Evaluation of 99th percentile and reference change values of a high-sensitivity cTnI method: A multicenter study

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ABSTRACT

Background: The Italian Society of Clinical Biochemistry (SIBioC) and the Italian Section of the European Ligand Assay Society (ELAS) have recently promoted a multicenter study (Italian hs-cTnI Study) with the aim to accurately evaluate analytical performances and reference values of the most popular cTnI methods commercially available in Italy. The aim of this article is to report the results of the Italian hs-cTnI Study concerning the evaluation of the 99th percentile URL and reference change (RCV) values around the 99th URL of the Access cTnI method.

Materials and methods: Heparinized plasma samples were collected from 1306 healthy adult volunteers by 8 Italian clinical centers. Every center collected from 50 to 150 plasma samples from healthy adult subjects. All volunteers denied the presence of chronic or acute diseases and had normal values of routine laboratory tests (including creatinine, electrolytes, glucose and blood counts). An older cohort of 457 adult subjects (mean age 63.0 years; SD 8.1 years, minimum 47 years, maximum 86 years) underwent also ECG and cardiac imaging analysis in order to exclude the presence of asymptomatic cardiac disease.

Results and conclusions: The results of the present study confirm that the Access hsTnI method using the DxI platform satisfies the two criteria required by international guidelines for high-sensitivity methods for cTn assay. Furthermore, the results of this study confirm that the calculation of the 99th percentile URL values are greatly affected not only by age and sex of the reference population, but also by the statistical approach used for calculation of cTn distribution parameters.

1. Introduction

Cardiac troponin I (cTnI) and T (cTnT) are the preferred biomarker for the differential diagnosis of acute coronary syndrome (ACS) [1–3]. International guidelines recommended that the increase in cTnI or cTnT levels over the 99th percentile upper reference limit (99th URL) should be considered as clinically relevant, and they also specifically indicated that this cut off value should be measured with an imprecision ≤ 10 CV % [1–3]. Only after the year 2006, some manufacturers set-up the first new generation of cTnI and cTnT immunoassays with improved...
analytical sensitivity with the aim to satisfy the quality specifications requested by international guidelines and consensus documents [4–14]. The 2018 Expert Opinion from the AACC and IFCC [2] recommends that high-sensitivity methods for cTnI and cTnT assay should satisfy two fundamental criteria. First, high-sensitivity methods should measure the 99th percentile URL with an imprecision (expressed as CV %) ≤ 10%. Second, high-sensitivity methods should measure cTn concentrations above the LoD of the method at least in 50% healthy subjects enrolled in large populations including > 300 men and 300 women [2]. The 99th URL values not only strongly depend on demographic and physiological variables of populations (i.e., the criteria for considering the reference population “healthy”), but also on the analytical performances of cTnI methods, as well as on the mathematical algorithms used for calculating the 99th percentile URL [15,16].

In 2016, the guidelines of European Society of Cardiology (ESC) on the management of acute coronary syndromes (ACS) in patients presenting without persistent ST-segment elevation (NSTEMI) suggested 0 h/1 h rule-in and rule-out algorithms when measurement of cardiac troponins is performed using high-sensitivity immunoassay methods [1]. These guidelines recommend the use of absolute change (i.e., expressed as difference in cTn concentrations, ng/L) rather than percentage variation for the assessment of the rise and/or fall of cTn values [1].

According to the claims of international guidelines [1–3], the Italian Society of Clinical Biochemistry (SIBioC) and the Italian Section of the European Ligand Assay Society (ELAS) have recently promoted a multicenter study (named Italian hs-cTnI Study) with the aim to accurately evaluate analytical performances and reference values of the most popular cTnI methods commercially available in Italy. The most important aims of this article are to report and discuss the results of the Italian hs-cTnI Study concerning the evaluation of the 99th percentile URL and reference change (RCV) values around the 99th URL of the Access method, for cTnI assay using UniCell DxI 800 automated platform (named Access hsTnI, REF B52699), recently distributed to the Italian clinical laboratories.

2. Materials and methods

2.1. Reference population and plasma sample collection

The Italian hs-cTnI Study is a multicenter clinical study. Heparinized plasma samples were collected from a total of 1306 healthy adult volunteers by 8 Italian clinical centers, including both University and Regional Hospitals, which have highly qualified workforce staff in emergency, cardiology and laboratory departments. Every center collected from 50 to 150 plasma samples from healthy adult subjects enrolled from the clinical and laboratory staff or volunteers blood donors with age from 18 to 86 years.

All volunteers denied the presence of chronic or acute diseases and had normal values of routine laboratory tests (including creatinine, electrolytes, glucose and blood counts) [17]. In particular, to more accurately evaluate cTnI concentrations of healthy subjects older than 47 years, plasma samples from 457 adult subjects collected in the MEHLP study were also assayed (mean age 63.0 years; SD 8.1 years, minimum 47 years, maximum 85 years). The MEHLP study is a screening study aimed to evaluate the cardiovascular subclinical disease in an asymptomatic general population with age > 45 years from the community of Montignoso (Massa, Italy), as reported in detail elsewhere [17]. All the subjects enrolled in the MEHLP study underwent an accurate health investigation by means of a thorough clinical examination and routine laboratory tests [17]. Furthermore, lifestyle habits and medical history were collected by questionnaires. Participants to the MHHELP study underwent also ECG and cardiac imaging analysis (computed tomography scan, carotid echography, echocardiography). Exclusion criteria were: presence of cardiac or systemic acute or chronic diseases, such as myocardial infarction, heart failure, coronary heart disease, hypertension, diabetes, kidney disease, obesity, tumour, hepatitis, BPCO, and use of drugs [17].

Every laboratory participating to the study stored at ~ 80°C two aliquots of about 1 mL of plasma samples collected from healthy subjects in tubes identified by alphanumeric barcodes. The stored tubes were sent to the reference laboratory of the study (Fondazione CNR Regione Toscana G. Monasterio, Pisa Italy) using a pack with dry ice within one month after the blood collection. Only age and the sex of adult healthy volunteers were known by the staff of the reference laboratory. In the reference laboratory the clinical samples were immediately stored at ~ 80°C and then the samples were measured within three months with the Access cTnI immunoassay.

The informed consent was obtained by all subjects enrolled in the study in accordance with the guidelines recommended by the respective local ethical committees.

2.2. cTnI immunoassay method

The Access hsTnI (REF B52699) assay is a two-site immunometric assay method (Beckman Coulter, Inc. Brea, CA 92821 USA). In the reference laboratory of the Italian hs-cTnI Study were previously evaluated the analytical characteristics and performances of the Access cTnI method [11]. The plasma samples were assayed in the reference laboratory according to the analytical procedures suggested by the manufacture using the DxI 800 platform [12]. Limits of blank (LoB), detection (LoD), and quantitation (LoQ) at 10% CV and 20% CV were calculated according to international standardized protocols [18,19], as previously described in details [11]. The imprecision profile was estimated by repeatedly measuring 10 heparinized plasma pools collected from healthy subjects and patients with cardiovascular diseases, as previously reported in detail [11]. These pools with mean cTn concentrations ranging from about 1 ng/L to about 50 ng/L were repeatedly measured in 40 different runs with at least 3 different calibrations using two or more lots of reagents throughout two months. To calculate the limit of quantification (LoQ), the relationship between the error of the measurement (expressed as CV values, Y-axis) and cTn concentrations (X-axis) was interpolated by means of a nonlinear regression curve [11].

The Reference Change Values (RCV) were evaluated according to Callum G. Fraser [20]. The bidirectional Z-score RCV between two results (CI 95%) were calculated by considering both the analytical variability of the method (CVα) and the intra-individual variability (CVβ), using the formula: RCV = 1.96 [2(CVα [2] + CVβ [2])] 1/2.

2.3. Statistical Analysis

For the evaluation and comparison of the analytical performance of tested cTnI immunoassay methods, standard statistical analyses were carried out using the JMP program (version 12.1.0, SAS Institute Inc., SAS Campus Drive, Cary, NC, USA). As cTnI circulating levels are not normally distributed, both non-parametric and parametric tests after logarithmic transformation (log-10) of data were used for statistical analysis. For the calculation of mean and standard deviation (SD) only robust methods were used. The identification of outlier values was performed by means of the based on the interquartile range (IQR) assuming a log-normal distribution [21]. An outlier value was evaluated by the formula: cTnI > Q3 + 3 IQR, as gating parameter; where, Q3 and IQR respectively are the third quartile and interquartile range (Q3–Q1) of cTnI distribution.

The calculation of cTnI distribution and 99th percentile URL values was performed with the JMP program using nonparametric method (Fig. 1A), as recommended by international guidelines [2]. Lognormal distribution using a robust method was also calculated for comparison (Fig. 1B). The 99th percentile and the respective confidence interval (CI) values (at 90% and 95%) were also calculated with adjusted bootstrap percentile method according to Carpenter & Bithell using
null
4. Discussion

4.1. Analytical parameters and RCV of cTnI immunoassays

The results of the present study confirm that the Access cTnI method, using DxI platform, shows analytical characteristics and performances recommended by all international guidelines for high sensitivity methods for cTn assay (Table 1) [11,23,24]. To best of our

Table 3
Mean, median, and percentile values of cTnI distribution values (ng/L) of the reference population.

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
<th>Median</th>
<th>25th percentile</th>
<th>75th percentile</th>
<th>97.5th percentile</th>
<th>99th percentile</th>
<th>99th perc. BS</th>
<th>method (CI%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole population (N = 1302)</td>
<td>3.3 ± 2.5</td>
<td>2.7</td>
<td>1.9</td>
<td>3.8</td>
<td>9.6</td>
<td>15.9</td>
<td></td>
<td>12.5 (11.8–14.2)²</td>
</tr>
<tr>
<td>Women (N = 650)</td>
<td>2.6 ± 1.8</td>
<td>2.2</td>
<td>1.6</td>
<td>3.2</td>
<td>6.5</td>
<td>13.6</td>
<td></td>
<td>8.9 (7.3–11.9)²</td>
</tr>
<tr>
<td>Men (N = 652)</td>
<td>3.8 ± 2.8</td>
<td>3.2</td>
<td>2.2</td>
<td>4.4</td>
<td>11.8</td>
<td>18.7</td>
<td></td>
<td>13.8 (12.4–16.7)²</td>
</tr>
<tr>
<td>WP ≤ 55 years (N = 732)</td>
<td>2.7 ± 1.9</td>
<td>2.3</td>
<td>1.6</td>
<td>3.2</td>
<td>8.0</td>
<td>14.8</td>
<td></td>
<td>11.0 (8.4–14.2)²</td>
</tr>
<tr>
<td>WP &gt; 55 years (N = 570)</td>
<td>4.0 ± 2.8</td>
<td>3.3</td>
<td>2.3</td>
<td>4.7</td>
<td>11.8</td>
<td>18.5</td>
<td></td>
<td>13.7 (12.3–17.3)²</td>
</tr>
<tr>
<td>Women ≤ 55 years (N = 361)</td>
<td>2.1 ± 1.4</td>
<td>1.9</td>
<td>1.3</td>
<td>2.6</td>
<td>5.0</td>
<td>12.8</td>
<td></td>
<td>6.8 (5.1–11.9)²</td>
</tr>
<tr>
<td>Men ≤ 55 years (N = 370)</td>
<td>3.2 ± 2.1</td>
<td>2.8</td>
<td>2.0</td>
<td>3.7</td>
<td>9.1</td>
<td>15.7</td>
<td></td>
<td>12.1 (8.9–15.1)²</td>
</tr>
</tbody>
</table>

BS: Bootstrap method

\* CI 90%.

\*\* CI 95%.

\*\*\* CI 99%

Fig. 2. Inter-relationship between log-cTnI concentration values (Y-axis) and age (X-axis). In the figure, the linear regressions with sex is also indicated (women: red color; men: blue color). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 4
Regression analysis between cTnI values (dependent variable) and age and sex (independent variables).

\[ R^2 = 0.227674 \quad (P < .0001) \]
\[ \text{Intercept} = 0.045 \quad (SE 0.025) \quad (P < .0001) \]
\[ \text{Age} = 0.007 \quad (SE 0.0005) \quad (P < .0001) \]
\[ \text{Sex (F)} = -0.082 \quad (SE 0.007) \quad (P < .0001) \]

SE: standard error

Fig. 3. The spline regression plot between cTnI concentrations (Y-axis) and age (X-axis) is reported in the figure. The spline regression analysis shows the trend between cTnI and age considering all the 1302 healthy subjects of the reference population. The curvilinear trend was fitted by considering the mean values of cTnI values of subjects with the same age interval.

4. Discussion

4.1. Analytical parameters and RCV of cTnI immunoassays

The results of the present study confirm that the Access cTnI method, using DxI platform, shows analytical characteristics and performances recommended by all international guidelines for high sensitivity methods for cTn assay (Table 1) [11,23,24]. To best of our
allowed significantly lower 99th percentile values than nonparametric
effect of outliers [22]. Indeed, the nonparametric bootstrap method
strap approach and “Tukey’s fences” method were used to reduce the
cTnI high-sensitivity methods. In this study both nonparametric boot-
method to detect outliers can greatly affect the 99th percentile URL of
healthy subjects.
to differences in age ranges, sample sizes, and sex-ratios of enrolled
(Table5). The between-study bias of 99th URL values are probably due
reported by Pretorius et al. [24] and manufacturer technical bulletin
in accordance with literature data because the 99th percentile URL
reported in Table 5. The results observed in the present study are well
in agreement with previous results [28,29], indicating that both calculation of 99th percentile and method to detect outliers can greatly affect the 99th percentile URL of
cTnI high-sensitivity methods. In this study both nonparametric boot-
approach and “Tukey’s fences” method were used to reduce the
effect of outliers [22]. Indeed, the nonparametric bootstrap method
allowed significantly lower 99th percentile values than nonparametric

<table>
<thead>
<tr>
<th>99th perc. women (CPM) (ng/L)</th>
<th>99th perc. men (CPM) (ng/L)</th>
<th>Mean ± SD and range of age (years)</th>
<th>Sample number (F/M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present study 13.6 (NP) 18.7 (NP)</td>
<td>M 51.1 ± 13.9; Range 18-86</td>
<td>650/652</td>
<td></td>
</tr>
<tr>
<td>8.9 (BS) 13.8 (BS)</td>
<td>(12.4-16.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretorius et al. [23] 9.6 (7.3-16.3)</td>
<td>F 33.6; M 43.9;</td>
<td>647/1185</td>
<td></td>
</tr>
<tr>
<td>Manufacturer 11.6 (8.4-18.3)</td>
<td>Range 21-99</td>
<td>595/494</td>
<td></td>
</tr>
<tr>
<td>19.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NP: nonparametric method; BS: bootstrap method.
⁎ CI 90%.
⁎⁎ CI 95%.

knowledge, this study report for the first time the RCV and absolute
critical change values of the Access cTnI method. The results reported in
Table 2 can be useful for the diagnosis of acute coronary syndrome in
patients presenting without persistent ST-segment elevation (NSTEMI),
the detection of myocardial injury in asymptomatic patients, and also
the evaluation of risk in the general population [1,3,4,26,27].

4.2. cTnI distribution values of reference population

The Fourth Universal Definition of Myocardial Infarction [3] states
that: “the term myocardial injury should be used when there is evidence
of elevated cardiac troponin values with at least one value above the
99th percentile URL”. Accordingly, the evaluation of 99th percentile
URL value of cTnI and cTnT has a fundamental relevance in the diag-
nosis of all cardiac diseases. Unfortunately, the accurate evaluation of
the 99th percentile value in the reference population presents some
important limitations, related to analytical performance of cTn
methods, demographic characteristic of reference populations, and
statistical approach used for calculation of distribution parameters
[15,16,28]. Indeed, large variations of 99th percentile URL values are
reported in the literature even for the same cTnI or cTnT immunoassay
method [15,16].

The results of the present study confirm that age and sex can
strongly affect the measurement of 99th percentile value, even when
the criteria for selection of reference population are well in accordance
with recommendations made by international guidelines [2,15]. In
particular, the results of the present study (as reported in Fig. 3) de-
monstrate that after the age of 55 years there is in both men and women
a progressive rise in cTnI concentrations, even when the healthy status
of the individuals enrolled in the study are well characterized using a
thorough clinical history together with laboratory (including assay of
BNP/NT-proBNP) and an accurate cardiac investigation (also including
ECC and echocardiography).

Sex-related 99th percentile URL values observed in this study, re-
ported by Pretorius et al. [24] and suggested by the manufacturer are
reported in Table 5. The results observed in the present study are well
in accordance with literature data because the 99th percentile URL
values of present study are included within the confidence interval
reported by Pretorius et al. [24] and manufacturer technical bulletin
(Table 5). The between-study bias of 99th URL values are probably due
to differences in age ranges, sample sizes, and sex-ratios of enrolled
healthy subjects.

The results of the present study are well in agreement with previous
results [28,29], indicating that both calculation of 99th percentile and
method to detect outliers can greatly affect the 99th percentile URL of
cTnI high-sensitivity methods. In this study both nonparametric boot-
strap approach and “Tukey’s fences” method were used to reduce the
effect of outliers [22]. Indeed, the nonparametric bootstrap method
allowed significantly lower 99th percentile values than nonparametric
one for both whole population and subgroups related to age and sex
(mean ± SD: 11.3 ± 2.6 ng/L vs 15.7 ± 2.3 ng/L; N = 7, P < .0001)
(Table 3). However, all 99th percentile values calculated with non-
parametric approach are within the 99% CI of those obtained with
nonparametric bootstrap method (Table 3). From a clinical point of
view, the use in the clinical practice of 99th percentile URL values,
calculated with nonparametric bootstrap method should theoretically
allow a better sensitivity but also a worse specificity both in detection
of myocardial injury and diagnosis of myocardial infarction [3]. Of
course, the relative cost/benefit of these two statistical approaches for
the calculation of the 99th percentile URL value of the Access cTnI
method should be evaluated by specifically designed clinical studies.

As previously reported in detail [11], the Access cTnI method sat-
fies the first fundamental criterium for high-sensitivity method for
cTnI assay. Indeed, the Access cTnI method measures the 99th per-
centile URL with an imprecision less than CV 10% (Table 1 and Fig. 1),
as requested by international guidelines [2]. The results, reported in
the present study, demonstrate that the Access cTnI method satisfies also
the second criterium for high-sensitivity method because it measures
cTn concentrations above the LoD in more than in 50% healthy subjects
of both men and women [2].

4.3. Strengths and limitations of the study

The strengths of this study are related to the experimental design.
Several Authors emphasized the need of both characterized reference
populations and standardized statistical approach to calculate cTnI 99th
percentiles [2,15,16,28,29]. Our study is a multi-center study which
enrolled a well selected healthy reference population according to in-
dications made by international guidelines and expert documents
[2,15]. The study reference population consisted of 1302 healthy adults
subjects with a large age range (18 to 86 years), and including 650
women and 652 men, well matched for age and sex-ratio. Furthermore,
for the calculation of 99th percentile URL values, the standard non-
parametric approach, as suggested by international guidelines [2], and
also the more complex, but also more accurate nonparametric bootstrap
method were used [22].

A relative limitation of this study is the low number of healthy
subjects with age > 55 years enrolled in this study (i.e., 570). This
number did not allow an accurate evaluation of the 99th percentile URL
values divided for men and women with age > 55 years. However, it is
well known that it is very difficult to enroll a great number of well
characterized healthy subjects with age > 70 years [15,16].

5. Conclusions

The results of the present study confirm that the Access hsTnI
method using the UniCell DxI 800 platform satisfies all the criteria and
quality specifications required by the most recent international guide-
lines for high-sensitivity methods for cTnI assay [2]. Furthermore, the
results of this study confirm that the calculation of the 99th percentile
URL values are greatly affected not only by age and sex of the reference
population, but also by the statistical approach used for calculation of
cTnI distribution parameters.

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