Clinica Chimica Acta 414 (2012) 112–119

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

State of the art of BNP and NT-proBNP immunoassays: The CardioOrmoCheck study

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ARTICLE INFO

Article history:
Received 22 June 2012
Received in revised form 19 July 2012
Accepted 26 July 2012
Available online 15 August 2012

Keywords:
BNP
NT-proBNP
Natriuretic peptides
Immunoassay
Quality control

ABSTRACT

To evaluate differences in analytical performance and clinical results of BNP and NT-proBNP immunoassays, a proficiency testing program, called CardioOrmoCheck study, has been organized since 2005 under the patronage of the Study Group of the Cardiovascular Biomarkers of the Italian Society of Clinical Biochemistry (SIBIOC). On average more than 100 Italian laboratories were involved in the annual 2005–2011 cycles.
In total, 72 study samples were distributed and measured by participant laboratories for a total of 6706 results. A great difference in between-method variability was found between BNP (43.0 CV%) and NT-proBNP (8.7 CV%) immunoassays. However, with the only exception of the POCT method for BNP assay, all immunoassay methods showed an imprecision ≤ 10 CV% at the cut-off levels (i.e. 100 ng/L for BNP and 400 ng/L for NT-proBNP assay, respectively). Furthermore, CardioOrmoCheck study demonstrated that the most popular BNP immunoassays are affected by large systematic differences (on average more than 2 folds between TRIAGE Beckman-Coulter and ADVIA Centaur Siemens methods), while the agreement between NT-proBNP methods was better.
CardioOrmoCheck study demonstrates that there are marked differences in analytical performance and measured values in particular among commercial methods for BNP assay. These findings suggest that it may be not reasonable to recommend identical cut-off or decision values for all BNP immunoassays.

1. Introduction

Cardiac natriuretic peptides, which include the atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) and their related peptides, constitute a complex family of peptide hormones produced and secreted by the heart [1–3]. The active peptides, ANP and BNP, are produced by cleavage of COOH-terminal part of the pro-hormone (proANP and pro-BNP), while the N-terminal fragments of pro-hormone, NT-proANP and NT-proBNP, are currently considered inactive [1–3].

The measurement of circulating BNP and its related-peptides is now considered to be a useful marker of myocardial function [1–6], and recent international guidelines recommend its use for the diagnosis, risk stratification, and follow-up of patients with chronic or acute heart failure [5–7]. Some meta-analyses [8,9] confirmed that both BNP and NT-proBNP immunoassays have a high degree of diagnostic accuracy and clinical relevance in both acute and chronic heart failure. However, very recent studies suggested that a great part of B-type natriuretic peptides measured in patients with cardiovascular disease is devoid of any biological activity [2,3,10–12].

In order to evaluate the differences in analytical performance and clinical results of the most popular BNP and NT-proBNP immunoassays, a proficiency testing study, based on an external quality assessment scheme and called CardioOrmoCheck, have been organized and carried out in Italy since January 2005. Previous, preliminary results of the CardioOrmoCheck study suggested that there are significant differences in analytical characteristics and measured values among the most popular commercial methods for B-type related natriuretic peptides, especially among the immunoassays considered specific for BNP. In particular, a 2.7 fold difference was on average found between the BNP values measured by the two methods reporting the highest and the lowest values, respectively [13]; while the NT-proBNP immunoassays showed only slight differences in both imprecision and measured values. These differences are probably due to the different crossreactions against the precursor proBNP and other related peptides shared by BNP immunoassays [14]; although some differences in the standard material used for the curve calibration cannot be excluded. According to these data [13,14], the most part of peptides measured by so-called BNP

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http://dx.doi.org/10.1016/j.cca.2012.07.017

immunoassays in patients with heart failure may be inactive. Moreover, significant differences in imprecision among methods were previously observed in the CardioOrmoCheck study [13]. These different analytical performances, concerning both measured values and analytical imprecision, may strongly affect the clinical usefulness of BNP immunoassay methods. However, in spite of these great differences in analytical and clinical performances, the most recent international guidelines [5,7] still suggest identical decisional values for all BNP (i.e., 100 ng/L) and NT-proBNP (i.e. 400 ng/L) immunoassay methods.

In the present article, authors reported the results obtained by the CardioOrmoCheck study throughout all the 7 years of the activity of the collaboratirve program (i.e., from 2005 to 2011 cycle). In particular, the greatly increased number of results available (i.e., 6706 results, 3269 for BNP and 3446 for NT-proBNP assay), compared to a previous report (i.e., 2354 results) [13], allowed a better evaluation of imprecision profiles of the most popular immunoassay methods of the survey and so an accurate estimation of CV confidence intervals, concerning the decisional values. Furthermore, the increasing number of study samples with different matrix (i.e., serum, EDTA or heparin plasma), tested in the study, allowed a more accurate evaluation of the possible matrix effect on the performance of BNP and NT-proBNP immunoassays.

2. Materials and methods

2.1. Collaborative study

The CardioOrmoCheck study is a proficiency testing program for the measurement of BNP and NT-proBNP, organized in Italy since 2005 under the patronage of the Study Group of the Cardiovascular Risk Biomarkers of the Italian Society of Clinical Biochemistry (SIBIOC) by three accredited (ISO 9001 accreditation) laboratories: QualiMedLab srl, working at CNR Institute of Clinical Physiology and Fondazione Regione Toscana G. Monasterio of Pisa, Department of Laboratory Medicine, University-Hospital of Padova, and Biomedical Research Centre of Castelfranco Veneto, Italy. On average, more than 100 Italian laboratories were involved in the annual 2005–2011 cycles. In particular, in the last cycle (year 2011), 130 Italian laboratories participated in the CardioOrmoCheck study: 55 laboratories used BNP, while 75 NT-proBNP methods. The major part (about 85%) of the participant laboratories was hospital labs, while the remaining part included private laboratories.

2.2. Sample preparation

In total, 72 study samples with different BNP/NT-proBNP concentrations were prepared by the central laboratories of the study (Supplemental File Table) according to the ILAC G13 guidelines, and measured by all participant laboratories for a total of 6706 determinations; some of these samples were repeatedly assayed by all laboratories to test also the within-laboratory variability. The list of BNP and NT-proBNP assay methods, more frequently used by participant laboratories, were reported in Table 1.

For the preparation of study samples, several plasma or serum specimens were pooled together to obtain a sample pool with final volume of about 100 mL, which was immediately stored at −20 °C. Different materials were used, such as plasma-EDTA, plasma Li-heparin, or serum (Supplemental File Table). Authors chose to prepare both plasma and serum samples in order to evaluate the relative commutability of different matrices and the possibly different degradation of peptides in plasma or serum.

All samples were tested for the absence of HBsAg, antiHCV, and antiHIV. Sample pools were prepared using residuals from samples collected from apparently healthy subjects (also divided according to gender) and patients with cardiac diseases with or without symptomatic heart failure. Blood samples collected from approximately 30–50 subjects/patients were included in every study sample. Subjects and patients gave the informed consensus for the use of their residual blood samples in the study.

Table 1
List of methods more frequently used by participant laboratories for the BNP and NT-proBNP assay.

BNP assays
1. BNP assay for ARCHITECT platform Abbott Diagnostics, Abbott Park, IL, USA. In the last cycle (year 2011), 8% of the laboratories used this method. A total of 314 results were produced by all the laboratories which used this BNP method, throughout all the cycles.
2. BNP TRIAGE Biosite method for Access and UniCel Dxl platforms, Beckman, Beckman Coulter, Inc., Fullerton, CA 92835, USA. In the last cycle (year 2011), 48% of the laboratories used this method. A total of 1099 results were produced by all the laboratories which used this BNP method, throughout all the cycles.
3. Triage Biosite BNP, Alere Inc., 51 Sawyer Road, Suite 200 Waltham, MA 02453-3448, USA. In the last cycle (year 2011), 13% of the laboratories used this method. A total of 759 results were produced by all the laboratories which used this BNP method, throughout all the cycles.
4. Advia Centaur BNP, Siemens Healthcare Diagnostics, TarryTown, NY 10591-5097, USA. In the last cycle (year 2011), 32% of the laboratories used this method. A total of 1016 results were produced by all the laboratories which used this BNP method, throughout all the cycles.

NT-proBNP assays
1. ECLIA NT-proBNP assay for Elecsys platform, Roche Diagnostics GmbH, D-68298 Mannheim. In the last cycle (year 2011), 32% of the laboratories used this method. A total of 1414 results were produced by all the laboratories which used this NT-proBNP method, throughout all the cycles.
2. ECLIA NT-proBNP assay for Modular platform, Roche Diagnostics GmbH, D-68298 Mannheim. In the last cycle (year 2011), 41% of the laboratories used this method. A total of 1199 results were produced by all the laboratories which used this NT-proBNP method, throughout all the cycles.
3. NT-proBNP assay for Dimension platform, Siemens Medical Solutions Diagnostics, TarryTown, NY 10591-5097, USA. In the last cycle (year 2011), 6% of the laboratories used this method. A total of 390 results were produced by all the laboratories which used this NT-proBNP method, throughout all the cycles.
4. NT-proBNP assay for Stratus system, Siemens Medical Solutions Diagnostics TarryTown, NY 10591-5097 USA. In the last cycle (year 2011), 5% of the laboratories used this method. A total of 126 results were produced by all the laboratories which used this NT-proBNP method, throughout all the cycles.
5. NT-proBNP assay for VIDAS system, bioMérieux Italia Spa, Bagno a Ripoli, Italy. In the last cycle (year 2011), 3% of the laboratories used this method. A total of 22 results were produced by all the laboratories which used this NT-proBNP method, throughout all the cycles.

Study samples were sent by mail as lyophilized materials. Lyophilization procedure was performed by Polymed (Sambuca, Firenze, Italy) within two weeks after preparation of sample pools. Stored sample pools, were defrosted, then distributed in approximately 150 vials (each containing a plasma/serum volume of 0.5 mL), and finally lyophilized, as previously reported [13]. The lyophilized materials were reconstituted with 0.5 mL of distilled water by participant laboratories before the assay. BNP and NT-proBNP concentrations of all the study samples were measured before and after the lyophilization by the reference laboratory (i.e., Laboratory of the Fondazione Regione Toscana G. Monasterio, Pisa) in order to evaluate the recovery of lyophilization procedure and the stability of BNP and NT-proBNP in the matrix samples. The recovery (mean ± SEM) after lyophilization procedure was significantly higher ($p = 0.0006$ by paired t test) for NT-proBNP assay (87.8 ± 4.4%) than for BNP assay (66.8 ± 4.3%). Furthermore, the recovery was not significantly different between EDTA plasma (mean ± SD recovery = 68.1 ± 24.7%), heparin plasma (52.2 ± 13.9%) or serum (64.5%) samples for BNP assay [13]. Samples with unreliable results were discarded. In particular, only the samples with BNP and NT-proBNP values, which showed peptide values similar to the pathophysiological characteristics of original samples (i.e., healthy subjects, patients with moderate or more severe heart failure) [1–3], were distributed as study samples in the survey.

2.3. Statistical analysis

Statistical analyses of the collected results were computed by the reference laboratory (i.e., Department of Laboratory Medicine, Fondazione

Regione Toscana G. Monasterio, Pisa), and then periodic and cumulative reports were prepared and sent by mail to each participant laboratory. It was also possible for the participant laboratories to find their individual results and the periodic and cumulative reports in a specific web site using a personal password (http://eqas.ifc.cnr.it). The periodic reports include a scoring system, reporting the performance of each single laboratory in comparison with those of other laboratories. Moreover, each report contains some quality parameters computed as follows:

1. Laboratory bias: mean percent deviations from the consensus mean of the results the laboratory obtained for all the study samples assayed in test period.
2. Laboratory imprecision: mean imprecision estimated from results reported by the laboratory for unidentified replicate samples. The mean imprecision is obtained by pooling the CVs for different replicate pools.
3. CV_T% (average between-laboratory agreement): pooled between-laboratory CV for all the study samples mailed out in the considered period.
4. BIAS% (average bias): the root mean square of all the laboratory biases.
5. CV_L% (average imprecision): median of all the laboratory imprecisions, accounting for the dispersion of the results of the laboratories with respect to their own means.
6. Method bias: mean of the percent deviations from the consensus mean of all the results reported by the users of the method.
7. Method imprecision: pooled CV computed from all results reported by the users of the method.

In particular, total variability (CV_T%) was estimated by averaging the CVs computed from the results of each study sample. This variability includes both systematic between-method differences and differences introduced by the laboratories. The imprecision of the methods was estimated by averaging the CVs of the results produced by the participants (using the same method) for the same study sample. Therefore, the reported average CVs, used for the calculation of imprecision profiles, were an estimate of the within-method, between-laboratory imprecision achieved by the method during the multicentre collaborative study. Outlier values were estimated according to the procedure recommended by Healy [15].

3. Results

3.1. Assay methods

The most important observation regarding the CardioOrmoCheck study is the inversion in the utilization trend of B-type natriuretic peptide immunoassays observed in the last years of the study. Indeed, while in the previous report [13] BNP assay methods were more utilized than NT-proBNP ones (53% vs 43%), at present time, NT-proBNP immunoassays resulted more utilized by Italian laboratories with 3446 results (51%) compared to 3260 (49%) results of BNP immunoassays. In particular, the ECLIA system for NT-proBNP, using the Elecsys and Modular platforms, was the most utilized method with a mean utilization ratio of 39% (on average 76% of utilization ratio considering only the NT-proBNP methods).

The MEIA method for the AxSYM system (Abbott Diagnostics) was gradually replaced in the last 3 years of the study cycles by the chemiluminescent assay using the ARCHITECT platform (Abbott Diagnostics). Some methods, including Immulite 2500, Vista and Stratus systems for NT-proBNP assay, were scarcely utilized by the participant laboratories, and so only a low number of results were available (Table 1). For this reason, the results of these methods were not considered in some statistical analyses of the present study.

3.2. Variability estimation

The mean total variability (i.e., including variability among all methods and laboratories) for BNP methods (43.0 CV%) was greatly higher than that for NT-proBNP methods (8.7 CV%). For BNP immunoassays, the mean variability, due to the difference between-methods (39.9 CV%), included the predominant part of total variability (corresponding to 86% of total variability), being the within-method variability on average 16.0 CV%. On the contrary, for NT-proBNP immunoassays the within-method and between-method variabilities were 6.5 CV% and 5.8 CV%, respectively; thus suggesting that the between-method variability contributes to the total variability less than within-method variability (44% vs 56%, respectively).

In order to better characterize the differences in imprecision between BNP and NT-proBNP immunoassays, we evaluated the imprecision profiles of the immunoassays methods, which were more popular in the CardioOrmoCheck study and presented an adequate number of data for the statistical analysis (more than 350 results) (Table 2). In particular, CV data of the MEIA method using AxSYM system and those of the chemiluminescent assay ARCHITECT platform for BNP assay, as well as the STRATUS, VISTA and VIDAS systems for NT-proBNP were not analyzed owing to an inadequate number of available data. For the other immunoassays (Table 1), the relationships (bivariate plots) between the imprecisions, expressed as CV % (Y-axis), and measured concentrations of the peptide (X-axis) were reported in Figs. 1 and 2 for BNP and NT-proBNP immunoassays, respectively.

As far as the BNP methods are concerned, a great variability in the imprecision was found between BNP immunoassays ($p<0.0001$ by repeated measures ANOVA). In particular, the TRIAGE POCT method for BNP showed significantly higher imprecision than the ADVIA Siemens and TRIAGE Beckman-Coulter automated systems ($p<0.0001$ by Scheffè post hoc test after ANOVA). Furthermore, we divided the 72 study samples in 3 groups according to the BNP concentrations: group A, BNP ≤ 50 ng/L; group B, BNP ranging from 51 to 100 ng/L; group C, BNP > 100 ng/L. The variation of CV values (dependent variable) was significantly explained ($p<0.0001$ by two way ANOVA) by differences in both BNP values, as measured by different immunoassays, and group concentrations. These data indicate that the imprecision of BNP immunoassays is strongly dependent by peptide concentrations with higher imprecision at lower values (Fig. 1). As a result, assuming a linear relationship between CV and logarithmic transformed values of the measured concentration, it was possible to estimate for the most common immunoassays in the survey the imprecision at the cut-off value of 100 ng/L, which is the decisional value recommended by international guidelines [5,7]; these data are reported in Table 2.

As far as the imprecisions of NT-proBNP immunoassays (Fig. 2) are concerned, a lower variability of imprecision was found compared to that of BNP immunoassays (Fig. 1). We divided the study samples in 3

Table 2
Imprecision data on the most used BNP and NT-proBNP immunoassays in the study.

System	Results	CV (CI 95%)[a] (%)
BNP immunoassays		
ADVIA Siemens	1016	10.2 (0.4–20)
POCT TRIAGE Alere	759	19.6 (2.3–36.9)
TRIAGE Beckman-Coulter	1099	9.5 (6-2–12.8)
NT-proBNP immunoassays		
ECLIA Modular Roche	1199	5.7 (5.3–6.2)
ECLIA Elecsys Roche	1414	5.7 (5.2–6.2)
Dimension 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) [5,7] by assuming a linear relationship between CV (dependent variable) and peptide concentration (independent variable) values. 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 control samples.

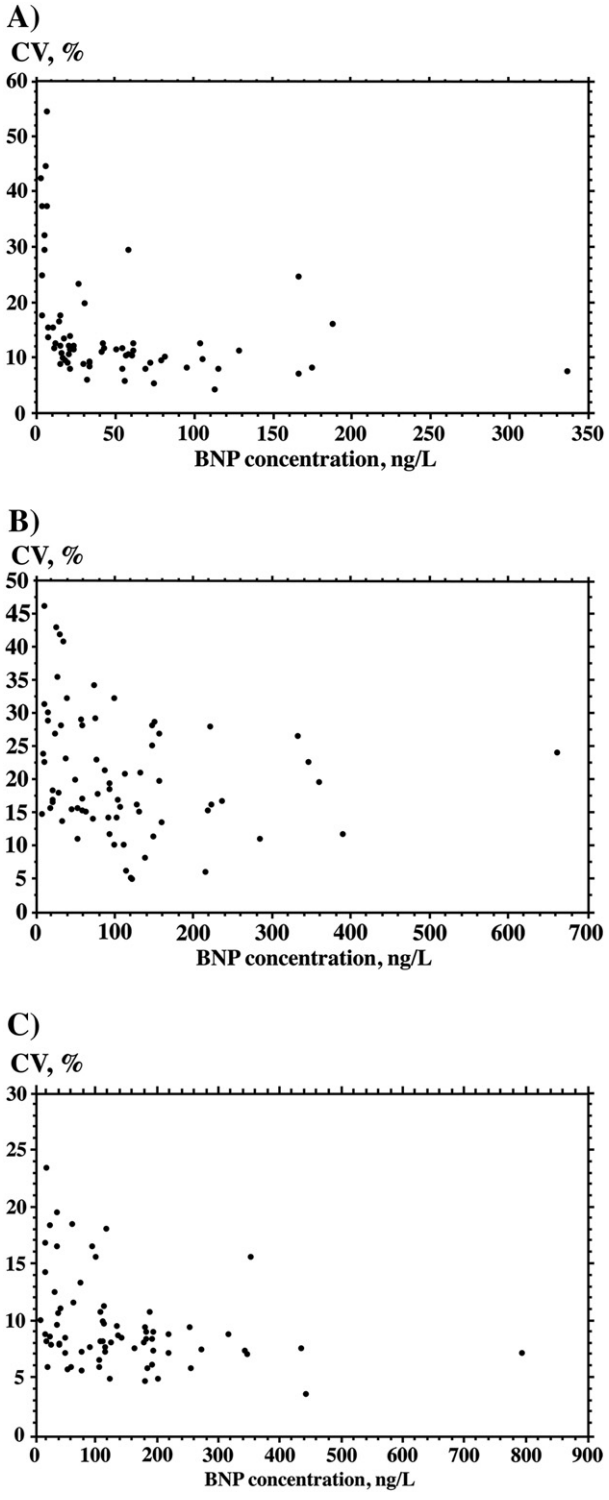

Fig. 1. Bivariate plots of relationship between CV values (Y-axis) and BNP concentrations (X-axis) measured by the most popular immunoassays in the survey. Part A. Relationship between CV values and BNP concentrations measured by ADVIA Siemens automated systems. Part B. Relationship between CV values and BNP concentrations measured by TRIAGE POCT Alere method. Part C. Relationship between CV values and BNP concentrations measured by TRIAGE Beckman-Coulter automated systems.

groups according to the peptide concentrations: group A, NT-proBNP ≤ 800 ng/L; group B, NT-proBNP ranging from 801 to 2000 ng/l; group C, NT-proBNP values > 2000 ng/L. We performed a two way, repeated measures, ANOVA using CV values as dependent variable and concentration groups and immunoassay methods as two independent variables. The mean imprecision of the automated

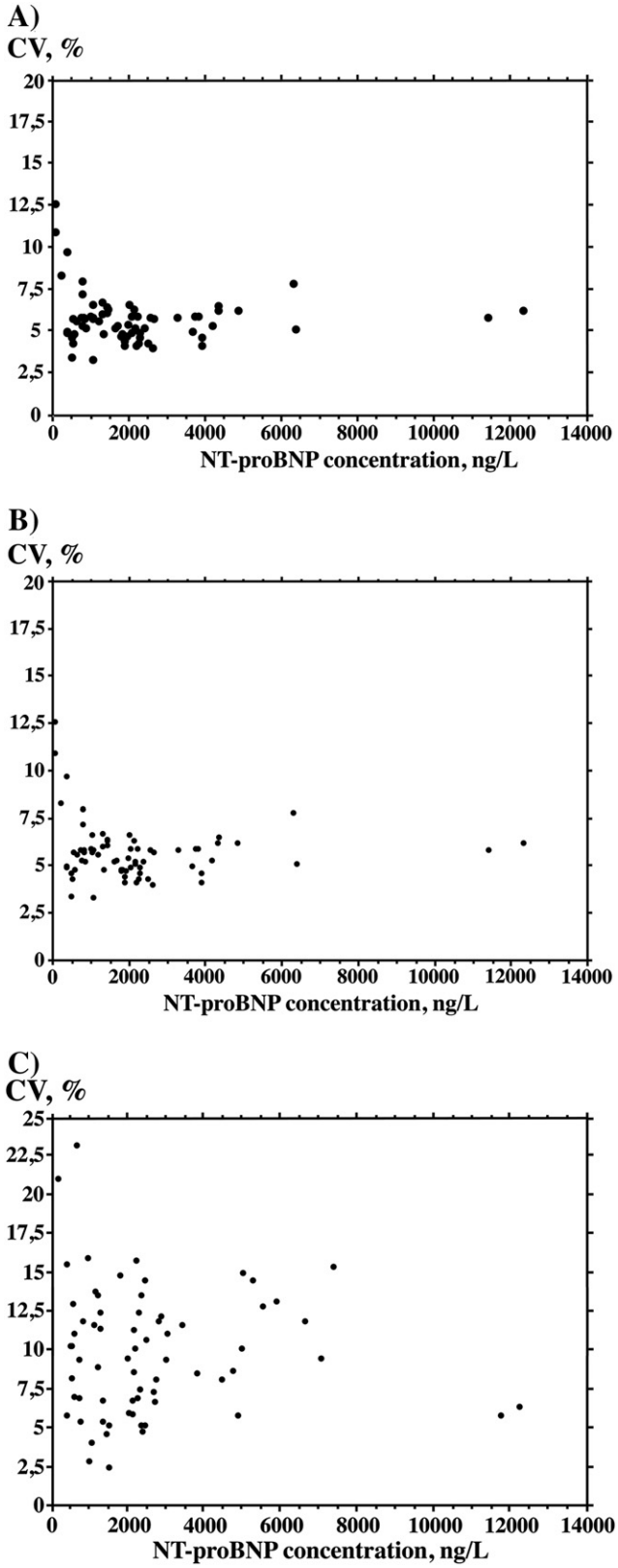

Fig. 2. Bivariate plots of relationship between CV values and NT-proBNP concentrations measured by the most popular immunoassays in the survey. Part A. Relationship between CV values and NT-proBNP concentrations measured by ECLIA method by Elecsys platform (Roche Diagnostics). Part B. Relationship between CV values and NT-proBNP concentrations measured by ECLIA method by Modular platform (Roche Diagnostics). Part C. Relationship between CV values and NT-proBNP concentrations measured by Dimension platform (Siemens Medical Solutions Diagnostics).

platform dimension was found to be significantly higher ($p<0.0001$ by Scheffé post hoc test) than those of ECLIA methods using both Elecsys and Modular platforms (Table 2). We found no association ($p=0.4623$) between the concentration groups (independent variable) and CV (dependent variable) by two ways, repeated measures ANOVA. Moreover, there were no significant correlations between CV values and log-transformed NT-proBNP concentrations for all the immunoassays. As a result, assuming that the imprecision at the cut-off level (i.e. 400 ng/L) is not significantly different to the mean imprecision, we estimated the 95% confidence intervals of the mean imprecision for each NT-proBNP immunoassay by pooling all data together (Table 2).

3.3. Evaluation of bias

As far as the BNP immunoassays are concerned, these methods gave results closely related with correlation coefficient values ranging from $R=0.920$ (correlation between the TRIAGE POCT method and ADVIA Siemens) to 0.981 (correlation between the TRIAGE Beckman-Coulter system and the TRIAGE POCT method) (Table 3). However, a significant difference ($p<0.0001$ by Scheffé post hoc test after repeated measures ANOVA using log-transformed data) was found between the mean BNP values measured by immunoassay methods (Fig. 3A). In particular, on average ADVIA systems (mean ± SD = 50.6 ± 56.9 ng/L) showed values about half ($p<0.0001$ by Scheffé test after ANOVA using log-transformed data) than those of TRIAGE POCT method (110.6 ± 109.8 ng/L), TRIAGE Beckman-Coulter systems (139.6 ± 127.2 ng/L), and MEIA Abbott systems (93.4 ± 100.1 ng/L), respectively. As an example, regression line and Bland–Altman plot concerning the BNP results obtained with ADVIA Centaur and TRIAGE Beckman-Coulter systems are reported in Fig. 4A and B, respectively. A significant bias, which increases linearly with the increase in BNP concentration, was obtained (Fig. 4B). Finally, the matrix effect on the results of BNP immunoassays was also tested by means of two way, repeated measures ANOVA. Plasma EDTA samples showed a significantly different ($p=0.0066$) behavior compared to the other matrices (i.e., serum and heparin samples). These findings indicate that EDTA plasma samples are preferable for a proficiency testing study, such as the CardioOrmoCheck.

As far as the NT-proBNP immunoassays are concerned, these methods gave results closely related: the two ECLIA methods showed a correlation R value corresponding to 0.948, while the Dimension methods showed R values corresponding to 0.938 with ECLIA Elecsys and to 0.982 with ECLIA Modular, respectively (Table 3). Finally, a significant difference ($p<0.0001$ by Scheffé post hoc test after repeated measures ANOVA using log-transformed data) was also found between the mean NT-proBNP values measured with the two ECLIA methods and Dimension platform system, although smaller than that seen for the BNP methods (Fig. 3B). Indeed, Dimension method showed NT-proBNP values on average about 17% higher than the two ECLIA methods. Finally, the different matrices tested in the study (serum, EDTA or heparin samples) did not significantly ($p=0.7688$) affect the results of NT-proBNP immunoassays, as tested by means of two way, repeated measures ANOVA.

Table 3
Correlation matrix between the values measured with the most popular BNP/NT-proBNP methods in study samples.

Part A. BNP immunoassay methods

Methods	ADVIA	POCT	BNP TRIAGE	MEIA
ADVIA	1.000	0.920	0.941	0.975
POCT	0.920	1.000	0.981	0.930
BNP TRIAGE	0.941	0.981	1.000	0.945
MEIA	0.975	0.930	0.945	1.000

Part B. NT-proBNP immunoassay methods

Methods	Elecsys	Modular	Dimension
Elecsys	1.000	0.948	0.938
Modular	0.948	1.000	0.982
Dimension	0.938	0.982	1.000

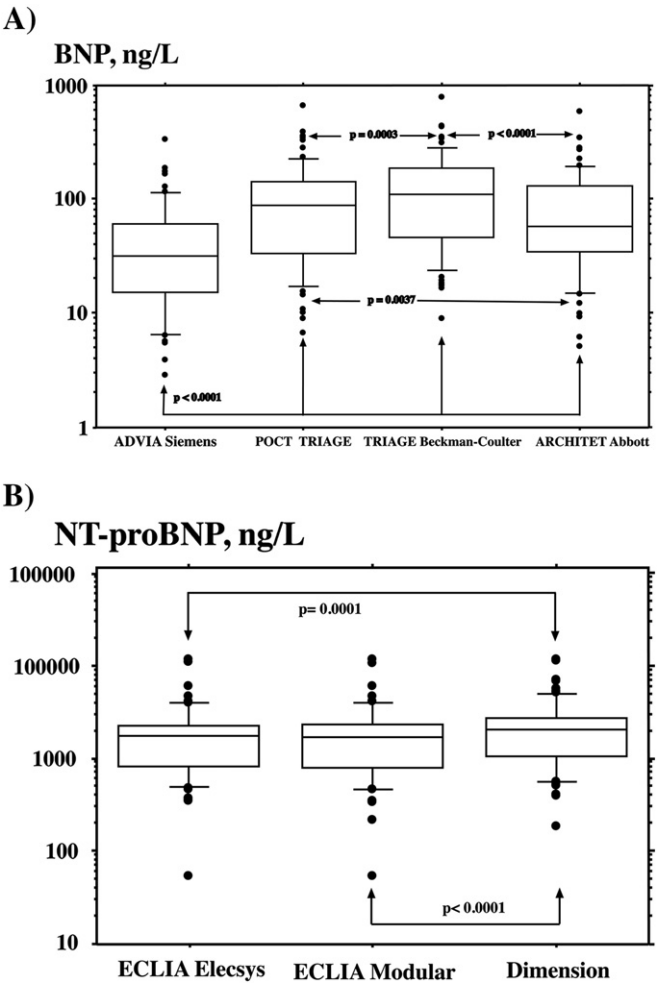

Fig. 3. Box (distribution) plot of BNP (Part A) and NT-proBNP (Part B) values measured by the most popular methods of the study. The data are reported as boxes indicating the 10th, 25th, 50th (median), 75th and 90th percentiles of BNP and NT-proBNP values measured in the 72 study samples; the outliners were indicated as separated black circles. The concentrations (Y-axis) are reported as log-scale. The levels of statistical significance (p values) are also indicated in the figure.

4. Discussion

4.1. Study protocol

Several issues should be considered when some immunoassay methods for the measurement of B-type related natriuretic peptides should be evaluated in a proficiency testing study [13]. In particular, some critical points are the low stability *in vitro* of B-type biologically active peptides, the possible matrix effects, and the evaluation of commutability of study samples. Considering these critical issues, to perform a reliable proficiency testing study for BNP and NT-proBNP immunoassay a formidable task should be considered.

Taking into account quality specifications [16] and recommendations by manufacturers [17], several different matrices derived from human blood samples should be used in a proficiency testing study [13] for BNP and NT-proBNP immunoassays. Indeed, the quality specifications for BNP assay recommend the use of EDTA plasma samples [16], while serum should be preferred for NT-proBNP assay [16,17]. Accordingly, one of the most important aims of the CardioOrmoCheck study was the evaluation of relative commutability of different matrices.

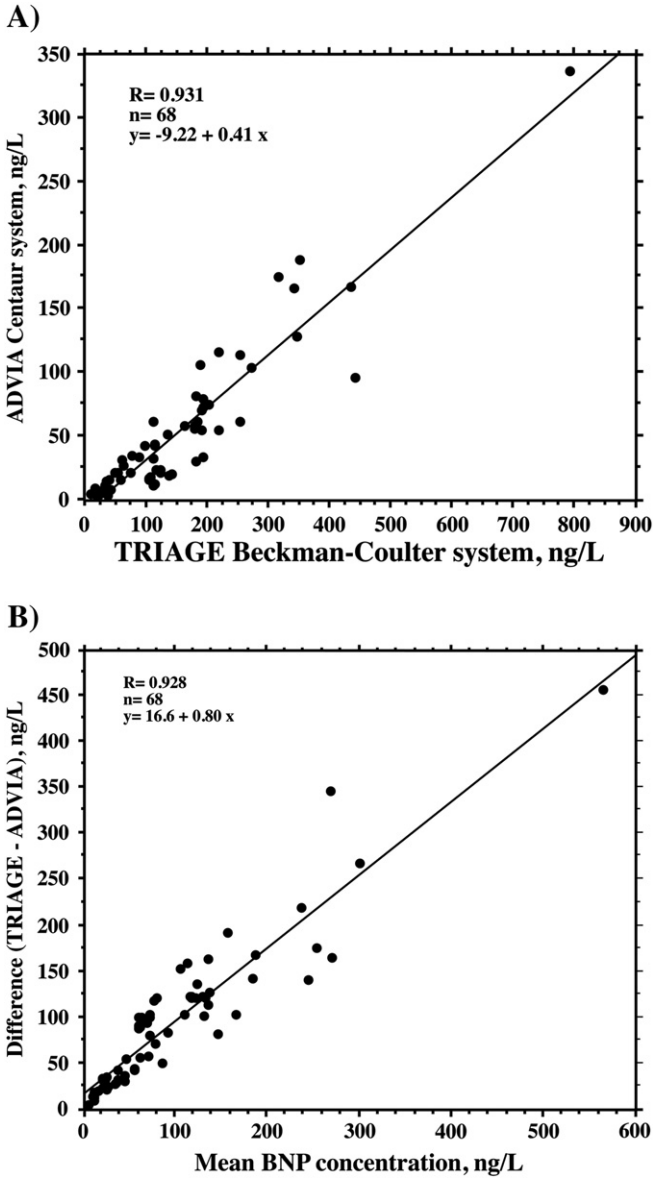

Fig. 4. Part A. Linear regression between the BNP values measured with TRIAGE Beckman-Coulter (X-axis) and ADVIA Centaur (Y-axis) systems in 68 study samples. Part B. Linear regression (Bland–Altman plot) between the mean BNP concentration, measured with TRIAGE Beckman-Coulter and ADVIA Centaur systems (X-axis), and the difference (Y-axis) between the BNP values measured by these two methods (TRIAGE − ADVIA) in 68 study samples.

At least to our knowledge, a primary reference material, which is certified as commutable for all BNP and NT-proBNP immunoassays, is not available. As a result, it is not possible to prepare secondary reference materials, calibrators or control samples with a certified concentration of the peptide [18–20]. It seems to be not reasonable to use in the present study a procedure recommended for the preparation of primary reference materials, such as the CLSI C53-A protocol [21]. Indeed, the aim of the present study is only to evaluate the intra- and between-assay variability of BNP and NT-proBNP immunoassays, which is an objective different to the more ambitious intendment to calibrate or to assess the trueness of these methods. According to this aim, as also suggested by other Authors [19,20], if commutable reference materials suitable for direct use are lacking, the only possible alternative for establishing traceability is to split human fresh samples with a laboratory performing the reference measurement procedure. However, the reference procedure for the measurement of B-type related peptides is also lacking. In a proficiency study, when the reference material and method are both lacking, the only remaining and practicable way is to compare the results of the different methods with the consensus mean, as made in CardioOrmoCheck study. Evidently, the "consensus mean" approach can allow only a relative estimate of bias between methods and laboratories. In addition, it is possible to test only the relative commutability of study samples, distributed in the proficiency testing program, in comparison with the original samples collected from healthy subjects or patients (but not with a reference material).

It is well known that some B-type related peptides, and especially the active peptide, BNP_{1-32}, are greatly degradable both *in vivo* and *in vitro* [2,3]. Preliminary data performed in the reference laboratory of the CardioOrmoCheck study (data not shown) indicated that it is not possible to organize a reliable proficiency study for BNP immunoassays, including more than 100 laboratories, using frozen plasma specimens, as study samples. As a result, in order to reduce the *in vitro* degradation and to make the distribution as well as the measurement of blood samples easier, only lyophilized plasma and serum samples were used in the CardioOrmoCheck study. As previously reported [13], the recovery after lyophilization procedure was significantly higher for NT-proBNP assay (on average 88%) than for BNP assay (on average 67%). The lower recovery after lyophilization and the greater instability *in vitro* of the BNP compared to NT-proBNP, may be, almost in part, the causes of greater variability found between BNP and NT-proBNP immunoassays in the present study. However, other authors [22–25] also reported a great variability in the ratio of BNP and NT-proBNP among both individual subjects and patients with cardiac diseases.

The term commutability has been defined by the CLSI C53-A protocol "as the interassay properties of reference material, calibrator, or trueness quality control materials that are comparable with those demonstrated by authentic clinical specimens" [21]. In the present study, a progressive increase in both BNP and NT-proBNP values of the consensus means from healthy subjects to patients with severe heart failure was found (Supplemental File Table), thus suggesting that the study samples, used in the CardioOrmoCheck study, retained (almost in part) the clinical information of the original blood samples. Furthermore, the good agreement between BNP or NT-proBNP methods, as expressed by the correlation coefficients (Table 3), found for the study samples, was very similar to those previously reported by several head-to-head comparisons of analytical characteristics and clinical results of immunoassay methods performed by the reference laboratory of the CardioOrmoCheck study [26–34] or other laboratories [22–25,35–37], using authentic specimens collected from healthy subjects and patients with cardiac or extracardiac diseases.

According to the data discussed in the previous paragraphs, the results of the present study confirm our preliminary data [13], suggesting that it is possible to prepare suitable study samples for a proficiency testing program by pooling human blood specimens collected from healthy subjects or patients with cardiac disease.

4.2. Results discussion

The CardioOrmoCheck study, including results concerning 7 years (from 2005 to 2011), indicates that the panorama of commercial BNP and NT-proBNP methods is rapidly changing. The immunoradiometric assay method for BNP assay by Shionogi, evaluated in the previous study [13], is at present no more commercialized, at least in Europe. On the other hand, the BNP method for ARCHITECT platform by Abbott, as well as VISTA, STRATUS, and IMMULITE systems by Siemens, and VIDAS system by BioMérieux for proBNP assay, not evaluated in the previous study [13], have been only recently distributed and so these "new" methods have been adopted by participating laboratories only in the last 2 or 3 cycles of the CardioOrmoCheck study. Unfortunately, the results concerning these "new" BNP and NT-proBNP methods are insufficient to allow a reliable statistical analysis.

Since BNP and NT-proBNP share completely different biochemical structure, molecular weight, biological activity and degradation pathways

[1–3,10–12], it is not surprising that BNP and NT-proBNP immunoassays show not only different analytical characteristics and quality specifications, but also different clinical results. Indeed, data of the CardioOrmoCheck study confirm that there are wide differences in both analytical performances and measured peptide values, especially among BNP methods. These differences in the performance between BNP and NT-proBNP immunoassays are theoretically expected because all the commercial NT-proBNP methods tested in the study (the two ECLIA methods and Dimension method) actually use antibodies and standard materials from the same source (i.e., Roche Diagnostics), while BNP methods use different antibodies and standard materials [14,16,22].

The greater number of results obtained in the present study allowed a more complete analysis of analytical performance of immunoassay methods than the previous one [13]. In particular, it was possible to evaluate the imprecision profiles and to calculate the imprecision at the recommended cut-off values for the most popular BNP and NT-proBNP immunoassays of the survey. The results of CardioOrmoCheck study demonstrate that the imprecision around the recommended cut-off values (corresponding to 100 ng/L for BNP and 400 ng/L for NT-proBNP, respectively) [5,7] varies among methods (Table 2). However, our data indicate that with the only exception of POCT method for BNP assay, all other BNP and NT-proBNP immunoassays show an imprecision at the cut-off values lower or around the 10% CV. On average, the NT-proBNP methods showed a lower variability compared to BNP immunoassays. These data are well in agreement with those previously reported in a study performed in the reference laboratory of the CardioOrmoCheck study, which compared the analytical characteristics of some BNP immunoassays with those of the ECLIA method for NT-proBNP [27]. This difference is probably in great part due to the different biochemical characteristics of BNP and NT-proBNP. NT-proBNP shows greater molecular mass and higher circulating levels, and it is more stable *in vivo and in vitro* than BNP. As a result, it is easier to set up a robust immunoassay method for NT-proBNP than for BNP.

As far as the bias between the BNP immunoassays is concerned, the results of the CardioOrmoCheck study confirm that the most popular BNP immunoassays are affected by large systematic differences (on average more than 2 fold between TRIAGE Beckman-Coulter and ADVIA Centaur Siemens methods, Figs. 3A and 4). Wide differences between the results of BNP immunoassays were also previously reported in a study performed in the reference laboratory of the CardioOrmoCheck study [27]. In particular, this study found lower BNP values measured by ADVIA Centaur and IRMA by Shionogi methods compared to the other immunoassays, such as the POCT TRIAGE methods and the MEIA methods for the AxSYM platform [27].

As far as the NT-proBNP immunoassays are concerned, Di Serio et al. [35] reported that some EDTA plasma samples (5 samples included in a set of 65) showed unexplainable higher NT-proBNP concentrations with Dimension platform compared to heparin plasma samples. As a result, these authors [35] suggested the use of heparin rather than EDTA plasma samples for the measurement of NT-proBNP. Prontera et al. [28] observed slightly but significant lower NT-proBNP values for EDTA plasma samples than for serum or heparin plasma samples with the ECLIA method. As a result, the significant bias found in the present study between the NT-proBNP values measured by the two ECLIA methods and the Dimension system in the study samples may be (almost in part) due to matrix effects. However, our data indicate that the different matrices of study samples (serum, EDTA or heparin samples) do not significantly affect the results of NT-proBNP immunoassays. It is important to note that Roche Diagnostics recently introduced a new ECLIA method for the NT-proBNP assay, which uses monoclonal antibodies instead of polyclonal antibodies of the previous method [28]. A recent study performed in the reference laboratory of the CardioOrmoCheck study [28] reported that the monoclonal ECLIA method showed very similar analytical characteristics with slightly lower NT-proBNP results (on average −2.5%) than the polyclonal ECLIA method. However, the data collected so far in the CardioOrmoCheck study are not sufficient to demonstrate a significant difference between the results of polyclonal compared to monoclonal ECLIA method.

4.3. Prospective and conclusive remarks

The data of the CardioOrmoCheck study indicate that the panorama of commercial BNP and NT-proBNP methods is rapidly changing. The most important observation regarding the CardioOrmoCheck study is the inversion in the utilization trend of B-type natriuretic peptide immunoassays observed in the last years of the study, indicating that in these years the NT-proBNP immunoassays resulted more utilized by Italian laboratories than the BNP immunoassays. As suggested by some authors [38,39], it is also conceivable that some methods, more specific for the intact prohormone peptide (proBNP), may soon become commercially available [40,41]. These data clearly suggest that there is not yet available an ideal method for the measurement of B-type natriuretic peptides. The proficiency testing study, such as the CardioOrmoCheck study, may support the comparative evaluation with the aim to find the most reliable methods and to achieve a better harmonization between immunoassay methods for BNP assay.

Moreover, the results of the CardioOrmoCheck study demonstrate that there are marked differences in analytical performance and measured values especially among commercial methods for BNP assay. These findings suggest that it may be not reasonable to suggest identical cut-off or decision values for all BNP immunoassays, as recommended by international guidelines [5,7]. Further studies are needed to confirm this observation.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2012.07.017.

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