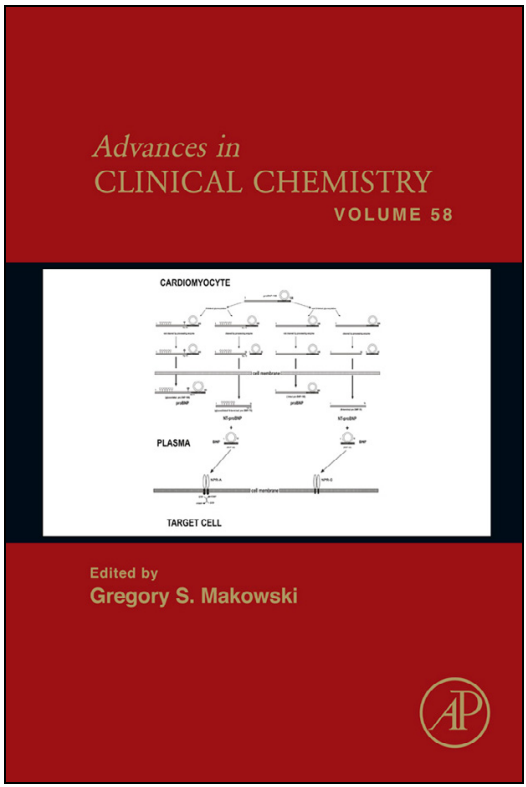

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CIRCULATING FORMS OF THE B-TYPE NATRIURETIC PEPTIDE PROHORMONE: PATHOPHYSIOLOGIC AND CLINICAL CONSIDERATIONS

Aldo Clerico,[1] Simona Vittorini, and Claudio Passino

Scuola Superiore Sant'Anna,[1] Fondazione G. Monasterio CNR—Regione Toscana, Pisa, Italy

1. Abstract

Recent studies reported that many different biochemical forms of B-type-related peptides circulate in human blood. In particular, a significant amount of the prohormone peptide (i.e., $proBNP_{108}$) can be detected in plasma of patients with heart failure. These data indicate that the posttranslational maturation processing of the B-type natriuretic peptide (BNP) precursor may not be efficient in heart failure. The aim of this chapter is to describe the biochemical pathways of $proBNP_{108}$ maturation and to discuss the pathophysiological relevance of alteration of the posttranslational maturation mechanisms in heart failure. An impaired cardiac endocrine function was proposed to explain the altered electrolyte and fluid homeostasis

[1]Corresponding author: Aldo Clerico, e-mail: aldo.clerico@sssup.it

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occurring in chronic heart failure. Recent studies demonstrated that a great part of BNPs assayed by immunoassay methods in healthy subjects and in patients with cardiovascular disease is devoid of biological activity. These findings suggest that an alteration in posttranslational maturation of BNP precursor may promote the resistance to biological action of BNP in patients with heart failure at a prereceptor level. These studies also open a new and more complex scenario regarding the circulating BNPs. The active hormone (i.e., $BNP_{1–32}$) may be produced even *in vivo* from the circulating precursor $proBNP_{108}$ by plasma enzyme degradation, such as the soluble form of corin, possibly able to process the circulating intact precursor of natriuretic hormones. As a future perspective, the simultaneous measurement of the $proBNP_{1–108}$ and the active peptide $BNP_{1–32}$ with more specific methods could allow a more accurate estimation of both production/secretion of B-type-related peptides from cardiomyocytes and the true activity of the cardiac endocrine function.

2. Background and Aim of the Study

All natriuretic peptides (including atrial natriuretic peptide (ANP), BNP, and C-type natriuretic peptide (CNP)) share a direct diuretic, natriuretic, and vasodilator effect as well as an inhibitory action on the inflammatory processes of both myocardium and smooth muscle cells [1–3]. Natriuretic peptides exert a protective effect on endothelial function and vascular remodeling [4–6]. These effects are mediated by two different guanylate cyclase-coupled receptors, NPR-A (more specific for ANP and BNP) and NPR-B (more specific for CNP)[7], while a third specific receptor NPR-C, not coupled to a guanylate cyclase, has essentially a clearance function for all natriuretic peptides [2,8].

A blunted natriuretic response after pharmacological doses of ANP and BNP has been observed in experimental animal models and in patients with chronic heart failure, suggesting a resistance to the biological effects of cardiac natriuretic hormones CNH [9–11]. As discussed in detail elsewhere [2,12,13], resistance to the biological action of CNH can be attributed at least to three different causes/mechanisms, acting at prereceptor, receptor, and postreceptor level, respectively (Table 1).

Several studies recently reported that many different biochemical forms of B-type-related peptides circulate in human blood [14–34]. In particular, large amounts of the BNP prohormone (i.e., $proBNP_{108}$) can be detected in plasma of patients with heart failure [14–21,24,31]. Dries *et al.* [21], using a specific immunometric assay [17], found detectable levels of $proBNP_{108}$ in the 99% of a large population of patients with chronic heart failure (i.e., 756

TABLE 1
CLASSIFICATION OF POSSIBLE MECHANISMS OF RESISTANCE TO BIOLOGICAL EFFECTS OF CNH

Pre-receptor level
(A) Presence of inactive peptides in plasma
(B) Increase in inactivation/degradation of active peptides
    1. Upregulation of NPR-C
    2. Increased activity of proteases
(C) Decreased renal filtration
Receptor level
(A) Downregulation of NPR-A and NPR-B in target tissues
(B) Altered CNH receptor binding or desensitization
Post-receptor level (activated counterregulatory mechanisms)
Altered intracellular signaling
    1. Decreased cGMP cellular accumulation (decreased production or increased degradation)
    2. Altered intracellular pathways downstream cGMP

participants). These data may indicate that the posttranslational maturation processing of BNP precursor is not efficient in heart failure [15,20,22,31–33]. As a result, a great part of BNPs assayed in healthy subjects and patients with cardiovascular disease may be devoid of biological activity. These findings indicate that an alteration in posttranslational maturation processing mechanisms of BNP precursor may promote the resistance to the biological action of BNP in patients with heart failure at the prereceptor level [2,22,32,33]. The aim of this chapter is to describe the biochemical pathways of $proBNP_{108}$ maturation and to discuss the pathophysiological relevance of alteration of these posttranslational maturation mechanisms in heart failure.

3. Biosynthesis of B-Type Natriuretic Peptides in Cardiomyocytes

In humans, BNP is synthesized as a 134-amino acid (aa) precursor protein (preproBNP) and is subsequently processed to form a 108-aa propeptide, named $proBNP_{1–108}$. The propeptide hormones of the cardiac natriuretic peptides can be enzymatically cleaved by proprotein convertases produced in the cardiomyocyte [32,35]. A cardiac serine protease, corin, and a ubiquitous serine protease, furin, have been proposed as possible convertases for $proBNP_{1–108}$ [36–38]. These proteases convert the $proBNP_{1–108}$ into the 76-aa N-terminal peptide (i.e., $NT\text{-}proBNP_{1–76}$) and the biologically active 32-aa C-terminal peptide (i.e., $BNP_{1–32}$) (Fig. 1).

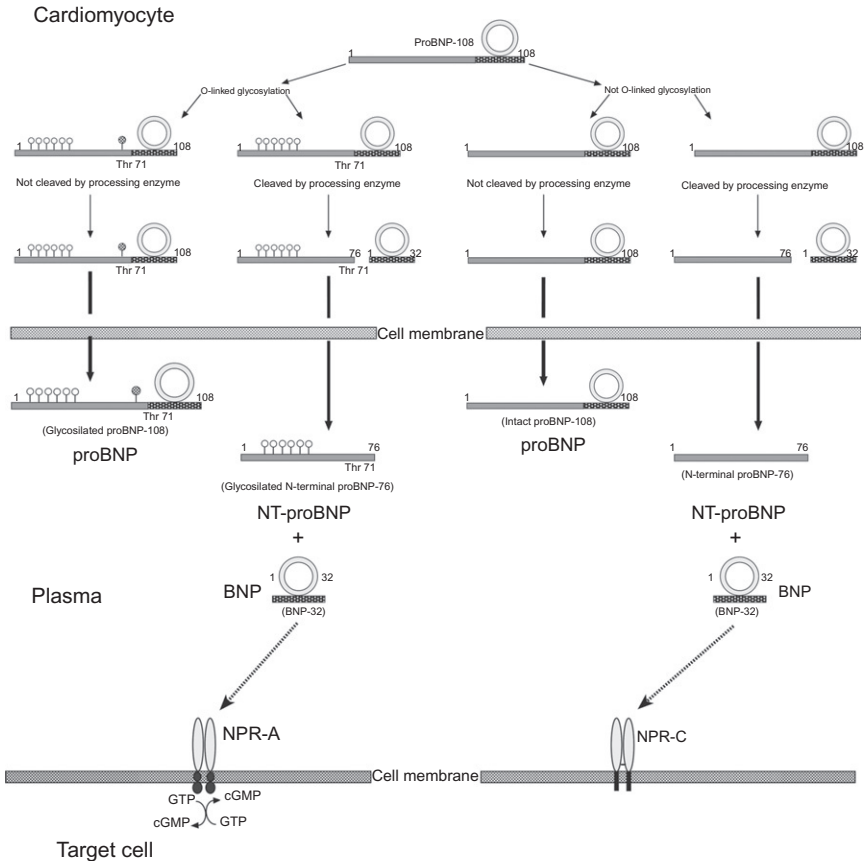

FIG. 1. Schematic representation of biosynthesis, secretion, and distribution of B-type-related natriuretic peptides. Some of the biosynthesized prohormone (proBNP-108) is *O*-glycosylated within the Golgi apparatus. If O-glycosylation does not occur, proBNP-108 can be cleaved to BNP-32 and NT-proBNP-76 by the processing enzymes within the *trans*-Golgi network. If O-glycosylation occurs, glycosylated-proBNP-108 cannot be cleaved, and uncleaved glycosylated-proBNP-108 is secreted into the circulation. Finally, a smaller part of intact prohormone is not glycosylated and cleaved, and so this peptide can be present into circulation in intact form as proBNP-108. As indicated in the figure, the glycosylation on the threonyl residue in position 71 (Thr 71) could regulate prohormone cleavage by either blocking or guiding endoproteolytical enzymes. Only $BNP_{1–32}$, which is the active hormone, is able to bind the specific receptors, NPR-A and NPR-C. NPR-A is a guanylate cyclase-coupled receptor, which mediates the biological effects of cardiac natriuretic peptides. NPR-C, not coupled to a guanylate cyclase, has essentially a clearance function for all natriuretic peptides.

Biosynthesized prohormone may be (or not) O-glycosylated within the Golgi apparatus; as a result, different maturation processing pathways are possible for $proBNP_{1–108}$ in cardiomyocytes [32,35,39] (Fig. 1). If O-glycosylation does not occur, $proBNP_{1–108}$ can be cleaved to $BNP_{77–108}$ and NT-$proBNP_{1–76}$ by the processing enzymes within the *trans*-Golgi network. If O-glycosylation occurs, glycosylated $proBNP_{1–108}$ cannot be cleaved, and uncleaved glycosylated $proBNP_{1–108}$ is secreted into the circulation. Finally, a smaller part of intact prohormone is not glycosylated and cleaved, and so this peptide can be secreted into circulation in intact form as $proBNP_{1–108}$. As indicated in Fig. 1, the glycosylation on the threonyl residue in position 71 (Thr 71) may regulate prohormone cleavage by either blocking or guiding endoproteolytic enzymes [32,35]. A very recent study [39] suggested that the predominant intracellular form of BNP was nonglycosylated $proBNP_{1–108}$, rather than $BNP_{1–32}$. Glycosylated $proBNP_{1–108}$, but not nonglycosylated $proBNP_{1–108}$, was detected as the major extracellular form in the culture supernatants of preproBNP-expressing cell lines and primary human cardiomyocytes. Finally, ablation of O-glycosylation of $proBNP_{1–108}$ at T71 residue, near the convertase recognition site, reduced the extracellular $proBNP_{1–108}$ and increased extracellular $BNP_{1–32}$ [39], suggesting that glycosylation in this point may act as regulatory site for precursor maturation.

4. Circulating B-Type-Related Peptides: Biochemical Characteristics

Some recent studies completely revised our vision concerning the biochemical characteristics of circulating forms of the B-type-related peptides. Only 5 years ago, the prohormones (i.e., proANP and proBNP) were thought to be predominantly stored in secretory granules and then nearly completely split in equimolar amounts into longer amino-terminal fragments (i.e., NT-proANP and NT-proBNP) and shorter C-terminal active peptide hormones (i.e., ANP and BNP) before secretion in the blood of healthy subjects [2,40]. On the contrary, some recent evidences suggested that $proBNP_{1–108}$ is the predominant B-type immunoreactive form in human blood of both the healthy subjects and the patients with congestive heart failure [17,18,21,24,31,39,41].

According to the biosynthetic pathways described in the previous paragraph, a huge number of circulating proBNP-derived fragments can be identified by chromatographic procedures in plasma of experimental animals and patients with heart failure [3,14–32,41,42]. Moreover, the $proBNP_{1–108}$ and NT-$proBNP_{1–76}$ (and probably also other shorter peptides derived from these peptides) are present in plasma in both glycosylated and nonglycosylated form, especially in plasma samples of patients with heart failure [19,22,28,30–32,39].

In particular, Seferian *et al.* [28] recently reported that the plasma pool of the endogenous NT-proBNP$_{1–76}$ contains a small portion (about 5%) of nonglycosylated or incompletely glycosylated peptide and that this portion can be detected by antibodies specific to the central part of the molecule.

A still open question is whether the circulating proBNP$_{1–108}$ is also present in polymeric form, such as trimer [25,26], or not [19]. Some more recent studies indicate that the high molecular forms of proBNP$_{1–108}$, found in older studies, were more probably due to highly glycosylated forms of the precursor peptide rather than due to the polymerization forms of the peptide [19].

5. The Measurement of Different Circulating Forms of the B-Type-Related Natriuretic Peptides: Analytical Characteristics and Methodologic Challenges

The presence of many circulating molecular forms of B-type-related peptides raises a question concerning the specificity of the immunoassay methods so far utilized for the routine measurement of these peptides in the clinical practice [43–45]. Indeed, as hypothesized several years ago [43], all immunoassays, which were considered specific for BNP$_{1–32}$ or NT-proBNP$_{1–76}$, actually show a variable degree of cross-reactivity with proBNP$_{1–108}$[44]. This issue actually raises a demanding methodological challenge [45]: the search for more specific immunoassay methods not only for the biologically active peptide BNP$_{1–32}$ but also for all the other B-type-related peptides, including the precursor proBNP$_{1–108}$.

Some different methodological approaches have been recently considered for the measurement of proBNP$_{1–108}$ [43]. In plasma or tissue extracts, proBNP$_{1–108}$ was usually isolated by means of chromatographic procedures, in particular, by HPLC, and then identified and measured by immunoassay or more accurately by mass spectrometry [14–32,39,43]. These methods are not only highly specific but also complex, time consuming, and not feasible for the clinical laboratory routine [43]. To solve these problems, at least three different methodological approaches have been developed with the aim to set up highly specific immunoassay methods for the measurement of proBNP$_{1–108}$ [17,23,46].

Goetze *et al.* [23] set up a RIA for proBNP$_{1–108}$ assay based on the measurement of plasma treated with trypsin, which cleaves all proBNP-related peptides to the small 1–21 fragment. The aim of these authors was to develop a processing-independent analysis for accurate quantification of proBNP$_{1–108}$ and its fragments in plasma. This method uses an antibody specific for a processing-independent epitope of human proBNP$_{1–108}$. This antibody was directed against the first 10 aa of proBNP peptide, and the same peptide, radioiodinated with chloramine-T, was used as tracer in the RIA. Using this method,

these authors determined the total concentration of $proBNP_{1–108}$ and of its products in healthy volunteers and HF patients, showing that $proBNP_{1–108}$ values were greatly higher in patients [23]. However, this RIA cannot be recommended for the clinical practice since it requires a preliminary treatment of plasma samples with trypsin, a very long incubation time (i.e., up to 5 days), and the use of radiolabeled material. Further, it is theoretically conceivable that this RIA measures all the peptides containing the N-terminal part of the precursor $proBNP_{1–108}$, including the NT-$proBNP_{1–76}$. As a consequence, for an accurate assay of circulating levels of the intact $proBNP_{1–108}$, NT-$proBNP_{1–76}$ concentration should be also accurately and independently measured.

Tamm *et al*. [46] developed a sandwich immunofluorescence assay for the quantification of $BNP_{1–32}$ and its precursor $proBNP_{1–108}$. Authors reported that this immunofluorescence assay holds a similar efficiency in recognizing the BNP, as well as the recombinant glycosylated and nonglycosylated forms of $proBNP_{1–108}$ [46]. This immunoassay method (named ''single-epitope sandwich assay'') is different from the conventional sandwich assay, requiring only one epitope for antigen immunodetection by two different monoclonal antibodies. In this novel immunoassay, the first antibody is used as a capture antibody and is specific for the region consisting of BNP amino acid residues 11–22, which is the most stable part of the peptide and includes the biologically active cysteine ring. The second antibody is used as a detection antibody and recognizes the immune complex, including the antigen (i.e., $BNP_{11–22}$) bound to the first antibody. In other words, the second antibody does not recognize the free antigen, but the primary immune complex, consisting of the first antibody and $BNP_{1–32}$, operates as antigen for the second antibody. Also this immunoassay method is not theoretically specific for the intact $proBNP_{1–108}$ peptide. Indeed, the single-epitope sandwich assay should also recognize all the peptides (even shorter than $proBNP_{1–108}$), sharing the region consisting of amino acid residues 11–22 of $BNP_{1–32}$ able to form a complex with the first antibody.

Giuliani *et al*. [17] selected a specific monoclonal antibody (named ''mAb Hinge76'') that recognizes the cleavage site of $proBNP_{1–108}$, an epitope found only in the precursor form (see Fig. 1). This monoclonal antibody recognizes the recombinant $proBNP_{1–108}$ in a dose-dependent manner, without any significant cross-reactivity with either recombinant NT-$proBNP_{1–76}$ or synthetic $BNP_{1–32}$. These authors set up a sandwich immunoassay for the measurement of proBNP, by combining the monoclonal antibody mAb Hinge76 with a polyclonal antibody directed against $BNP_{1–32}$ (i.e., against the COOH-terminal of $proBNP_{1–108}$). An automated version of this method on the BioPlex^{TM} 2200 analyzer was then set up, and their analytical characteristics have been evaluated [47]. More recently, some studies reported the clinical results obtained with this immunoassay in the general population [25]

and in patients with heart failure [48] or chronic renal disease [49] by measuring proBNP concentrations with this immunoassay method.

The setup of direct (i.e., without preliminary chromatographic purification) accurate immunoassay methods for the biologically active cardiac hormones, ANP and BNP, is recognized to be as a very challenging task, due to the low plasma concentration, molecular weight, and plasma half-life (both *in vivo* and *in vitro*) of these hormones, as compared to their hormone precursors proANP and proBNP peptides (Table 2) [43]. Considering these technical drawbacks, it is not surprising that all commercial immunoassay methods, used in clinical routine and usually considered specific for $BNP_{1–32}$, are significantly interfered by the precursor peptide proBNP, although with different degree of cross-reactivity [44].

6. Pathophysiological Relevance of Circulating proBNP

The measurement of B-type-related peptides has attracted the attention of clinicians when the pivotal role of cardiac endocrine function in the pathophysiology of heart failure was established [2,8,20,22,33,40,50]. Further, the introduction of the assay of B-type-related natriuretic peptides in clinical practice has resulted in a significant improvement in accuracy of the diagnostic and prognostic stratification workup in patients with cardiac diseases [50–57].

A deficient biological action of cardiac endocrine hormones has been proposed to explain the altered electrolyte and fluid balance occurring in chronic heart failure [2,11,13]. This phenomenon, defined as the "endocrine paradox" of the heart [15,22], is characterized by extremely high circulating levels of hormones, produced by cardiomyocytes, with powerful diuretic/natriuretic and vasodilator activity in patients with congestive heart failure, showing signs of fluid retention and vasoconstriction. As discussed in detail

TABLE 2
BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERISTICS OF BNP, NT-PROBNP, AND PROBNP PEPTIDES

	BNP	NT-proBNP	proBNP
Molecular mass	3462 Da	8457 Da[a]	11,900 Da[a]
Amino acids	32	76	108
Biological function	Active hormone	Inactive	Prohormone
Half-life	15–20 min	>60 min	>60 min
Glycosylation	Not glycosylated	Highly glycosylated *in vivo*	Highly glycosylated *in vivo*

[a] The molecular mass (MM) of NT-proBNP and proBNP depends on the degree of glycosylation of the peptide; in the table are reported the MM of nonglycosylated peptides.

elsewhere [2,58], resistance to the biological action of cardiac natriuretic hormone system can be attributed to different mechanisms, acting at prereceptor, receptor, and/or postreceptor level (Table 1).

Considering the possible causes of resistance at the prereceptor level, recent issues suggested that in patients with heart failure, there may be an inadequate posttranslation maturation of the biosynthetic precursors of BNP system [15,22,25–31,39]. Several studies indicated that a great part of BNPs measured in patients with heart failure is devoid of biological activity [15,16,20,30,36,38,39,59–61]. In addition to bioactive $BNP_{1–32}$, a huge number of circulating proBNP-derived fragments can be identified by chromatographic procedures in human plasma, including the intact and glycosylated forms of precursor $proBNP_{1–108}$ and N-terminal truncated BNP form 3–32 [15,22,25–31,39]. As compared to inactive peptides $proBNP_{1–108}$ and NT-proBNP, the active peptide $BNP_{1–32}$ has a shorter plasma half-life (about 15–20 min vs. 1 or 2 h) and consequently lower plasma concentration (Table 2).

On the other hand, the above reported studies [15,16,20,30,31,36,38,39,59–61] open also a new and more complex scenario regarding the circulating BNPs. The active hormone (i.e., $BNP_{1–32}$) may be produced also *in vivo* from the circulating precursor $proBNP_{108}$ by plasma enzyme degradation [16–32,43–45,59–61]. Human blood contains a soluble form of corin, possibly able to process the circulating intact precursor of natriuretic hormones [61,62]. In particular, Dong *et al.* [61] recently reported that soluble corin is measurable in human blood and that plasma corin levels are significantly lower in heart failure patients than in healthy controls, and inversely correlated to the severity of the disease [61]. Finally, Semenov *et al.* demonstrated that synthetic or recombinant human proBNP can be processed to active BNP in the circulating blood, when injected in the femoral vein of rats [63]. The hypothesis of the peripheral processing of circulating $proBNP_{1–108}$ clearly assumes that the peripheral maturation of the precursor $proBNP_{1–108}$ would be submitted to regulatory rules, possibly altered with heart failure progression [16,20,22,32,48,59]. However, further studies are needed to clarify the pathophysiological and clinical relevance of alteration in maturation of prohormone precursors of cardiac natriuretic hormones in patients with heart failure.

7. The Need of More Specific Immunoassays for the Measurement of $proBNP_{1–108}$ and $BNP_{1–32}$ Peptides

The results of the CardioOrmocheck study [64] recently confirmed that the most popular BNP immunoassays are affected by large systematic differences (up to 2.7 folds), while the agreement between NT-proBNP methods,

which use the same standard materials and couple of antibodies, was better (up to 1.2 folds). In particular, this multicenter proficiency testing study reported that AxSYM and ARCHITET platforms (Abbott Diagnostics), ACCESS and UniCell Dxi platforms (Beckman Coulter Inc.), and the POCT method (TRIAGE Biosite, Inverness Medical) shared on average similar BNP values, while the fully automated ADVIA Centaur platform and an IRMA method (by Shionogi & Co., Ltd.,), which use the same antibodies, showed on average significantly lower (of about 50%) results compared with the other methods. Moreover, Luckenbill *et al.* [44] reported that there are significant differences in cross-reactivity between $proBNP_{1–108}$ and commercially available BNP and NT-proBNP immunoassays. These data [44,63] suggest that the large variation in BNP values measured by commercial methods is largely due to differences in cross-reactivity with $proBNP_{1–108}$ and its split products. On the other hand, a very recent study by Nishikimi *et al.* [65] suggested that most endogenous plasma NT-$proBNP_{1–76}$ is glycosylated and undetectable with commercially available assay tests (i.e., ECLIA method for fully automated platforms by Roche Diagnostics), which are not able to detect the glycosylated peptide [44], and that the relative glycosylation level is increased by hemodialysis in patients with chronic renal failure.

Therefore, the above-mentioned studies [44,64,65] clearly demonstrate that commercial methods, considered specific for $BNP_{1–32}$ and NT-$proBNP_{1–76}$, are actually not specific for these peptides. Since only $BNP_{1–32}$ is believed to be biologically active, at the present time, the commercially available assays cannot accurately estimate the true biological activity of the cardiac natriuretic system. In other words, there is a substantial discrepancy between biological and immunological activities when commercial methods are employed for the determination of plasma BNP concentration.

A fully automated immunoassay specific for $proBNP_{1–108}$ has some theoretical advantages as biomarker (i.e., more stable molecule, higher molecular weight, lower biologic variability) compared to the measurement of the active hormone BNP (Table 2). As a future perspective, the simultaneous measurement within the same plasma sample with two methods, one specific for the intact precursor $proBNP_{1–108}$ and the other for active peptide $BNP_{1–32}$, could allow a more accurate estimation of both production/secretion of B-type-related peptides from cardiomyocytes and the true activity of the cardiac endocrine function, compared to the single assay of either peptide. Information obtained by simultaneous measurement of proBNP and BNP with specific assays should likely extend our present knowledge of pathophysiological mechanisms linking together heart failure progression and cardiac endocrine dysfunction [33]. Indeed, a recent study in ambulatory patients with chronic systolic heart failure showed that the combined assessment of conventional BNP and proBNP immunoassays provides additional

information in determining the risk of adverse clinical outcomes, particularly in patients with low BNP values [21]. However, other studies are needed to definitively demonstrate the clinical usefulness of this new immunoassay [17] for $proBNP_{1–108}$ measurement.

In conclusion, more specific methods for the measurement of both $proBNP_{1–108}$ and $BNP_{1–32}$ peptides are welcome in order to achieve a more complete and accurate estimate of cardiac endocrine function. Further, designed studies will be necessary to evaluate if these new specific assays for $BNP_{1–32}$ and $proBNP_{1–108}$, used either alone or in combination, will perform better in terms of diagnostic and prognostic accuracy as compared to the currently commercially available BNP and NT-proBNP assays.

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