic information (**Central Illustration**). Vodovar et al. (41), in a subanalysis of the GREAT (Global Research on Acute Conditions Team) study (which explored the hypothesis that BNP may act as an endogenous NEPi), provided information about NEP activity and concentration in patients with acute HF (and also in a subgroup of chronic HF patients); they had not set out to examine NEP as a surrogate biomarker. The investigators found elevated circulating levels of sNEP that were moderately higher in patients with the highest BNP levels, but they did not explore their prognostic value. **Table 2** dissects the clinical and assay differences between studies to date that have reported data on sNEP in HF.

NEP may eventually emerge as a novel biomarker (94). However, the various assays currently used to measure NEP levels require further refinement and

TABLE 2 Clinical Studies Using sNEP for HF Prognostication

First Author (Ref. #)	HF Setting	n	Age, yrs	LVEF, %	NT-proBNP, ng/l	Female, %	Assay	Lot No.	Matrix	sNEP, pg/ml
Bayés-Genís et al. (90)	HFrEF	1,069	66.2	33.5	1,302	28.2	SK00724-01 Aviscera Bioscience (Santa Clara, CA)	20111893	Serum	642
Vodovar et al. (41)	ADHF	469	74	45	5,682	47	SEB785Hu USCN Life Science (Wuhan, China)	NA	Plasma	314
Bayés-Genís et al. (93)	ADHF	350	72.6	46.6	3,953	53.1	SK00724-01 Aviscera Bioscience	20112070	Serum	670
Goliasch et al. (92)	HFpEF	144	72	62	1,148	65	SEB785Hu USCN Life Science	NA	Plasma	2,862

ADHF = acutely decompensated heart failure; HF = heart failure; HFpEF = heart failure with preserved ejection fraction; HFrEF = heart failure with reduced ejection fraction; LVEF = left ventricular ejection fraction; NA = not available; NT-proBNP = N-terminal pro-B-type natriuretic peptide; sNEP = soluble neprilysin.

FIGURE 5 Comparison of 4 Commercial Immunoassays for sNEP
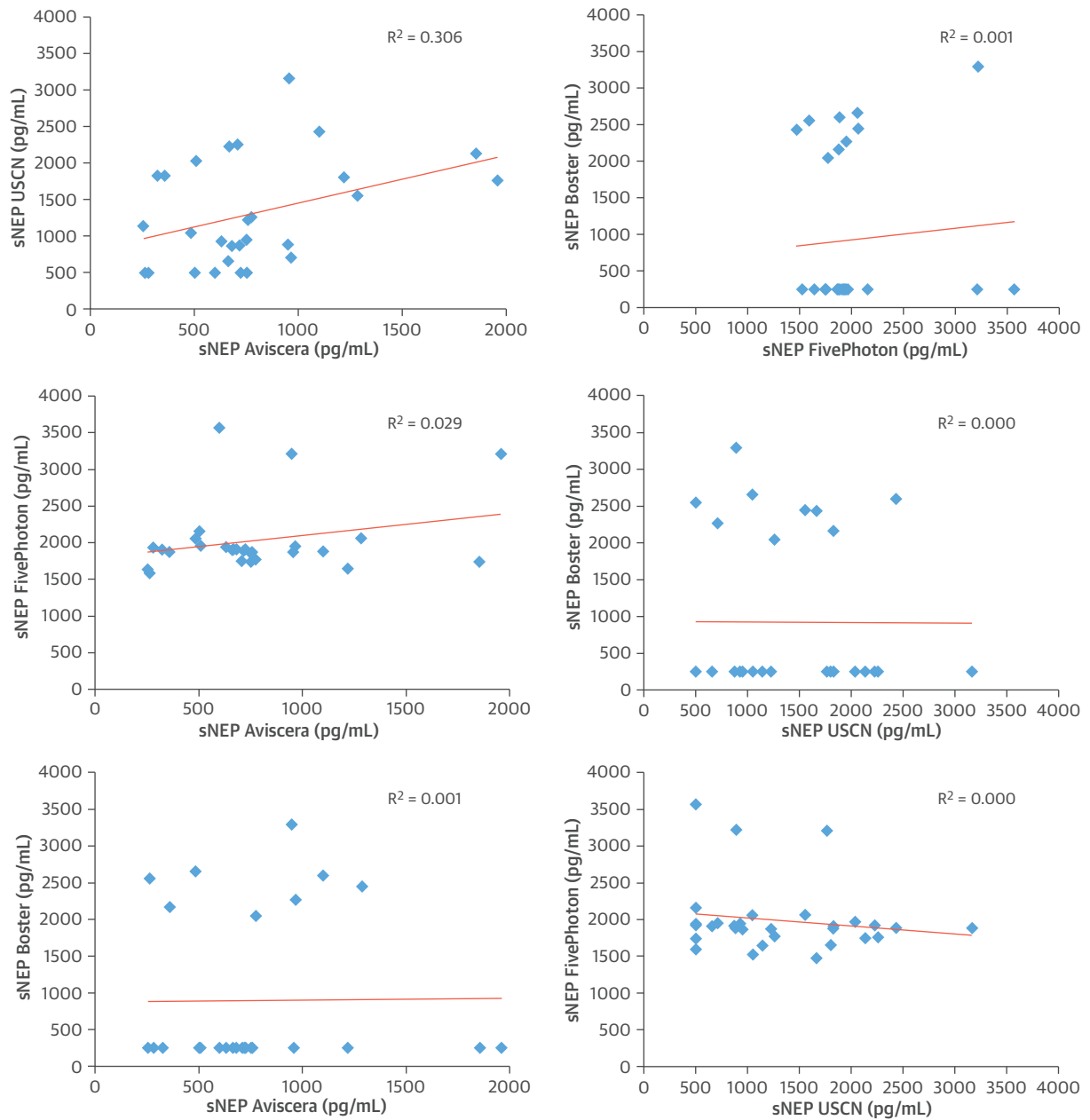
Thirty serum samples were processed in duplicate, each from a different aliquot stored at –80°C. The 4 commercial assays used were sNEP Aviscera (Aviscera Bioscience, Santa Clara, California), sNEP USCN (USCN Life Science, Wuhan, China), sNEP FIVEphoton (FIVEphoton Biochemicals, San Diego, California), and sNEP Boster (BosterBio, Pleasanton, California). An alarming lack of correlation was observed between assays. sNEP = soluble neprilysin.

validation. Comparing results from different studies, it is crucial to understand potential pitfalls regarding both analytic and pre-analytic issues. First, regarding preservation, all sNEP studies reported to date used samples stored at –80°C, but there are no published data concerning sNEP stability. Our in-house experience shows that sNEP is stable after 24 h at 5°C, but we acknowledge that this issue is, as yet, ill-characterized. Second, all currently available assay kits target wide areas of the protein's extracellular domain (52-750 amino acids) and might also react with nonsoluble NEP. Indeed, NEP is a surface marker

of mature neutrophils (95); thus, appropriate centrifugation and sample separation are key to prevent unwanted membrane-bound NEP contamination. Third, deciding on serum as the sample source is not a trivial issue. After appropriate centrifugation, serum samples are in principle noncellular, whereas plasma samples may contain residual cells that could add confusion to an already complicated analysis (96). Moreover, improperly performed venipuncture may contribute to cellular lysis, releasing not only internal proteins but also parts of membrane-bound elements. Thus, although measurements with the clinically approved diagnostic kits yield similar analytic recoveries in serum and plasma, in the case of sNEP, information is scarce.

If pre-analytic considerations are complex, the analytic process of measuring sNEP is simply puzzling. The lack of reference materials and reference measurement procedures seems logical at this early stage of scientific interest, but data comparisons using the different commercially available immunoassays reveal an alarming lack of transferability (Figure 5). This scenario cannot be explained by 1 single factor but rather by an accumulation of many. Immunogen sequence, antibody clonality, and calibrator choice are not well detailed by the manufacturers, as shown in Table 3. These may be key issues, in addition to different dilution patterns and recommended assay temperatures.

As previously described in the present review, NEP belongs to the family of zinc-dependent metallopeptidases. These include the endothelin-converting enzymes 1 and 2, the Kell blood group protein, and the PEX gene product (97). Because most of these proteins have structural similarities to NEP, cross-reactivity studies are indeed a major issue. Moreover, the amino-acid sequences of the membrane metalloendopeptidase-like proteins MMEL and NEP2 are also highly homologous with NEP. NEP2 has

2 alternatively spliced forms: a membrane-bound and a soluble-secreted variant. The latter, also known as soluble secreted endopeptidase (98), might be an interesting biomarker itself, but it can also be a highly confusing factor for sNEP analyses.

The ideal biomarker assay should be identified by a combination of 2 monoclonal antibodies specific to a well-defined region of sNEP, with null cross-reactivity with the aforementioned similar molecules. Unfortunately, these requirements are not fulfilled by any of the marketed immunoassays. So which assay should we choose? According to data found in published reports, an effective limit of quantification of at least 0.250 μg/ml seems a minimum requirement for sNEP studies, as this limit has an intraseries coefficient of variation of <10% and an interseries coefficient of variation of <15% along the measurement range. Performance characteristics of the 4 commercially available assays fulfilling these characteristics are listed in Table 3. The assay from Aviscera Bioscience (Santa Clara, California) may, at present, be preferred because of its adequate cross-reactivity studies, although it uses polyclonal antibodies.

The pioneering studies that investigated sNEP in clinical HF cohorts used a modified Aviscera sandwich immunoassay. Serum was chosen to minimize pre-analytic issues and matrix complexity. To improve analytic sensitivity and to obtain a lower limit of sample quantification, several modifications were made to incubation time, assay temperature, mixing intensity, and dilution pattern. Of note, after our publication, Aviscera Bioscience made modifications to the recommended procedures, including the recommended dilution patterns. Unfortunately, we recently detected a relevant analytic issue with a new large lot from Aviscera Bioscience (number 20112126), which displayed very poor sensitivity, despite calibration curves similar to those of previous

TABLE 3 Performance Characteristics of the 4 Commercially Available Assays for sNEP

	Coating Antibody	Detection Antibody	Assay Range, pg/ml	Intraassay CV, %	Interassay CV, %	Protocol	Controls Provided	Cross-Reactivity Studies
SK00724-01 Aviscera Bioscience (Santa Clara, CA)	Polyclonal	Polyclonal	125–8,000	6	12	2-step (sandwich)	Positive control, 500 pg/ml	Human ECE-1 and ECE-2 Human NEP-2 Mouse Kell
SEB785Hu USCN Life Science (Wuhan, China)	Not specified	Not specified	31–2,000	10	12	2-step (sandwich)	None	NA
EK0851 Boster Bio (Pleasanton, CA)	Monoclonal	Polyclonal	125–8,000	5.6	7.3	2-step (sandwich)	None	NA
FIVEphoton Biochemicals (San Diego, CA)	Monoclonal	Polyclonal	5–1,000	NA	NA	Single-step	None	NA

CV = coefficient variation (highest reported) according to the manufacturer; ECE = endothelin-converting enzyme; NEP = neprilysin; other abbreviations as in Table 2.

lots. Such inconsistency is a major problem with polyclonal antibody-based assays, and it is not easy to avoid. We also explored the FIVEphoton Bio-chemicals (San Diego, California) immunoassay, namely because it incorporates a monoclonal coating antibody, which may grant it better specificity, and a series of low-concentration calibrators for a better quantification range. Surprisingly, in-house analyses with this kit diverged substantially from data derived with other commercially available sNEP kits (**Figure 5**). Morover, Goliash et al. (92) and Vodovar et al. (41) used the USCN Life Science (Wuhan, China) kit, which provides the closest relationship with Aviscera Bioscience. Logic suggests that any assay with monoclonal antibodies should be more specific than a polyclonal approach, but questions about the exact immunogen targeted and potential cross-reactivities have arisen.

CONCLUSIONS

It is mandatory that immunoassays for sNEP quanti-fication follow a stringent path from "research only" to US Food and Drug Administration/Conformité Européene approved before their use is considered in clinical practice. The correct quantification of sNEP remains a challenge that needs to be overcome to suppress potential biases regarding the interpre-tation of the different studies. Is our selected immunogen a stable molecule, or are we detecting fragments thereof? Do neutrophil populations influence sNEP outcomes? Proteomic studies, combining high-performance separation techniques and mass spectrometry, seem the next logical step for valid characterization of the target protein.

Membrane-bound NEP has been well characterized in diverse tissues, where it is relatively abundant, but serum screening for new soluble fragments at concentrations as low as picograms per milliliter is not trivial. Human plasma contains >1,000 identi-fied proteins that create a complex network (99), and sNEP separation, fragmentation, and correct characterization may take time.

We borrow the epilogue written by Erdös and Skidgel in 1989 to conclude the present review (15): "Neprilysin may be considered as a sort of hero in a 19th century novel. Neprilysin had a humble begin-ning, cleaving the B chain of insulin after having been discovered in the proximal tubules. . . . Subsequently, neprilysin acquires important functions in high pla-ces, such as cleaving substance P in the lung or enkephalin in the brain. . . . As is typical of a Victorian character, it also has a darker side connected with sex: neprilysin has an unexplored, unexplained function by existing in high concentrations in the male genital tract. Nevertheless, it finally returns to its site of origin in triumph by being a key renal enzyme, cleaving the glamorous substrate [natri-uretic peptides], the peptide hormone from the heart. Obviously, further chapters to this story will be added in the future." Indeed, neprilysin, both as a biotarget and as a biomarker, has a splendid future.

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