Opinion Paper

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New issues on measurement of B-type natriuretic peptides

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Abstract: The measurement of the active hormone of B-type natriuretic peptide (BNP) system actually has several analytical limitations and difficulties in clinical interpretations compared to that of inactive peptide N-terminal proBNP (NT-proBNP) because of the different biochemical and pathophysiological characteristics of two peptides and quality specifications of commercial immunoassay methods used for their measurement. Because of the better analytical characteristics of NT-proBNP immunoassays and the easier pathophysiological and clinical interpretations of variations of NT-proBNP levels in patients with heart failure (HF), some authors claimed to measure the inactive peptide NT-proBNP instead of the active hormone BNP for management of HF patients. The measurement of the active peptide hormone BNP gives different, but complementary, pathophysiological and clinical information compared to inactive NT-proBNP. In particular, the setup of new more sensitive and specific assays for the biologically active peptide BNP1-32 should give better accurate information on circulating natriuretic activity. In conclusion, at present time, clinicians should accurately consider both the clinical setting of patients and the analytical characteristics of BNP and NT-proBNP immunoassays in order to correctly interpret the variations of natriuretic peptides measured by commercially available laboratory methods, especially in patients treated with the new drug class of angiotensin receptor-neprilysin inhibitors.

Keywords: ARNi; biomarker-guided therapy; B-type natriuretic peptide (BNP); heart failure; immunoassay; inhibition; LCZ696; neprilysin; NT-proBNP.

Analytical characteristics of BNP and NT-proBNP methods

The measurement of the active hormone of B-type natriuretic peptide (BNP) system actually presents several analytical limitations and difficulties in clinical interpretations compared to that of inactive peptide N-terminal proBNP (NT-proBNP) because of the different biochemical and pathophysiological characteristics of two peptides (Table 1) and the quality specifications of commercial immunoassay methods. Indeed, the inactive peptide NT-proBNP fits better the analytical and clinical characteristics required for an ideal laboratory biomarker than the active peptide BNP. NT-proBNP is more stable in vivo and in vitro: it has a higher molecular mass and a longer biological plasma half-life and a lower intra-individual biological variation (Table 1). Furthermore, data from external quality assessment studies demonstrated that immunoassays methods for NT-proBNP share on average better analytical performances (including better sensitivity and imprecision) than those of BNP [1–4]. Furthermore, these studies also reported that there are large systematic differences (up to twofolds) between the BNP values measured by the most popular immunoassay methods, whereas the NT-proBNP methods show a between-method variability lower than 20% because they use calibrators and materials from the same manufacturer [3, 4]. Finally, from a clinical point of view, the circulating levels of NT-proBNP show a greater and progressive increment (on average, more than 120-folds compared to healthy subjects) from early to more severe disease in patients with heart failure (HF), as assessed by NYHA functional class, than those of BNP (i.e. on average, only an increment of about 50-folds) (Figure 1 and Table 2), measured by an immunoradiometric assay (IRMA) [5, 6]. Of course, the ratio between NT-proBNP and BNP is greatly method dependent [5–8]; as a
Controls (n = 85)

NYHA I (n = 25)

NYHA II (n = 91)

NYHA III (n = 48)

NYHA IV (n = 29)

Increase of biomarkers compared to control group

Figure 1: Progressive increase in BNP and NT-proBNP levels from less to more severe stages of HF, assessed by NYHA functional class. The plasma concentration values of NT-proBNP, measured by the ECLIA method (Roche Diagnostics, Germany), and BNP, measured by the immunoradiometric assay (IRMA) Shionoria (Shionogi & Co, Japan), are expressed as the ratio between the mean biomarker concentration values found in HF groups and that found in healthy control subjects. Original BNP and NT-proBNP values measured in healthy subjects and patients with heart failure are reported in Table 2 and were previously discussed in detail by Prontera et al. [2].

Table 1: Biochemical and physiological characteristics of BNP, NT-proBNP and proBNP peptides.

<table>
<thead>
<tr>
<th></th>
<th>BNP</th>
<th>NT-proBNP</th>
<th>proBNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular mass</td>
<td>3462 Da</td>
<td>8457 Da*</td>
<td>11,900 Da*</td>
</tr>
<tr>
<td>Amino acids</td>
<td>32</td>
<td>76</td>
<td>108</td>
</tr>
<tr>
<td>Biological function</td>
<td>Active hormone</td>
<td>Inactive</td>
<td>Pro-hormone</td>
</tr>
<tr>
<td>Half life</td>
<td>15–20 min</td>
<td>&gt;60 min</td>
<td>&gt;60 min</td>
</tr>
<tr>
<td>Glycosylation</td>
<td>Non-glycosylated</td>
<td>Highly glycosylated in vivo</td>
<td>Highly glycosylated in vivo</td>
</tr>
</tbody>
</table>

*The molecular mass (MM) of NT-proBNP and proBNP depends to the degree of glycosylation of the peptide; the MM of non-glycosylated peptides is reported in the table.

result, the data reported in Figure 1 are only an example. The wider clinical range of NT-proBNP should theoretically allow a better discrimination between the clinical phases of HF than BNP [8]; however, usually there are no differences in the diagnostic use of BNP and NT-proBNP immunoassays [1].

Two recent studies from the PARADIGM-HF (Prospective Comparison of ARNI with ACEI to Determine Impact on Global Mortality and Morbidity in Heart Failure) trial [9, 10] reported conflicting results between BNP and NT-proBNP levels. PARADIGM-HF study evaluated the clinical effects of a new composite drug (denominated LCZ696 or Entresto), first-in-class composite angiotensin receptor-neprilysin inhibitors (ARNi) [11]. The action mechanism of this drug is complex, combining the effect of the angiotensin II receptor blocker and that of the neprilysin inhibitor. The enzyme neprilysin causes degradation not only of natriuretic peptides but also of a variety of components affecting the mechanisms of action for several other circulating biological active peptides, including enkephlins, bradykinins, angiotensins, endorphins, insulin and gastrin [12]. The rationale for the use of a drug containing a neprilysin inhibitor in HF patients is that this proteolytic enzyme can degrade the biologically active natriuretic peptides, especially CNP and ANP and to a lesser extent BNP [11–13]. For this reason, a drug, containing a substance inhibiting natriuretic peptide degradation, may increase the circulating levels of the biologically active natriuretic hormones (Figure 2) and, by doing so, may improve the clinical conditions of HF patients by increasing diuresis and natriuresis and also by reducing cardiac stress [11]. The results of the PARADIGM-HF study indicated that plasma BNP levels were higher during treatment with LCZ696 than with enalapril, but on the contrary, circulating levels of NT-proBNP and cardiac troponin T (cTnT) were lower during treatment with LCZ696 than with enalapril after the first weeks of treatment [9]. Authors explained these conflicting results obtained in the PARADIGM-HF study by considering the combined action of LCZ696 drug. Indeed, BNP (but not NT-proBNP) is a substrate for neprilysin [11, 13]; as a result, the increase in BNP levels after LCZ696 administration should reflect the inhibiting action of the drug on neprilysin, even if BNP1-32 is degraded with minor efficiency by neprilysin than ANP and CNP [11–13]. On the contrary, the decrease in NT-proBNP levels should reflect the beneficial effects of the drug on myocardial function and vascular hemodynamics, especially because of the inhibition in renin-angiotensin-aldosterone system activity [11, 13]. Indeed, the reduction of cardiac stress during LCZ696 treatment should reduce the production...
and secretion of natriuretic peptides from cardiomyocytes, hence producing a fall in circulating levels of NT-proBNP, too [14]. Considering these pathophysiological premises, clinicians should accurately evaluate the clinical setting in order to correctly interpret the variations of natriuretic peptides, measured by commercially available laboratory methods. In particular, clinicians should take into account that an increase in BNP levels in HF patients could be caused by inhibiting effect of LCZ696, but also by deterioration of clinical conditions in patients who do not respond to treatment. On the other hand, an increase in plasma NT-proBNP is usually due to deterioration of clinical conditions of HF patients [1, 14].

Because of the better analytical characteristics of NT-proBNP immunoassays and the easier pathophysiological and clinical interpretations of variations of NT-proBNP levels in HF patients, some authors claimed to assay the inactive peptide NT-proBNP instead of the active hormone BNP for management of HF patients under treatment with LCZ696 [15, 16]. On the contrary, we do not think that it is yet the time to celebrate a requiem for the death of BNP assay, and we would like to explain in the present article why BNP assay is still alive, and why the measurement of the active natriuretic hormone may still be useful in both experimental research and clinical practice.

### Rationale for BNP measurement

More than 30 years ago, De Bold et al. [17] reported that atrial extracts contain some biological active peptides, which promote a rapid and massive diuresis and natriuresis when injected in rats. After only few years, several endogenous peptide hormones with natriuretic and vasodilator activity have been identified in the human blood and peripheral tissues [1, 14]. Atrial natriuretic peptide (ANP), BNP and their related peptides are predominantly produced by atrial and ventricular cardiomyocytes, constituting the cardiac endocrine function [14]. From a pathophysiological point of view, cardiac endocrine function is an essential component of the integrated systems of the body, and thus, it plays a pivotal role in fluid, electrolyte and hemodynamic homeostasis [14]. A continuous exchange of information flows from the cardiac endocrine system to nervous and immunological systems and to other organs, including kidney, endocrine glands, liver, adipose tissue, immunocompetent cells and vice versa. This close link between cardiac natriuretic peptide system and counter-regulatory systems could explain the increase in circulating levels of BNP/NT-proBNP in some

### Table 2: BNP and NT-proBNP values (mean ± SD) measured in normal subjects and patients with heart failure (HF), divided according to New York Heart Association (NYHA) functional class groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>BNP</th>
<th>NT-proBNP</th>
<th>NT-proBNP/BNP molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy subjects</td>
<td>85</td>
<td>12.5 ± 10.7 (3.6 ± 3.1)</td>
<td>55.9 ± 37.7 (6.60 ± 4.45)</td>
<td>1.8</td>
</tr>
<tr>
<td>All HF patients</td>
<td>193</td>
<td>287.7 ± 350.5 (81.1 ± 101.3)</td>
<td>3087.4 ± 5439.2 (364.3 ± 641.8)</td>
<td>4.5</td>
</tr>
<tr>
<td>HF NYHA I</td>
<td>25</td>
<td>71.3 ± 103.3 (20.6 ± 29.8)</td>
<td>588.3 ± 950.8 (69.4 ± 112.2)</td>
<td>3.4</td>
</tr>
<tr>
<td>HF NYHA II</td>
<td>91</td>
<td>185.1 ± 263.0 (53.6 ± 76.0)</td>
<td>1424.3 ± 1676.3 (168.1 ± 197.8)</td>
<td>3.1</td>
</tr>
<tr>
<td>HF NYHA III</td>
<td>48</td>
<td>412.8 ± 283.7 (119.2 ± 82.0)</td>
<td>4959.0 ± 6976.2 (585.2 ± 823.2)</td>
<td>4.9</td>
</tr>
<tr>
<td>HF NYHA IV</td>
<td>29</td>
<td>598.0 ± 582.2 (172.8 ± 152.6)</td>
<td>7362.4 ± 8477.7 (868.8 ± 1000.49)</td>
<td>5.0</td>
</tr>
</tbody>
</table>

These data were used for the preparation of Figure 1. Values are expressed in both conventional units (ng/L) and SI units (pmol/L) within brackets. These data were previously published by Prontera et al. [2]. Plasma NT-proBNP was measured with the ECLIA method (Roche Diagnostics, Germany), and plasma BNP with the immunoradiometric assay (IRMA) Shionoria (Shionogi &Co, Japan) [2].

### Figure 2: Schematic representation of biosynthesis, secretion and distribution of B-type-related natriuretic peptides.

Human BNP is synthesized as a 134-amino acid precursor protein (pre-proBNP), including a signal peptide of 26 amino acids, and is subsequently processed to form a 108-aa pro-peptide (proBNP). The proBNP can be enzymatically cleaved both in cardiomyocytes and in plasma by some pro-protein convertases to form the 76 amino acids N-terminal peptide (NT-proBNP) and the biologically active 32 amino acid C-terminal peptide (BNP). In plasma, proBNP, NT-proBNP and BNP are further degraded by some proteolytic enzymes.
non-cardiac-related clinical conditions, including kidney, liver, pulmonary, metabolic and endocrine disorders [14].

Although even the most recent international guidelines reported only an interlocutory judgment about the natriuretic peptide-guided HF management [18–20], the monitoring of ambulatory patients with acute (decompensated) or chronic HF is frequently done, especially in specialized HF centers [21, 22]. From a clinical point of view, both BNP and NT-proBNP levels usually monitor the effectiveness of the pharmacological treatment of HF patients: the HF patients, “responder” to treatment, usually show a progressive reduction in circulating levels (more than 30%) and also a better clinical outcomes [22, 23]. However, it is important to note that pathophysiological interpretations of plasma concentration variations of these two peptides (BNP vs. NT-proBNP) should be conceptually different in both healthy subjects and patients with cardiac disease [1, 14].

NT-proBNP is an inactive peptide produced in an equimolar ratio together with BNP by action of some cellular or circulating proteolytic enzymes (such as corin and furin) on the precursor pro-hormone (proBNP) (Figure 2) [1, 14, 24–28]. As a result, NT-proBNP assay is not a reliable index of the biological (natriuretic) activity of cardiac endocrine system in severe HF because the concentration of less active peptides in patients with severe HF (such as NT-proBNP and proBNP) is greatly higher than that of the active peptide BNP1–32 (Figure 1) [1, 29–33]. Moreover, a recent study [34] suggested that the commercial immunoassays, commonly used for NT-proBNP assay, employ non-glycosylated calibrator materials and mostly antibodies directed against epitopes with potential O-glycosylation site occupancy. Because the most part of circulating inactive peptides related to NT-proBNP and proBNP are O-glycosylated [7, 18, 35], these immunoassays cannot measure some glycosylated peptides [34, 36]. Moreover, the commercially available immunoassays for NT-proBNP are interfered by the intact peptide proBNP (especially non-glycosylated peptide) [36] and also by some other shorter peptides derived from the proteolytic degradation of this pro-hormone [1] (Figure 2). In conclusion, the commercially available immunoassays for NT-proBNP do not allow an accurate measurement of this peptide.

On the other hand, plasma levels of the active hormone BNP1–32 are directly related to peripheral hormonal response of the cardiac endocrine function throughout the binding to specific natriuretic peptides receptors (NPR-A), which are present in all tissues of the body, including the central nervous system [14]. In particular, considering the natriuretic activity, circulating active peptide BNP1–32 can stimulate the specific natriuretic receptor (i.e. NPR-A) on renal tubular cells, inducing natriuresis. As a result, the specific measurement of the active peptide BNP1–32 (like that of ANP) should be considered a direct index of cardiac endocrine system activity [14]. Another reliable index of natriuresis induced by cardiac natriuretic peptides is the assay of cyclic GMP (cGMP) in the urine: this cyclic nucleoside is the second messenger of the natriuretic hormone system. cGMP is released in tubular renal cells as consequence of ANP and BNP binding to NPR-A sites on cell membranes of tubular renal cells [37].

Quality specifications for an accurate BNP assay

Recent studies revealed that the cardiac B-type natriuretic system is far more complex than we ever envisaged [1, 14, 24]. In addition to the peptide hormone BNP and the inactive peptide NT-proBNP, a huge numbers of other circulating peptides derived from the pro-hormone proBNP can be identified by chromatographic procedures in human plasma, including the intact and glycosylated forms of proBNP itself [29–33, 38] (Figure 2). Furthermore, the active hormone BNP may be produced even in vivo from the circulating intact precursor proBNP through enzymatic cleavage by some plasma proteases (such as corin) [35, 36, 39] (Figure 2). Indeed, a recent study, using an in vivo experimental rat model, demonstrated that the split of proBNP into BNP and NT-proBNP can actually occur in circulation [40]. From a clinical point of view, the peripheral processing of circulating proBNP could likely be submitted to regulatory mechanisms, which might be impaired in patients with HF, opening new perspectives in the treatment of HF [41].

The active hormone BNP, including 32 amino acids (BNP1–32), can be accurately detected and measured with mass spectrometry methods [1]. In particular, some studies [32, 42–44] reported that the true plasma BNP1–32 concentration measured with mass spectrometry methods in patients with severe HF is much lower than BNP values usually measured by commercially available immunoassay methods. At present time, the mass spectrometry methodology cannot be used in clinical laboratories for measurement of BNP because of the high cost of instrumentation, the long laboratory turnaround time, the complex analytical procedure, which generally include a preliminary extraction and/or chromatography processing of sample, and the need of highly qualified laboratory staff for analysis.
On the other hand, there are large systematic differences (up to twofolds) between the commercial immunoassay methods so far available for BNP assay [45–48]. These differences are in part attributable to specific interferences related to the presence in clinical samples of proBNP and its degradation products [1, 36, 49]. According to these evidences [1, 36, 44–49], the commercially available immunoassay methods, considered specific for the active hormone BNP, present an obvious paradox. As discussed in the previous paragraph, BNP immunoassays should specifically measure only the active peptide hormone because clinicians could be interested in evaluating the “true biologically active status” of the cardiac endocrine function [1, 14]. Unfortunately, none of the commercially available methods is able to accurately provide this important pathophysiological information because all BNP immunoassays are greatly affected by some less active peptides, especially glycosylated and non-glycosylated proBNP, which are the predominant peptides present in blood samples of patients with severe HF [1, 29–33, 38, 49]. Furthermore, the commercially available immunoassay methods for BNP measure not only the peptide BNP1-32, but also several degradation products with varying biological activities, derived from the active peptide due to action of some proteolytic enzymes (Figure 2) [1, 14, 24]. Several proteolytic enzymes can degrade natriuretic peptides in plasma and tissues, including neprilysin, dipeptidyl peptidase IV, insulin degrading enzyme and peptidyl arginine aldehyde protease, but their action on BNP seems to be lower than that observed on ANP and CNP [50–52].

Fortunately, a very recent study [53] may offer a solution to this clinically relevant analytical problem. For the first time, Lewis et al. [53] were able to set up an ELISA method that measures BNP1-32 in plasma without interference by proBNP. This two-site ELISA uses a specific polyclonal antibody that recognizes the amino-terminal end of BNP1-32 and does not cross-react with proBNP, in conjunction with a C-terminal antibody, which recognizes BNP26-32. Detection by this assay requires both amino-terminus and carboxy-terminus of BNP to be intact. As expected, this new ELISA for BNP1-32 measured much lower BNP values than the commercial BNP method using the fully automated ARCHITECT platform. In 22 healthy subjects, the median (interquartile range) BNP concentration measured by the specific ELISA was 0.29 (0.2–0.6) ng/L, whereas the median value measured by ARCHITECT method was 17.3 (8.7–22.5) ng/L; even larger was the difference in BNP values measured by these two methods in a group of HF patients: 40.7 (2.0–90.1) ng/L (n = 42) vs. 1778 (1134–2853) ng/L (n = 39), respectively [53]. Interestingly, despite greatly lower values measured, this ELISA for BNP1-32 showed a percent increase in median BNP concentrations from the groups of healthy controls to HF patients slightly higher than that of ARCHITECT method (59% vs. 44%). Of course, the analytical performance and clinical results of this ELISA should be evaluated and confirmed in other studies using larger populations of both healthy controls and HF patients before to definitely state that we have found an immunoassay method able to measure the “true” concentration of the active hormone BNP1-32.

HF therapy with ARNi: Is it the time for the renaissance of BNP assay?

The results reported by Lewis et al. [53] confirmed that on average, the BNP1-32 concentration in healthy subjects is very low (median under 1 ng/L). The setup of a new generation of commercial BNP immunoassay methods with a relevant increment in both analytical sensitivity and specificity is needed in order to detect these very low analyte concentrations. This is a very difficult challenge, but the very recent experience on the development of highly sensitive methods for cardiac troponins suggests that this mission is not impossible [1].

More sensitive and specific immunoassay methods for the active hormone BNP1-32 will allow a more accurate estimation of circulating natriuretic activity in patients with HF, including those under treatment with ARNi. The time courses of plasma BNP and NT-proBNP concentrations of patients enrolled in the PARADIGM-HF study [9] levels tell us the same story, although seen from two different perspectives. BNP, measured with ADVIA Centaur method [54], increased at 4 weeks of LCZ696 treatment with a slighty decrease after 8 months of treatment, whereas NT-proBNP levels, measured by ECLIA Elecsys method [55], were significantly lower after 4 weeks compared to baseline and then they further decreased after 9 months of therapy. The rapid decrease in NT-proBNP levels confirms the clinical evidences, suggesting that the majority of patients under LCZ696 treatment shows a clinical benefit from the drug, whereas the rapid increase followed by a trend to decrease of BNP levels indicates that this improvement in clinical conditions is almost in part due to the increase in natriuretic activity due to neprilysin inhibition on BNP and ANP degradation. Therefore, BNP and NT-proBNP assay allows complementary (not contradictory) information on clinical conditions of patients treated with ARNi. It is important to note that the same complementary information is respectively allowed by
cTnT assay (suggesting a reduction in heart stress and damage) and urinary cGMP assay (indicating an increased natriuretic activity under pharmacological treatment) [9].

At this point, it is important to discuss whether a more sensitive and specific BNP assay, as that described by Lewis et al. [53], could add further and/or more accurate information on pathophysiological and clinical conditions of HF patients under LCZ696 treatment. As a matter of fact, proBNP cross-reacts by about 14%–17% with BNP in the ADVIA BNP assay [36, 56], which is the laboratory method used in the PARADIGM-HF trial for BNP assay [9]. All the other commercially available BNP immunoassays are also (or even more) cross-reacted by proBNP than ADVIA BNP assay (from about 20% to 40%) [36, 56]. From a physiological point of view, proBNP is able to bind the specific natriuretic receptor NPR-A, but it stimulates guanylyl cyclase-A (GC-A) with reduced potency (i.e. 13-fold less) than BNP [37, 57]. Considering that the proBNP and its related peptides are the predominant peptides present in plasma of HF patients [29–33, 38], the measurement of BNP with the immunoassays at present commercially available gives an inaccurate estimation of biologically active B-type natriuretic peptides present in patients with severe HF. Therefore, the results of a more sensitive and specific assay for BNP should theoretically allow a better correlation between immunoreactivity and biological activity (i.e. natriuretic activity) [14]. Furthermore, a more specific BNP assay should display a better correlation with rapid variations in pathophysiological conditions compared to other less specific immunoassay methods significantly interfered by proBNP because the active peptide has a greatly lower plasma half-life than less active pro-hormone (Table 1). In particular, it is conceivable that a more sensitive and specific method, such as that by Lewis et al. [53], should show a greater decrease in BNP levels compared to the ADVIA Centaur method after 8 months of treatment similarly to that observed with NT-proBNP levels in the PARADIGM-HF trial [9].

New perspectives and conclusions

Because of complex metabolic pathways and pathophysiological mechanisms in which B-type natriuretic peptides are involved, as well as to the differences in quality specifications of immunoassay methods, clinicians should give great care to the pathophysiological interpretation of plasma BNP and NT-proBNP variations in HF patients. Indeed, the measurement of the active peptide hormone BNP should theoretically give different, but complementary, pathophysiological and clinical information than that of the inactive NT-proBNP. Unfortunately, the clinical interpretation of variations of natriuretic peptides in HF is made still more difficult because current BNP methods cross-react with proBNP, and also they measure several BNP fragments instead of the intact hormone; on the other hand, NT-proBNP assays are cross-reacted by proBNP and its degradation products [1, 49].

Therefore, it is needed to set up some more sensitive and specific immunoassays for the active peptide BNP as that described by Lewis et al. [53]. These new and specific methods should give more accurate information on the circulating natriuretic activity [1]. On the other hand, the measurement of the pro-hormone proBNP with highly specific immunoassays [29–31, 58] may be useful as an estimate of less active B-type natriuretic peptides produced by cardiomyocytes and circulating as predominant peptides in plasma of patients with severe HF [1, 29–31, 49].

In conclusion, at present time, clinicians should accurately consider both the clinical setting of patients and the analytical characteristics of BNP and NT-proBNP immunoassays in order to correctly interpret the variations of natriuretic peptides measured by commercially available laboratory methods, especially in patients treated with ARNI.

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