Critical Reviews in Clinical Laboratory Sciences
Publication details, including instructions for authors and subscription information:
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State of the art of immunoassay methods for B-type natriuretic peptides: An update

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Published online: 30 Dec 2014.

Critical Reviews in Clinical Laboratory Sciences

http://informahealthcare.com/lab
ISSN: 1040-8363 (print), 1549-781X (electronic)

Crit Rev Clin Lab Sci, 2015; 52(2): 56–69
 DOI: 10.3109/10408363.2014.987720

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REVIEW ARTICLE

State of the art of immunoassay methods for B-type natriuretic peptides: An update

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Abstract

The aim of this review article is to give an update on the state of the art of the immunoassay methods for the measurement of B-type natriuretic peptide (BNP) and its related peptides. Using chromatographic procedures, several studies reported an increasing number of circulating peptides related to BNP in human plasma of patients with heart failure. These peptides may have reduced or even no biological activity. Furthermore, other studies have suggested that, using immunoassays that are considered specific for BNP, the precursor of the peptide hormone, proBNP, constitutes a major portion of the peptide measured in plasma of patients with heart failure. Because BNP immunoassay methods show large (up to 50%) systematic differences in values, the use of identical decision values for all immunoassay methods, as suggested by the most recent international guidelines, seems unreasonable. Since proBNP significantly cross-reacts with all commercial immunoassay methods considered specific for BNP, manufacturers should test and clearly declare the degree of cross-reactivity of glycosylated and non-glycosylated proBNP in their BNP immunoassay methods. Clinicians should take into account that there are large systematic differences between methods when they compare results from different laboratories that use different BNP immunoassays. On the other hand, clinical laboratories should take part in external quality assessment (EQA) programs to evaluate the bias of their method in comparison to other BNP methods. Finally, the authors believe that the development of more specific methods for the active peptide, $BNP_{1–32}$, should reduce the systematic differences between methods and result in better harmonization of results.

Abbreviations: BNP: B-type natriuretic peptide, $proBNP_{77–108}$; **DPPIV**: dipeptidyl peptidase IV; **EQA**: external quality assessment; **HF**: heart failure; **IDE**: insulin degrading enzyme; **IRMA**: immunoradiometric assay; **NEP**: neutral endopeptidase; **NT-proBNP**: 1–76 NH_2-terminal peptide of proBNP, $proBNP_{1–76}$; **PAAP**: peptidyl arginine aldehyde protease; **POCT**: point of care testing; **ProBNP**: $proBNP_{1–108}$; **RIA**: radioimmunoassay

Keywords

Cardiac endocrine function, cardiovascular risk, heart failure, immunoassay methods, natriuretic peptides

History

Received 21 August 2014
Revised 28 September 2014
Accepted 11 November 2014
Published online 26 December 2014

Background

The measurement of BNP (B-type natriuretic peptide) and its related peptides is now considered the first-line biomarker for the management of patients with heart failure (HF)[1,2]. Indeed, several meta-analyses have confirmed that immunoassay methods for the measurement of B-type cardiac natriuretic peptides such as BNP and NT-proBNP show a high degree of diagnostic and prognostic accuracy[3–6] in patients with either acute or chronic HF. Furthermore, several authors have suggested that BNP/NT-proBNP assays are useful for monitoring pharmacological treatment in HF patients[6–10], as well for screening for asymptomatic left ventricular dysfunction in populations at risk of developing HF[6]. Recent international guidelines recommend the use of BNP and NT-proBNP immunoassay methods for the diagnosis and risk stratification of patients with acute and chronic HF with the highest level of evidence[2,11,12]. However, some concerns persist about the utilization of BNP/NT-proBNP in clinical practice, and there are difficulties in the clinical interpretation of results obtained with different immunoassay methods. Indeed, the most recent guidelines[2] recommended the use of these biomarker in guided therapy in patients with acutely decompensated HF only with a low level of evidence (class of evidence: IIb). Furthermore, methodological aspects regarding the specificity

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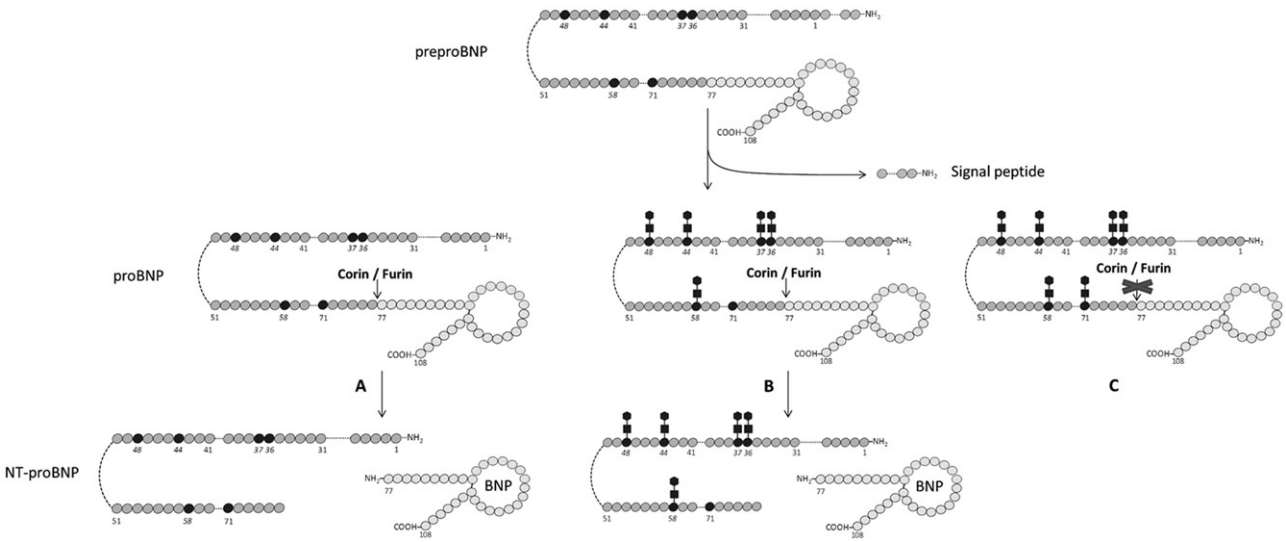

Figure 1. Schematic representation of biosynthesis of glycosylated or non-glycosylated B-type related natriuretic peptides. (A) Human BNP is synthesized as a 134-amino acid precursor protein (pre-proBNP) that includes a signal peptide of 26 amino acids; pre-proBNP is subsequently processed to form a 108-amino acid pro-peptide, proBNP. The proBNP can be enzymatically cleaved by pro-protein convertases such as corin and furin, which are produced in the cardiomyocytes and are mainly located in the trans-Golgi network and on the plasma membrane, respectively[27]. ProBNP is thus processed to form the 76-amino acid N-terminal peptide (NT-proBNP) and the biologically active 32-amino acid C-terminal peptide (BNP), both of which are secreted into plasma. (B) Some of the proBNP can be post-translationally *O*-glycosylated within the Golgi apparatus at Thr36, Ser37, Ser44, Thr48, Ser53 or Thr58[22]. Proteolytic cleavage by corin or furin occurs also with *O*-glycosylated proBNP; thus a glycosylated form of NT-proBNP can be released into plasma. (C) When *O*-glycans bind to Thr71, proBNP is not processed by furin and corin; thus glycosylated proBNP will be secreted into the circulation[25].

of immunoassays need to be considering for the appropriate use of BNP/NT-proBNP measurements in the clinical setting. Recent studies have demonstrated that several circulating peptides related to the precursor of the active hormone, proBNP, are present in human plasma; a major portion of these peptides may have reduced or even no biological activity[13–18].

Considering these recent findings, quality specifications, in particular the specificities of immunoassays and the decision levels[19,20], and recommendations[1,2] for the measurement of B-type cardiac natriuretic peptides, need to be updated. Therefore, the aim of this article is to describe the state of the art of the immunoassay methods for B type-related natriuretic peptides. In particular, the methodological correlation and the clinical consequences deriving from current knowledge on the production, secretion and peripheral metabolism of B type-related natriuretic peptides will be discussed in detail.

Recent advances in the understanding of the production and degradation pathways of BNP

Human BNP is synthesized as a 134-amino acid precursor protein (preproBNP) and is subsequently processed to form a 108-amino acid propeptide, $proBNP_{1–108}$. Some proprotein convertases such as corin and furin, produced in the cardiomyocytes, may process $proBNP_{1–108}$ to form two separate peptides: the 76-amino acid N-terminal peptide, $proBNP_{1–76}$, usually called NT-proBNP, and the biologically active 32-amino acid C-terminal peptide, $proBNP_{77–108}$, usually called BNP (Figure 1)[13,14,21].

Recent studies suggest that these biochemical pathways may be regulated by the glycosylation status of proBNP[13,14,21–27]. Indeed, the biosynthesized pro-hormone ($proBNP_{1–108}$) can be partly *O*-glycosylated within the Golgi apparatus[21–25]. The glycosylation sites on the proBNP peptide chain are illustrated in Figure 1. Also indicated in Figure 1 is glycosylation of the threonyl residue in position 71 (Thr71), which is close to the site of enzymatic cleavage (position 76 of proBNP), and which may regulate pro-hormone cleavage by either blocking or guiding endoproteolytical enzymes[13,21,26–28].

A number of studies[21–28] show that proBNP produced in cardiomyocytes has different fates: (1) non-glycosylated $proBNP_{1–108}$ can be processed to BNP and NT-proBNP by the enzymatic cleavage within the trans-Golgi network; (2) some glycosylated $proBNP_{1–108}$, especially the peptide glycosylated on Thr71, is secreted into the circulation as intact peptide; and (3) proprotein convertases are not able to process all the proBNP produced, especially when production is greatly increased as it is in patients with severe HF. As a result, part of the intact pro-hormone, even non-glycosylated, is not cleaved but is secreted directly into the circulation as intact $proBNP_{1–108}$.

Circulating B-type natriuretic peptides

Three principal peptides can be found in the plasma of healthy subjects: precursor proBNP ($proBNP_{1–108}$), inactive peptide NT-proBNP ($proBNP_{1–77}$) and active peptide hormone $BNP_{1–32}$. The main biochemical and physiological characteristics of these three peptides are reported in Table 1.

Circulating BNP is rapidly degraded *in vivo* by peptidases such as dipeptidyl peptidase IV (DPPIV)[29–31] and neutral endopeptidase (NEP)[32]; it is unclear whether other plasma enzymes such as meprin[32,33] can degrade this peptide hormone in humans[27,32]. Moreover, some studies have suggested that insulin-degrading enzyme (IDE) is able to degrade BNP to smaller peptides[34–36]. Finally, Belenky et al.[37] have suggested

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

	BNP	NT-proBNP	proBNP
Molecular mass	3462 Da	8457 Da[a]	11900 Da[a]
Amino acids	32 (part 77–108 of pro-BNP, $proBNP_{77–108}$)	76 (part 1–76 of pro-BNP, $proBNP_{1–76}$)	108 ($proBNP_{1–108}$)
Biological activity	Hormone	Inactive	Pro-hormone
Half-life	15–20 min	>60 min	>60 min
Biological variation	40–70%	20–50%	Unknown
Glycosylation	Non-glycosylated	Highly glycosylated	Highly glycosylated

[a]The molecular mass of NT-proBNP and proBNP depends on their degree of glycosylation; the table reports the molecular mass of non-glycosylated peptides.

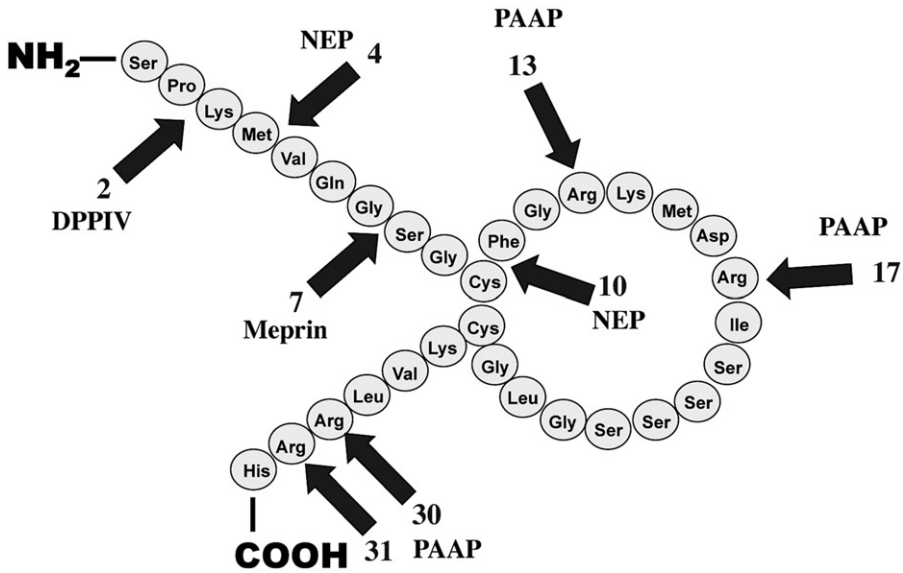

Figure 2. Degradation sites of BNP. This figure illustrates the BNP peptide chain including the specific degradations sites for each kind of enzymes. The numbers within brackets indicate the sites of degradation for each enzyme. The ring between the two cysteine residues is important for biological activity, because this portion of the peptide is bound by the specific natriuretic receptor. Indeed, the rupture of the cysteine ring completely inactivates the peptide hormone.

that peptidyl arginine aldehyde protease (PAAP) can degrade BNP at sites in the peptide chain where arginine is present, because specific inhibitors of this enzyme greatly reduce the degradation of the hormone *in vitro*. Figure 2 is a schematic illustration of the BNP peptide chain that includes the suggested cleavage sites for each enzyme that has been demonstrated to degrade the peptide hormone.

Moreover, a large number of circulating proBNP-derived fragments can be identified by chromatographic and mass spectrometric analysis in the plasma of experimental animals and patients with HF[14,15,16,21,38–42]. ProBNP and NT-proBNP (and probably other shorter peptides derived from these precursors) are present in plasma in both glycosylated and non-glycosylated forms, especially in plasma samples of patients with HF[18,23,27,40,41].

Recent studies have suggested that intact precursor proBNP is the major BNP-immunoreactive form found in human blood, especially in patients with congestive HF[14,23,29,38,39]. These findings suggest that the active hormone, BNP, could potentially be produced *in vivo* from circulating proBNP through enzymatic cleavage by plasma proteases such as corin[43–45]. Indeed, the processing of human proBNP to active BNP has been demonstrated to occur *in vivo* in an experimental rat model[46]. Several studies[43–46] suggest that the proBNP should be considered a circulating prohormone. Other studies suggest that there are two distinct proBNP pools from which BNP can be produced, one in the cardiomyocytes and the other in the plasma (Figure 3)[47,48]. BNP could potentially also be produced from proBNP *in vitro* by corin, which is present and stable for 72 h at 4 °C in plasma samples[49]. However, to the best of our knowledge, there have been no studies with the specific aim to evaluate the *in vitro* production of BNP from proBNP in plasma samples.

Peripheral processing of circulating proBNP may potentially be regulated by specific biochemical mechanisms that could be affected in patients with HF[13,49]. This hypothesis, if confirmed, would open new perspectives in the treatment of HF. Indeed, the pathophysiological mechanism of peripheral processing of circulating proBNP could be considered as novel pharmacological targets for drugs inducing and/or modulating the maturation of the prohormone into the active hormone, BNP[50].

Methodological considerations for B-type natriuretic peptide related immunoassays

The three principal B-type related natriuretic peptides, precursor proBNP, inactive peptide NT-proBNP and active peptide hormone BNP, can be measured in the plasma of healthy subjects and patients with HF using immunoassay techniques. Immunoassay methods that are considered specific for each of these peptides have been developed[13,47,48].

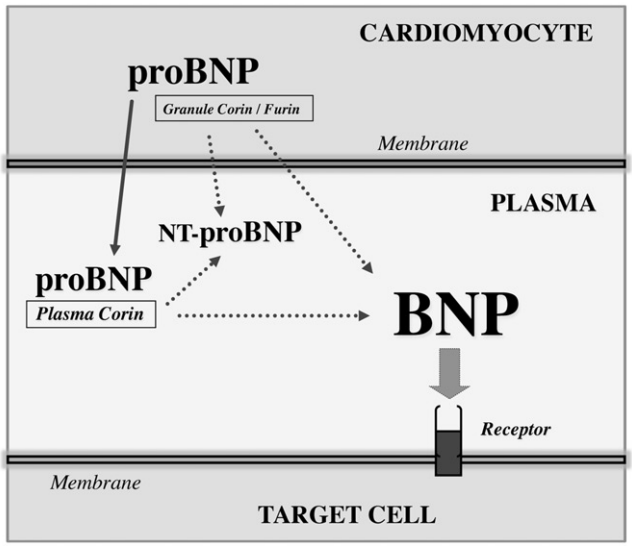

Figure 3. Processing of proBNP in cardiomyocytes and in plasma. There are two proBNP pools from which BNP can be produced: one is in the cardiomyocytes and the other is in plasma. Recent studies suggest that BNP may be produced *in vivo* from the circulating intact precursor proBNP through enzymatic cleavage by plasma proteases such as corin.

Quality specifications for B-type natriuretic peptide immunoassays have been reported and discussed in detail by expert panels[1,19,20,51]. We will discuss in the following paragraphs some important methodological issues concerning these immunoassay methods.

Immunoassay methods for BNP and NT-proBNP

A detailed history of the development of immunoassay methods for BNP and NT-proBNP have been reported in previous reviews[52,53]; in this article, we will describe only the most important and recent findings in this field. It was hypothesized several years ago[52] and demonstrated recently[54] that all immunoassays which are considered specific for BNP or NT-proBNP share some cross-reactivity with proBNP. Considering that proBNP is the major BNP-immunoreactive form found in human blood, especially in patients with congestive HF, this cross-reactivity is clinically relevant. According to Luckenbill et al.[54], proBNP is the most important cross-reacting peptide in BNP immunoassays, especially in samples from HF patients. Glycosylated proBNP has been found to cross-react more than the non-glycosylated form of the peptide, with cross-reactivities of 19–38% and 5–14%, respectively, in the four most common commercial immunoassay methods for BNP[54]. Also glycosylated proBNP, but not non-glycosylated proBNP, cross-reacts in immunoassays methods specific for NT-proBNP by 29–249%. These findings raise important methodological issues as well as lead to difficulties in the interpretation of clinical results[47,48,55].

It is important to note that all NT-proBNP immunoassays employ the same antibodies and calibrator materials, which are distributed by Roche (Roche Diagnostics, Basel, Switzerland)[19,20,47]. As a result, only small systematic differences between the commercially available NT-proBNP immunoassays methods have usually been observed; on average, the total variation between methods is less than 10%[56,57]. However, commercially available immunoassay methods for BNP employ different antibodies and standard materials (Table 2)[19,20,47,58,59], and have large (up to two-fold) systematic differences between some methods[56,57,60–66]. Some characteristics of antibodies and standard materials used by the common immunoassays for BNP are reported in Table 2[19,20,58,59].

The great heterogeneity of B-type natriuretic peptides circulating in human blood can in part explain the systematic differences among the results provided by immunoassay methods considered specific for BNP[56,57,60–66]. In Figure 4, the binding sites (epitopes) of some antibodies reported in Table 2 are shown on the BNP peptide chain. Comparison of epitopes of the antibodies used in BNP immunoassays (Figure 4 and Table 2) with the degradation sites on the hormone peptide chain (Figure 2) may allow a better understanding of the large systematic difference between BNP immunoassays. The most common commercial methods for BNP measurement used in the clinical laboratories are sandwich-type immunometric assays[19,20,47,56–66]. These methods usually employ two polyclonal or monoclonal antibodies specific for two separate epitopes of the BNP peptide chain (Figure 4 and Table 2). One of these antibodies is always specific for the intact cysteine ring, to detect the biologically active form, and the other antibody is specific for the NH_2-terminus or, alternatively, for the C-terminus of the peptide chain (Table 2).

As a result, the immunoassay methods for BNP may be divided in two types: those that are specific for the C-terminus and the NH_2-terminus of the BNP peptide chain. The former type of immunoassay methods should not measure BNP-related peptides degraded at the NH_2-terminus (such as $BNP_{3–32}$ or $BNP_{10–32}$), while the latter should not measure BNP-related peptides truncated at the C-terminus (such as $BNP_{1–27}$)[37]. Indeed, Belenky et al.[37] first demonstrated these two different types of BNP immunoassay methods. The assays more specific for the C-terminus are the immunoradiometric assay (IRMA) method (Shionogi Diagnostic Division, Osaka, Japan), and the Siemens ADVIA method for the Centaur platform (Siemens Health Care Diagnostics, Munich, Germany); note the Shionogi IRMA and ADVIA Centaur methods use the same antibodies. The assays more specific for the NH_2-terminus are the Alere Triage BNP methods (Alere Diagnostics, Waltham, MA).

Recent results of the CardioOrmoCheck study[56,57,60], an external quality assessment (EQA) program which distributed samples from healthy subjects or HF patients and quality control materials, showed that the Shionogi IRMA method, the Siemens ADVIA method for the Centaur platform and the ST AIA-PACK method for the AIA platform (Tosoh Corporation, Tokyo, Japan) measured up to 50% lower values for BNP values in comparison with other immunoassays such as the Alere Triage BNP POCT (point of care testing) method (Alere Diagnostics, Waltham, MA), the Alere Triage BNP test for the Beckman Access and UniCell DxI platforms (Beckman Coulter Diagnostics, Brea, CA), the microparticle enzyme-linked immunoassay method for the AxSYM platform and the chemiluminescent microparticle immunoassay method for the Architect platform (both from Abbott Diagnostics, Abbott Park, IL). It is important to note that the Shionogi IRMA method, the Siemens ADVIA method

Table 2. Characteristics of antibodies and standard materials used by the most popular BNP and NT-proBNP immunoassays (modified from references[19,58,59]).

Methods	Capture antibody	Detection antibody	Standard material
BNP immunoassays			
Architect platform (Abbott)	NH_2 terminus and part of the ring structure (Scios), murine monoclonal AB, aa 5–13	COOH terminus, murine monoclonal AB, aa 26–32	Synthetic BNP
Triage POCT method (Alere)	NH_2 terminus and part of the ring structure (Scios), murine monoclonal AB, aa 5–13	BNP (Biosite), murine Omniclonal AB, epitope not characterized (probably N-terminus 1–10)	Recombinant BNP
Triage method for Access and DxI platforms (Beckman Coulter)	NH2 terminus and part of the ring structure (Scios), murine monoclonal AB, aa 5–13	BNP (Biosite), murine Omniclonal AB, epitope not accurately characterized (probably N-terminus 1–10)	Recombinant BNP
ADVIA method for Centaur platform (Siemens)	COOH terminus (BC-203), murine monoclonal AB, aa 27–32	Ring structure (KY-hBNPII) (Shionogi), murine monoclonal AB	Synthetic BNP
IRMA (Shionogi)	COOH terminus (BC-203), murine monoclonal AB, aa 27–32	Ring structure (KY-hBNPII) (Shionogi), murine monoclonal AB	Synthetic BNP
AIA platform (Tosoh)[a]	COOH terminus (BC-203), murine monoclonal AB, aa 27–32	Ring structure (KY-hBNPII), murine monoclonal AB	Synthetic BNP
NT-proBNP immunoassays			
ECLIA method using monoclonal antibodies for Elecsys and Modular platforms (Roche)	Murine monoclonal AB, aa 27–31	Sheep monoclonal AB, aa 42–46	Synthetic $NTproBNP_{1–76}$
NT-proBNP assay for Dimension RxL, Stratus CS, Dimension VISTA, Dimension EXL with LM platforms (Siemens)	NH_2 terminus monoclonal sheep AB, aa 22–28	Central molecule, sheep monoclonal AB, aa 42–46	Synthetic $NTproBNP_{1–76}$
NT-proBNP assay for Immulite platforms (Siemens)	NH_2 terminus polyclonal sheep AB, aa 1–21	Central molecule, polyclonal sheep AB, aa 39–50	Synthetic $NTproBNP_{1–76}$

[a]The data regarding the AIA platform (Tosoh)are personal communications from TOSOH EUROPE N.V., Tessenderlo, Belgium.

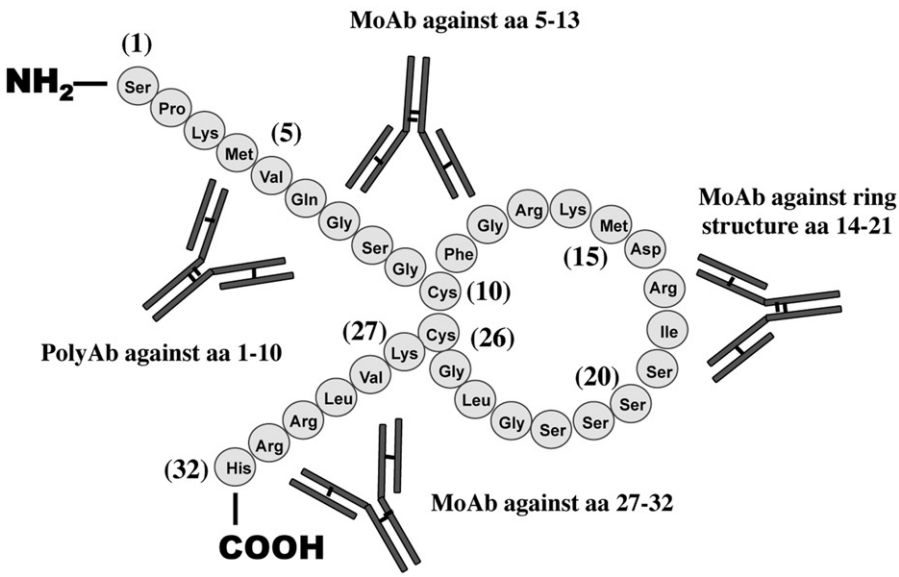

Figure 4. Epitopes of antibodies for BNP immunoassays. The epitopes of the antibodies used in the most common BNP immunoassays (reported in Table 2) are indicated on the BNP peptide chain.

for the Centaur platform and the Tosoh ST AIA-PACK method use the same antibodies and standard materials that are supplied by Shionogi (Table 2). Figure 5 shows the results of the 2013 and 2014 cycles of the CardioOrmoCheck study for the most common BNP immunoassays in Italy. These data confirm the systematic differences observed between the BNP immunoassay methods reported in previous studies that used plasma samples collected from healthy subjects and patients with cardiovascular diseases for the comparison between methods[56,57,60,61,64–66].

Specific immunoassay methods for proBNP

There are different methodological approaches to the measurement of proBNP[47,48,52,53,62]. In plasma or tissue extracts, proBNP was usually isolated by means of chromatographic

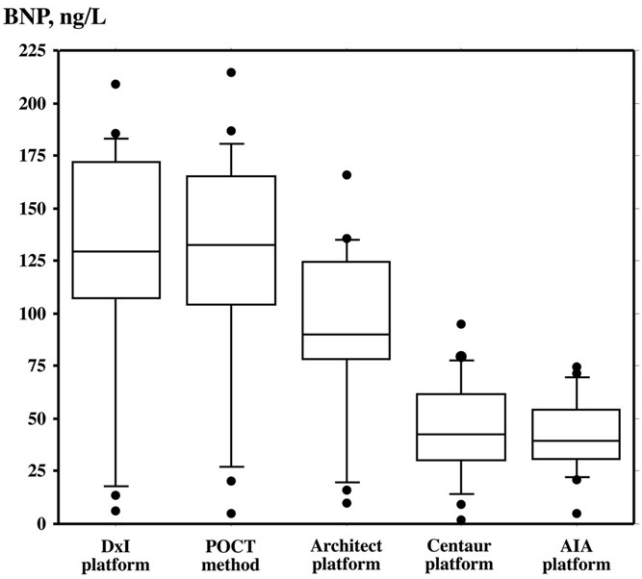

Figure 5. The box (distribution) plot of BNP values, measured by the most common methods used by the laboratory participants in the CardioOrmoCheck study[56,57,60], is reported. The data are reported as boxes indicating the 10th, 25th, 50th (median), 75th and 90th percentiles of BNP values. The outliers are indicated as separate black circles. The BNP data concern 19 study samples distributed in the CardioOrmoCheck EQA program in 2013 and 2014, and include 912 results obtained by about 100 Italian laboratories. The mean BNP values measured with the Siemens Centaur and Tosoh AIA platforms were significantly lower ($p < 0.001$ by *post hoc* Scheffé test after repeated measures ANOVA) than those measured with the Beckman DxI and Abbott Architect platforms and with the Alere Triage BNP POCT method, respectively. Indeed, the mean (±SD) BNP values measured on the Siemens Centaur platform (44.3 ± 24.2 ng/L) and Tosoh AIA platform (42.1 ± 17.6 ng/L) were on average less than one-half of those measured on the Beckman DxI platform (123.1 ± 62.4 ng/L), Abbott Architect platform (92.6 ± 41.5 ng/L) and the Alere Triage BNP POCT method (125.3 ± 55.2 ng/L). No significant differences were found among the BNP values measured on the DxI platform, Architect platform and Alere POCT device.

procedures, in particular by HPLC, and then identified and measured by immunoassay or, more accurately by mass spectrometry[14–18,22,24,38,39,41]. These methods are highly specific for the peptide, but are also complex, time-consuming and not available in the routine clinical laboratory. To solve these problems, at least three different methodological approaches have been developed with the aim to set up a highly specific immunoassay method for the measurement of proBNP[42,67,68].

Goetze et al.[67] developed a radioimmunoassay (RIA) for proBNP assay based on the measurement of plasma treated with trypsin, which cleaves all proBNP-related peptides to the $BNP_{1–21}$ fragment. This method used an antibody specific for a processing-independent epitope of human proBNP; it was directed against amino acids 1–10 of the proBNP peptide, and the same peptide, radio-iodinated with chloramine-T, was used as the tracer. This RIA measured all the peptides containing the N-terminal part of the precursor $proBNP_{1–108}$, including NT-proBNP. The authors determined the total concentration of proBNP and its products in healthy volunteers and HF patients, and showed that proBNP values were much higher in the HF patients compared to the volunteers[67]. From an analytical point of view, this RIA is not suitable for routine clinical use because it requires preliminary treatment of plasma samples with trypsin, a very long incubation time (up to 5 days), and the use of radiolabeled material. From a pathophysiological point of view, this RIA provides an estimation of the overall production by cardiomyocytes of the precursor proBNP, including both the intact circulating $proBNP_{1–108}$ and the parts of the precursor split before secretion (or in plasma) into NT-proBNP (and BNP)[47].

Tam et al.[68] developed a sandwich immunofluorescence assay for the quantification of BNP and its precursor, proBNP. They reported that this immunofluorescence assay recognized BNP as well as the recombinant glycosylated and non-glycosylated forms of proBNP with the same efficiency[68]. This immunoassay method (namely, ''single-epitope sandwich assay'') was different from the conventional sandwich assay because it required only one epitope for antigen immunodetection by two different monoclonal antibodies. In this novel immunoassay, the first antibody, the capture antibody, was specific for $BNP_{11–22}$, which is the most stable part of the peptide and which includes the biologically active cysteine ring. The second antibody, the detection antibody, recognized the immune complex, i.e. the antigen $BNP_{11–22}$ bound to the first antibody[68]. This single-epitope sandwich assay should recognize all the peptides that are shorter than proBNP, which include the region consisting of amino acids 11–22 of BNP, and that are able to form a complex with the first antibody[47].

Giuliani et al.[42] selected a specific monoclonal antibody that recognizes the cleavage site of $proBNP_{1–108}$, an epitope found only in the precursor form (Figure 1). This monoclonal antibody recognized 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[42] developed a sandwich immunoassay for the measurement of proBNP by combining the monoclonal antibody with a polyclonal antibody directed against $BNP_{1–32}$. An automated version of this method was set up on the BioPlex™ 2200 Analyzer Multiplex System (Bio-Rad Laboratories, Hercules, CA) and its analytical characteristics were evaluated[69]. More recently, some studies reported the clinical results obtained in the general community[70] and in patients with HF[71] or chronic kidney disease[72] by measuring proBNP concentrations using this immunoassay method. However, at the present time, this automated method for proBNP is not commercially available in North America and Europe.

Pathophysiological and clinical interpretations of immunoassay results

Although symptomatic HF patients (class C and D of ACC/AHA guidelines)[2] usually have extremely high circulating levels of natriuretic cardiac hormones that have powerful diuretic/natriuretic and vasodilator properties, they actually suffer from fluid retention and vasoconstriction. This phenomenon, sustained by reduced biological activity of cardiac natriuretic hormones and by altered peripheral turnover of these peptides[13,73,74], has been described as the ''endocrine paradox in heart failure'' (increased circulating levels of B-type related natriuretic peptides, measured by

immunoassay methods, but reduced activity of the cardiac natriuretic endocrine system): the diuretic/natriuretic and vasodilator action of increased production of cardiac natriuretic hormones by cardiomyocytes is hampered by peripheral resistance to their biological effects (in particular the natriuretic action) in HF patients[75,76].

These data are in accordance with the recent findings that the post-translational processing of ventricular proBNP is impaired in HF patients[13,21,28,48]. Consequently, a large proportion of B-type natriuretic peptides, as measured by immunoassay methods in these patients, may have reduced or even no biological activity. Indeed, in addition to bioactive $BNP_{1–32}$, a large number of circulating proBNP-derived fragments can be identified by chromatographic procedures in human plasma.

In particular, several studies have reported that the intact and glycosylated forms of the precursor proBNP should be considered to be the predominant B-type related peptide circulating in HF patients with severe disease (patients in III and IV NYHA functional class)[14,23,29,38,39,77]. Only the B-type natriuretic peptides that have an intact cysteine-ring are able to bind to the specific natriuretic receptor, and so these peptides are also biologically active (Figure 2)[13,21,50]. The peptide chain of $proBNP_{1–108}$ also includes the specific cysteine ring, and so the pro-hormone is able to bind to the specific natriuretic receptor. However, $proBNP_{1–108}$ activates the guanylyl cyclase-A of the specific natriuretic receptor with reduced potency that results in reduced biological activity compared to that of the hormone $BNP_{1–32}$[78]. Considering that proBNP is the predominant B-type related peptide circulating in HF patients with severe disease, its reduced biological activity may explain in part the endocrine paradox in heart failure[75,76].

On the other hand, several studies reported that the true levels of the peptide hormone $BNP_{1–32}$, identified and measured by means of mass spectrometry in patients with severe HF, may be much lower than those measured by commercially available immunoassay methods[38,39,77]. Some peptides derived from the degradation of $BNP_{1–32}$ may also have reduced or no biological activity[32], especially those without an intact cysteine-ring (Figure 2). Some of these peptides may be detected by some immunoassay methods that are considered specific for the biologically active hormone BNP. Unfortunately, at present time, there are no data about the effective biological activity of these BNP-derived peptides.

A fully automated immunoassay method specific for $proBNP_{1–108}$[42,69] would be very useful, as $proBNP_{1–108}$ has some advantages as a biomarker (i.e. more stable molecule, higher molecular weight and lower biological variability) compared to the measurement of the active hormone, BNP (Table 1). A recent study in ambulatory patients with chronic systolic HF 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[79]. As a future perspective, the simultaneous measurement in the same plasma sample by two methods, one specific for the intact precursor $proBNP_{1–108}$, and the other for active peptide $BNP_{1–32}$, may allow more accurate estimation of both the production/secretion of B-type related peptides from cardiomyocytes and the true activity of cardiac endocrine function compared to the single assay of either peptide. However, at the present time, there are no commercially available immunoassay methods that are able to measure *only* the active peptide, $BNP_{1–32}$. For the moment, this difficult analytical task, the accurate measurement of the peptide $BNP_{1–32}$, can be achieved only by means of complex and time-consuming methods such as immunoassays that include a complex preliminary chromatographic step[52,53] or mass spectrometry procedures, that are not suitable for the routine clinical laboratory[18,38,39,77]. Therefore, the development of a new generation of immunoassay methods and further clinical studies are needed to address the important issue of the endocrine paradox in heart failure[75,76].

Comparison between the results found by immunoassay methods for B-type-related natriuretic peptide: reference values and decision limits

From a pathophysiological point of view, it would be better to measure the active peptide, BNP, instead of the inactive peptides, NT-proBNP and proBNP, in order to evaluate the ''true biologically active status'' of the cardiac endocrine function[13,47,75,76,80]. However, to date, none of the commercially available methods is able to provide such information accurately. BNP immunoassay methods show large systematic differences due to the interferences of some inactive peptides, especially the glycosylated and non-glycosylated forms of the precursor peptide, proBNP[14,23,29,38,39,77]. According to international guidelines[19], manufacturers should test and clearly declare the cross-reactivities of glycosylated and non-glycosylated proBNP in their BNP immunoassay methods. As BNP immunoassay methods show very different analytical performance[37,53–57,60,61,64–68,81–89], the reference values and decision limits should be related to gender and age[12,51,90–92].

As far as reference values are concerned, circulating levels of cardiac natriuretic peptides are strongly gender-dependent in healthy subjects[13,90,93]. In particular, plasma levels of both BNP (Figure 6 and Table 3)[90–92] and NT-proBNP (Table 4)[94,95] are very high in the first 4 days of life; then peptide values fall rapidly during the first week with a further slower progressive reduction throughout the first month of life. BNP/NT-proBNP concentrations remain steady, without any significant changes, from 31 days to 12 years of age[90–95]. Plasma BNP/NT-proBNP concentrations increase progressively throughout adolescence in girls, reaching values in normal cycling women about 2-fold higher than men of the same age (Figure 7)[13,90–95]. In healthy adult subjects after 50 years of age, there is a gradual increase in both BNP (Figure 6) and NT-proBNP values[90,93]. In order to explain these variations, the possible influence of sex steroid hormones on the cardiac natriuretic hormone system[90,93], as well as changes in the cardiovascular system with age[96,97], may need to be considered. Higher plasma BNP/NT-proBNP levels of women during the fertile adult period compared to men could be explained by the action of sex steroid hormones, as previously discussed in detail[13,93,98]. In healthy adult subjects, there is a gradual increase in both BNP and NT-proBNP after 65 years of age. The increase of circulating

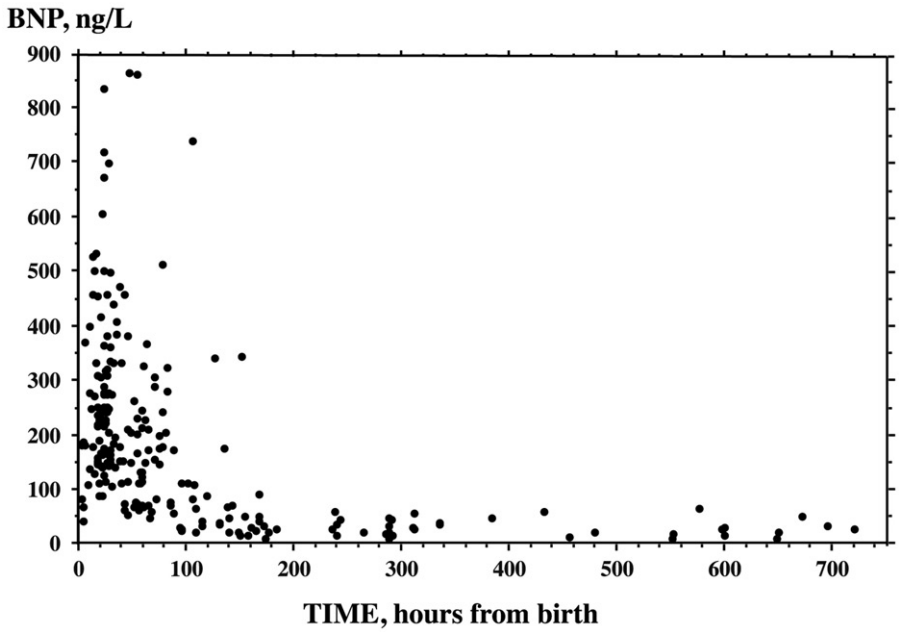

Figure 6. Plasma BNP values measured using the Alere Triage BNP method on the Beckman Access (REF 98 200) in 293 healthy newborns and infants in the first month of life in the authors' laboratory (modified from references[91,92]).

Table 3. Distribution of BNP values (ng/L), grouped according to 4 time periods from the birth to 12 years of life, in 253 healthy newborns and infants.

Groups (time periods from birth)	Number of individuals	Mean ± SD	Median	Range	97.5th percentile	*p* value
0–2 days	68	280.3 ± 167.5	243.5	41–866	758.7	<0.0001[a]
3–30 days	75	136.1 ± 149.3	75.0	10–763	741.4	<0.0001[b]
1–12 months	46	20.3 ± 10.7	19.0	5–45	43.9	
1–12 years	64	15.7 ± 8.9	13.0	4–46	39.8	
All groups (0–12 years)	253	123.4 ± 160.1	38.0	4–866	622.0	

Tests were measured in the author's laboratory with the Alere Triage BNP method on the Access platform (Beckman Coulter).
Range: minimum and maximum values.
[a]Significantly higher than all the following time period values.
[b]Significantly higher than the values observed throughout the next time periods (i.e. 1–12 months and 1–12 years).

Table 4. References values for NT-proBNP (ng/L) immunoassays in healthy neonates, infants and children measured by the ECLIA method using the Elecsys platform (Roche Diagnostics; data from Nir et al., modified from references[94,95]).

Age	Number of subjects	Median (ng/L)	Range (ng/L)	95th percentile (ng/L)	97.5th percentile (ng/L)
0–2 days	43	3183	260–13 224	11 987	13 222
1–5 days	20	1937	28–5309		
3–11 days	84	2210	28–7250	5918	6502
>1 month to ≤1 year	50	141	5–1121	646	1000
>1 to ≤2 years	38	129	31–657	413	675
>2 to ≤6 years	81	70	5–391	289	327
>6 to ≤14 years	278	52	5–391	157	242
>14 to ≤ 18 years	116	34	5–363	158	207
4 months–15 years	58	111	5–391	349	

levels of cardiac natriuretic hormones with age may be due to gradual decline in myocardial function and other organ function (including the kidney) that is typical of senescence[90,96,97]. As a result, BNP/NT-proBNP assays may be considered as biochemical markers of increased risk of cardiac morbidity in old age[90]. As well, the increase in the circulating levels of cardiac natriuretic peptides with age may be due to a decrease in their clearance rate. Indeed, age modulation of the maximum binding capacity of clearance (C-type) receptors for cardiac natriuretic peptides was reported in the platelets of elderly persons[99].

Considering the large systematic differences between BNP methods[37,54–57,60,61], one could argue that decision limits and cut-off values should be method-dependent. However, the

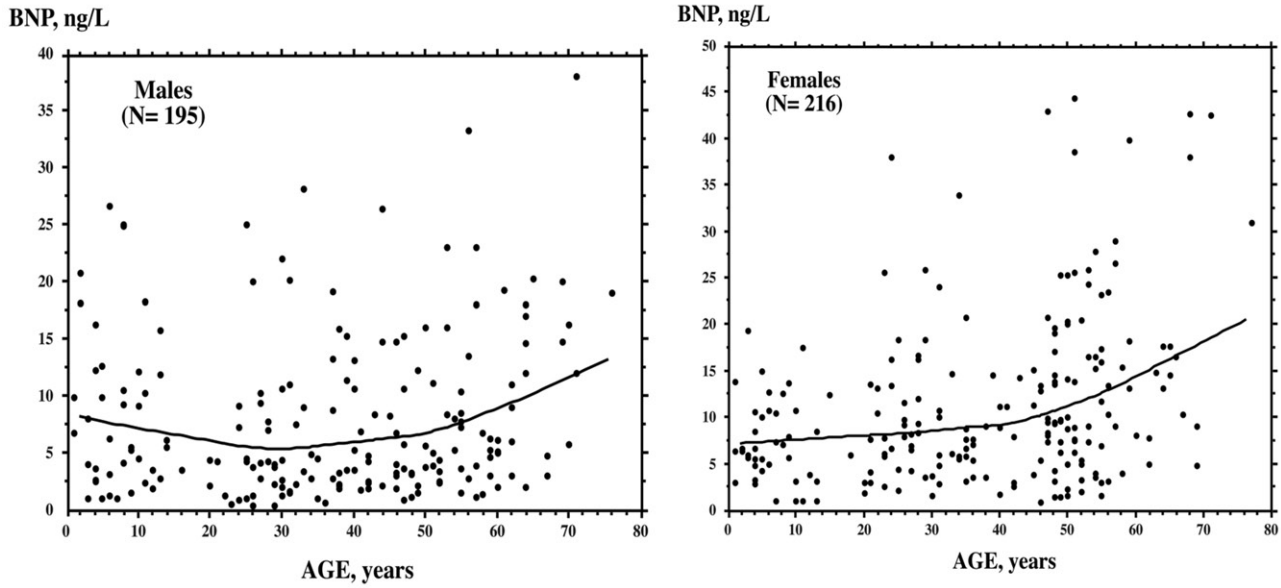

Figure 7. Variation of BNP levels in healthy males ($n = 195$) and females ($n = 216$). The age-dependent trend (continuous line) was assessed by smoothing analysis using 66% of locally weighted scatterplot smoother (LOWESS). Plasma BNP was measured in the authors' laboratory with a two-site Shionogi IRMA assay). Data modified from reference[90].

most recent international guidelines[1,100] suggest identical decision values for all BNP immunoassays: (1) a lower decision limit of 100 ng/L for exclusion of HF, (2) a grey zone of 100–500 ng/L and (3) an upper decision limit >500 ng/L for confirmation of a diagnosis of HF in patients with acute dyspnoea admitted to the Emergency Department. These decision limits are appropriate for the Alere Triage BNP methods for both the Alere POCT device and the Beckman DxI and Access platforms[101,102], but they are probably much too high for the Siemens ADVIA method for the Centaur platform and Tosoh AIA-PACK method for the AIA platform, which on average show BNP values that are about 30–50% lower than those measured with the Alere Triage BNP method[56,57,60,61,83,84]. Unfortunately, to the best of our knowledge, there are no studies specifically designed to evaluate and compare the diagnostic accuracy of the most common immunoassays method for BNP in the same population of patients with acute dyspnoea admitted to the Emergency Department.

Since there is predominantly one source of antibodies and calibrators for NT-proBNP assays)[1,19,20], NT-proBNP immunoassays usually have lower systematic differences than BNP immunoassays[56,57,60]. However, Di Serio et al.[103] reported that EDTA plasma samples showed unexplained higher NT-proBNP concentrations with the Dimension platform (Siemens Health Care Diagnostics, Munich, Germany) compared to heparin plasma samples. As a result, these authors[103] suggested the use of heparin rather than EDTA plasma samples for the measurement of NT-proBNP. Prontera et al.[104] observed slightly but significant lower NT-proBNP values for EDTA plasma samples than for serum or heparin plasma samples with the ECLIA method using the Elecsys platform (Roche Diagnostics, Basel, Switzerland). The results of these two studies[103,104] indicate that some differences between the values of NT-proBNP measured with different platforms (such as the Roche Elecsy or the Siemens

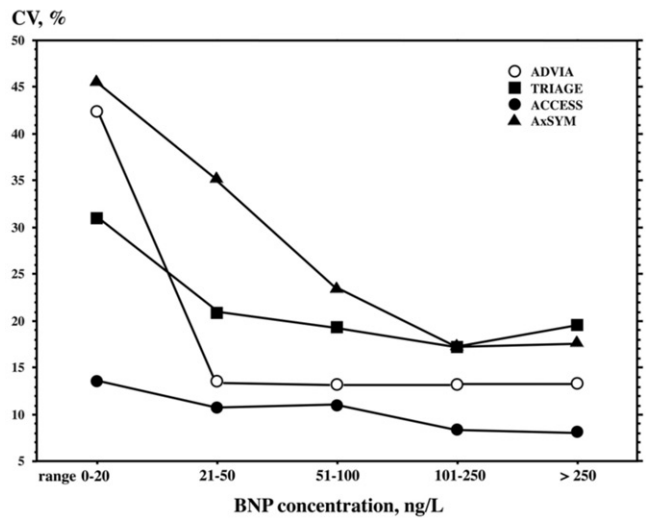

Figure 8. Imprecision profiles of the most common BNP immunoassays in the Italian EQA scheme, CardioOrmoCheck Study[56]. CV values represent the mean between-laboratory variability for each method. Samples were grouped together according to five concentration ranges for BNP immunoassays in order to estimate the average CV values. The data reported in the figure were obtained in the authors' laboratory and concern the results of the EQA cycles from 2005 to 2008, in which about 100 Italian laboratories participated, and were modified from reference[56].

Dimension platforms) may occur when different matrices such as serum or plasma samples are used. Furthermore, it is important to note that Roche recently introduced a new ECLIA method for the NT-proBNP assay that uses monoclonal antibodies instead of the polyclonal antibodies of the previous method; the monoclonal method on the Elecsys platform showed very similar analytical characteristics with slightly lower NT-proBNP results (on average 2.5%) than the polyclonal method[104]. The results of the CardioOrmoCheck study indicated that the total variability between NT-proBNP

Table 5. Imprecision data on the most used BNP and NT-proBNP immunoassays in the CardioOrmoCheck study (annual cycles from 2005 to 2011; data modified from the reference[57]).

System	Results	CV (%) (CI 95%)[a]
BNP immunoassays		
ADVIA system for Centaur platform (Siemens)	1016	10.2 (0.4–20)
POCT TRIAGE (Alere)	759	19.6 (2.3–36.9)
TRIAGE system for DxI and Access platforms (Beckman–Coulter)	1099	9.5 (6.2–12.8)
NT-proBNP immunoassays		
ECLIA system for Modular platform (Roche)	1199	5.7 (5.3–6.2)
ECLIA system for Elecsys platform (Roche)	1414	5.7 (5.2–6.2)
Dimension platform (Siemens)	390	9.9 (8.9–10.9)

[a]For BNP immunoassays, the CV values and the 95% respective confidence intervals (CI) were calculated at the cut-off (decisional) value (i.e. 100 ng/L)[1] by assuming a linear relationship between CV (dependent variable) and peptide concentration (independent variable) values[57]. For NT-proBNP immunoassays, the imprecision values reported in the table represent the mean CV values and the respective confidence intervals, calculated by pooling together all 72 control samples[57].

immunoassays methods are lower (on average always less than 20%) than those observed between BNP immunoassays. These findings are largely expected because all the NT-proBNP methods use the same antibodies and calibrator materials[56,57]. Taking into account that the results of the NT-proBNP methods are well harmonized, it is reasonable to use the same reference values and decision limits for these methods, as has been suggested by several international guidelines[1,2,11,12,19,20]. Furthermore, the reference values[105–107] and decision limits of NT-proBNP immunoassays have been evaluated in several studies[108–111]. The most recent international guidelines[1] recommend only one decision value of 300 ng/L of NT-proBNP for exclusion of HF in patients with acute dyspnea, but different upper decision limits to rule in HF, according to age: >450 ng/L for age <50 years, >900 ng/L for ages 50–75 years and >1800 ng/L for age >75 years.

However, from a clinical point of view, it is important to underline that all the international guidelines state that the commercially available BNP and NT-proBNP immunoassays usually give clinically comparable results when used for diagnosis, prognosis and follow-up of HF patients[1,2,11,12,19,20,51]. Of course, changes over time in patients with acute or chronic HF depend on biological and analytical variability[106,107,112]. NT-proBNP shows a higher plasma half-life and consequently also lower intra-individual biological variability than BNP (Table 1)[106,107,112–114]. However, only marked deviations from baseline (usually more than 30% for both BNP and NT-proBNP) correlate with hemodynamic improvement or deterioration in patients with acute or stable HF[110,115,116]; as a result, frequent blood sampling seems to be unnecessary[1]. Although the most recent international guidelines[1] recommend that laboratories should report reference change values based on published biological variation and analytical imprecision, this recommendation is usually disregarded in clinical laboratory practice for two reasons: there are large differences in reference change values reported in the literature, and the mathematical calculation of reference change values is difficult to perform because circulating levels of BNP and NT-proBNP have a log-normal distribution of[106,107,112–114]. A practical approach (i.e. a rule of thumb) in the management of patients with acute or chronic HF, suggested by some guidelines[1,51] and by several authors[7–10,106,116], may be to consider only changes >30% as clinically relevant.

The analytical performance, in particular analytical sensitivity and precision, of the POCT methods for BNP/NT-proBNP[64,65,117,118] is lower on average than immunoassay systems using automated platforms (Figure 8 and Table 5)[56,57,60,61,64,66], but the methods may nevertheless satisfy analytical goals[118]. The introduction of a new generation of POCT methods for BNP/NT-proBNP with better analytical performance into home-monitoring strategies could help patients and physicians to avoid unnecessary hospitalization or anticipate admission to the clinical ward when it is actually needed[119]. In this regards, a POCT method using capillary blood samples for the assay of BNP has recently been developed[120,121]. Preliminary data from our laboratory[121] suggest that BNP values measured by this POCT method in fresh capillary whole blood collected by fingerstick from adult subjects or by heel-prick in neonates and children are close correlated to those measured in venous plasma samples from the same subjects and patients.

Future perspectives and conclusions

In general, according to evidence-based laboratory medicine principles, a biomarker should be evaluated in several phases[122]. Biomarkers that do not change the management of a disease are probably unable to significantly affect patient outcome and are thus seldom cost-effective[122,123]. Randomized trials are the gold standard for establishing the effectiveness of biomarker-guided strategies[124]. Unfortunately, there are few examples of such trials in cardiology[124]. Indeed, the lack of well-designed randomized clinical trials may explain the relatively low degree of evidence (i.e. class IIa, level B) assigned to BNP-guided therapy in patients with chronic HF, even in the most recent guidelines[2]. However, some pivotal randomized clinical trials on BNP-guided therapy are now in progress[9]. The results of these studies will hopefully spread more light on the usefulness of this strategy in HF patients and promote the adoption of BNP-guided therapy in the management of HF patients.

From a pathophysiological point of view, a future perspective on the assay of B-type natriuretic peptides may be the contemporaneous estimation of both the production/

secretion of B-type related peptides from cardiomyocytes and the overall biological activity of cardiac endocrine function. This goal could be achieved by testing plasma samples using two methods simultaneously, one specific for intact precursor $proBNP_{1–108}$ and the other for active peptide $BNP_{1–32}$[47,48,55]. However, the clinical usefulness of these new and more specific methods will have to be accurately evaluated by randomized clinical trials that compare NT-proBNP and proBNP methods according to evidence-based medicine principles[122–124].

In conclusion, clinicians should consider that there are large systematic differences between methods when they compare results obtained from different laboratories that use different BNP assays. On the other hand, clinical laboratories should take part in EQA programs to evaluate the bias of the method used in comparison to other BNP methods. Finally, the authors believe that the development of more specific methods for active peptide $BNP_{1–32}$ should reduce the systematic differences among current methods and result in better harmonization among results.

Declaration of Interest

The authors report no declarations of interest.

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Notice of Correction:
The version of this article published online ahead of print on 30 Dec 2014 contained an error on page 10 of the article. The sentence ''NT-proBNP shows a lower plasma half-life. . .'' should have read ''NT-proBNP shows a higher plasma half-life. . .''. The error has been corrected for this version.