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The European Biological Variation Study (EuBIVAS): weekly biological variation of cardiac troponin I estimated by the use of two different high-sensitivity cardiac troponin I assays

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Abstract

Background: Cardiac troponins (cTn) are specific markers for cardiac damage and acute coronary syndromes. The availability of new high-sensitivity assays allows cTn detection in healthy people, thus permitting the estimation of biological variation (BV) of cTn. The knowledge of BV is important to define analytical performance specifications (APS) and reference change values (RCVs). The aim of this study was to estimate the within- and between-subject weekly BV (CV_I, CV_G) of cTnI applying two high-sensitivity cTnI assays, using European Biological Variation Study (EuBIVAS) specimens.

Methods: Thirty-eight men and 53 women underwent weekly fasting blood drawings for 10 consecutive weeks. Duplicate measurements were performed with Singulex Clarity (Singulex, USA) and Siemens Atellica (Siemens Healthineers, Germany).

Results: cTnI was measurable in 99.4% and 74.3% of the samples with Singulex and Atellica assays, respectively. Concentrations were significantly higher in men than in women with both methods. The CV_I estimates with 95% confidence interval (CI) were for Singulex 16.6% (15.6–17.7) and for Atellica 13.8% (12.7–15.0), with the observed difference likely being caused by the different number of measurable samples. No significant CV_I differences were observed between men and women. The CV_G estimates for women were 40.3% and 36.3%, and for men 65.3% and 36.5% for Singulex and Atellica, respectively. The resulting APS and RCVs were similar for the two methods.

Conclusions: This is the first study able to estimate cTnI BV for such a large cohort of well-characterized healthy individuals deriving objective APS and RCV values for detecting significant variations in cTnI serial measurements, even within the 99th percentile.

Keywords: analytical performance specifications; biological variation; high-sensitivity cardiac troponin I; reference change value.

Introduction

Cardiac troponin (cTn) assays are the mainstay for the diagnosis of acute myocardial infarction (AMI) [1–3] as well as markers of cardiac damage [4, 5] and prognostic indicators of all-cause mortality [6]. It is critical to understand if the observed variations in cTn concentration are really associated with a pathological condition or may just be attributable to analytical and biological variation (BV). The availability of new high-sensitive [7] or even "ultra-sensitive" [8, 9] methods allows for measurement of

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cTnI in the majority of healthy subjects, allowing for the use of cTn as a risk indicator in the general population, for which there is increasing evidence [4, 6, 10]. This enhances the need for relevant and robust estimates of BV also in a long-term setting in healthy individuals to aid in the interpretation of changes in cTn, as well as establishing reference change value (RCV) and analytical performance specifications (APS) for the use of cTn in such a manner [11]. Currently available BV data for cTnI BV have been summarized by Nordenskjöld et al. [12] and are mostly based on [1] analysis with a non-commercial analytical system [13], [2] small cohorts of potentially unhealthy individuals [12, 14, 15] or [3] based on sampling with a short time interval (24 h) [16]. The scope of our study was to estimate the weekly BV of high-sensitive cTnI with two recently released commercial methods targeting different epitopes, Siemens Atellica and Singulex Clarity (Singulex is as of June 2019 no longer on the market), on the well-characterized population of the European Biological Variation Study (EuBIVAS) [17–20]. The study has been conducted in accordance with the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) recommendations [21].

Materials and methods

Individuals

Ninety-one volunteers from five European countries (Italy, Spain, The Netherlands, Norway and Turkey) participated in the EuBIVAS. Their characteristics are reported in Supplementary Tables 1 and 2, and their health status and the exclusion criteria applied are described elsewhere in detail [17]. Briefly, 38 men (22–59 years) and 53 women (21–69 years), all Caucasians, underwent weekly fasting blood drawings for 10 consecutive weeks (April–June 2015). All the centers followed the same preanalytical protocol and used the same blood drawing devices and collection tubes (Becton Dickinson, NJ, USA). Serum and EDTA plasma samples were aliquoted and sent, frozen in dry ice, to the coordinating center San Raffaele Hospital in Milan and stored in a freezer at −80 °C until distribution to Clinical laboratory of Policlinico of Milan, Italy (EDTA plasma) and to the Department of Laboratory Medicine Hospital Universitario La Paz Madrid, Spain (serum) for the cTnI analysis (April–July 2018).

The EuBIVAS protocol was approved by the Institutional Ethical Review board of San Raffaele Hospital in agreement with the World Medical Association Declaration of Helsinki and by the Ethical Board/Regional Ethics Committee for each center. All participants signed a written consent form.

cTnI analysis

Two different analytical systems were applied, with analysis being performed in two different centers.

Center 1. Policlinico of Milan. The analyses were performed on the Sgx Clarity System (Singulex, Alameda, CA, USA), using EDTA plasma as sample material. All samples were measured in duplicate, with all samples obtained from the same subject being measured using a single reagent pack in a single analytical run. The same single reagent lot was used for analysis of samples from all subjects. Due to the analytical throughput of the analyzer, a maximum of eight subjects per day were measured (160 measurements/day). Sgx Clarity cTnI Controls (Singulex, Alameda, CA, USA) were used as quality control materials.

Center 2. Hospital Universitario La Paz Madrid. The analyses were performed on Siemens Atellica (Siemens Healthineers, Erlangen, Germany), using serum as sample material. All samples were measured in duplicate. All samples obtained from the same subject were measured using a single reagent pack in a single analytical run. All samples were measured for 3 consecutive days (30 subjects, 600 measurements/day) with the same reagent lot. Liquichek Cardiac Markers Plus (Bio-Rad, Hercules, CA, USA) was used as quality control material.

The technical characteristics of the two analytical methods are provided in Supplementary Table 3.

Data analysis

Calculation of the within-subject BV (CV_I) estimates was performed using CV-ANOVA, an ANOVA method where data first are transformed using the CV transformation [22]. The CV-ANOVA was adopted for the analysis as this straightforward, non-parametric procedure has been shown to be a robust, largely distribution-free procedure for estimating CV_A and CV_I in three-level nested random models [22]. Outlier identification and removal were performed for replicates and samples on the CV-transformed data, by assessing homogeneity of analytical CV (CV_A) (between-replicates) using the Bartlett test [23] and homogeneity of CV_I using the Cochran test [24]. To evaluate differences in concentrations between participants from the different countries, data were visually inspected (data not shown). To examine if there was a general trend in the overall concentration over the study period, as described for other measurands in the EuBIVAS [19], and if individuals were at steady state, we calculated the regression of the mean of the 180 duplicate analysis from every blood drawing 1, 2 …. 10 (pooled mean group sample concentrations) versus the blood drawing number [1–10]. Subjects were considered in steady state if the 95% confidence intervals (CI) of the slope of the regression line included zero. Larger individual systematic changes were identified by the homogeneity test of the CV_I (Cochran test). The between-subject BV (CV_G) was estimated by ANOVA on natural log-transformed data after applying the Dixon Q test to detect outliers between individuals and the Shapiro-Wilk test to verify the normality assumption [25].

APS for the analytical imprecision (CV_{APS}), analytical bias (B_{APS}), RCV and the number of samples needed to reach the homeostatic set point were calculated according to the formulas reported in the Supplementary Appendix.

Data analyses were performed using Excel 2010 and IBM SPSS statistics, version 23.

Results

The Atellica assay has a higher limit of detection (LoD) than Singulex (Supplementary Table 3), with consequences for the number of subjects with measurable cTnI concentrations. With the Singulex method, just one subject out of 91 had one sample < LoD in both replicates; in total 99.4% of samples had measurable concentrations (non-reportable results 10 out of 1781 analyses). With the Atellica method, only 1286 results out of 1731 measurements (74.3%) were higher than the LoD. So, for the Atellica method, six individuals had no measurable values and another 17 had <5 measurable samples. All these 23 with non-measurable cTnI were excluded from the calculation, 21 of whom were women.

About 6% of the data for each of the two methods were eliminated as outliers prior to calculation of CV_I estimates (see Supplementary Table 4 for details). The eliminated samples were the same for both methods, except for a few samples due to the different LoD. Only two individuals (one man and one woman) had samples with concentrations higher than the 99th percentile for both methods. The woman (24 years) had cTnI concentrations >99th percentile in the last two collected samples when cTnI was measured with the Singulex assay. Using the Atellica assay, seven out of her 10 samples were above the 99th percentile for women of 38.6 ng/L, varying from 40 to 114 ng/L. The man presented a cTnI peak in one sample (1380 ng/L for Singulex and 7000 ng/L for Atellica) and four other samples just above the 99th percentile for men with both methods. This study subject was an athlete doing weightlifting and the peak sample was taken after strenuous training exercise; the creatine kinase in the same sample gave a result of 18,500 U/L. Both subjects were excluded from the calculations. All the other subjects included in the calculation were in steady state. Different numbers of individuals were eliminated as outliers based on the Dixon test for the two methods prior to the calculation of CV_G: one woman for Singulex and two women and four men for Atellica (see Figures 1 and 2 and Supplementary Table 4). The results of CV_A, CV_I and CV_G estimates obtained for both methods are reported in Table 1. When including different number of study subjects as basis for the CV_I estimates due to the higher LoD for Atellica, the CV_I estimates obtained with the Singulex Clarity and Atellica systems were significantly different (i.e. the 95% CI did not overlap) only for the whole study population (upper part of Table 1). Results for the male subgroups were similar, for the female subgroup slightly different, but with overlapping 95% CI. If, however, only including the Singulex results from the study subjects who were used as basis for the Atellica estimates, the Singulex CV_I estimates decreased and, even if Atellica estimates remained lower, results were no longer statistically different (overlapping 95% CI for all subgroups, see Table 1). Mean cTnI values were highly different between the two methods, on average about 3 times higher for the Atellica system (Table 1). The calculated APS and RCVs were similar for the two methods, as was

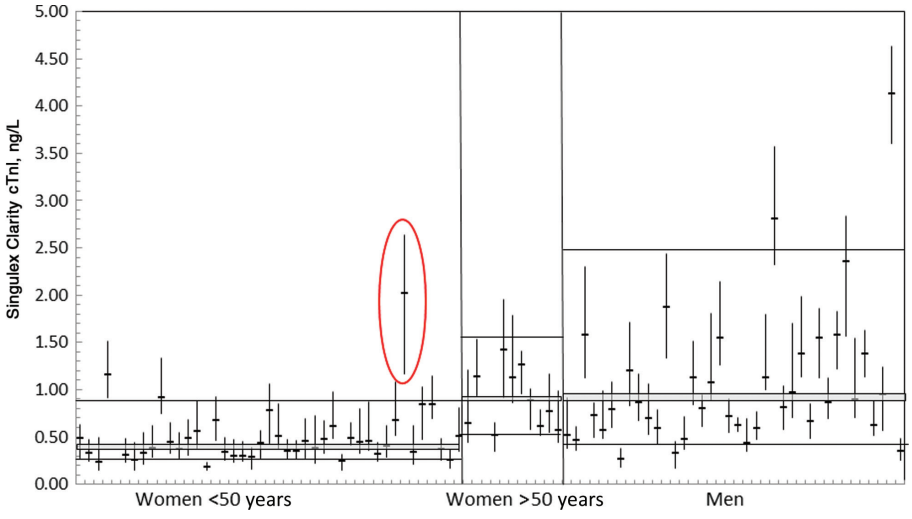

Figure 1: cTnI Singulex Clarity.
Range minimum to maximum and median value (hyphen) for each individual ordered by age. The gray bar indicates the median ± CI; continuous lines indicate the 5th and 95th percentiles for women and men. The figures report all the individuals whose samples were included to derive CV_I estimates, and those not included in the calculation of CV_G are circled.

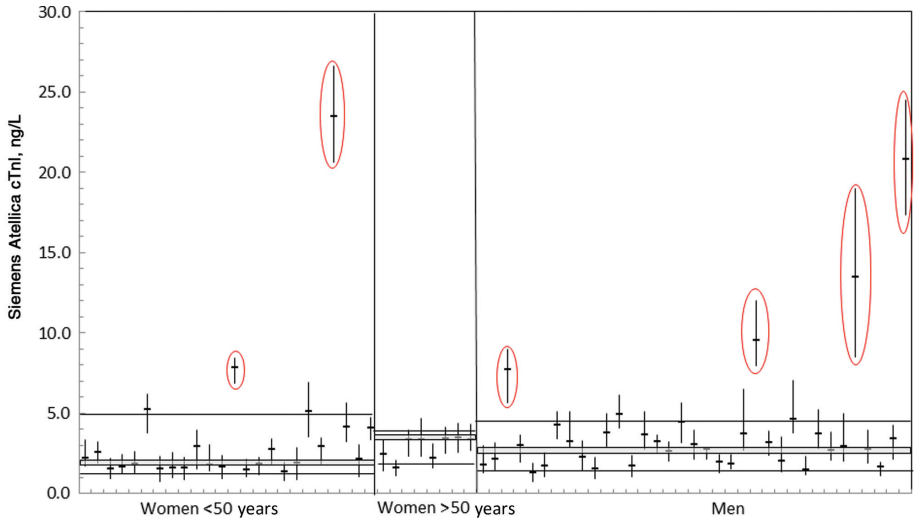

Figure 2: cTnI Siemens Atellica.
Range minimum to maximum and median value (hyphen) for each individual ordered by age. The gray bar indicates the median ± CI; continuous lines indicate the 5th and 95th percentiles for women and men. The figures report all the individuals whose samples were included to derive CV_I estimates, and those not included in the calculation of CV_G are circled.

the case also for the index of individuality (II) and the number of samples needed to reach the homeostatic set point (±10%) (Table 2). Only for men measured on Singulex was the II much lower as a consequence of the higher CV_G.

Discussion

The availability of high-sensitivity cTnI methods with a very low LoD and good precision at low cTnI concentrations allows the delivery of CV_I estimates in healthy individuals. This is important because it allows the definition of APS based on BV and the definition of RCV based on biology. The CV_A estimates derived from duplicate analysis of the study samples are higher than the calculated CV_{APS} but it must be taken into consideration that this CV_A estimate is obtained at a mean cTnI concentration <1 ng/L and <3 ng/L for Singulex Clarity and Siemens Atellica, respectively, concentrations which are about 10 times lower than the respective 99th percentile of the healthy population (see Supplementary Table 3). Although significant differences were observed between CV_I estimates based on the two analytical systems for the overall group and the female subgroup, this is likely explained by the different level of sensitivity of the two analytical methods and not by the different epitopes that they recognize. Both systems can be categorized as highly sensitive methods [3, 26], but for Atellica, in line with the manufacturer's specifications, about 75% of the samples were above the LoD and 25% were below (not measurable), while for Singulex more than 99% were measurable. The individuals with non-measurable samples were mostly women (21 out 53, 40% of females' results) and only two were men (5.4% of the 37 males' results). Thus, the female study population is substantially different for the two methods and this explains the different CV_I estimates observed between women and consequently for the overall estimate. In line with this, when the estimates were based on results from the same study subjects (second part of Table 1), the Singulex estimates decreased, and the difference was no longer significantly different. Another possible contributor to the differences in estimates is the sample materials on which the analyses were performed: serum for Atellica and EDTA plasma for Singulex.

Our data confirm that women present with lower cTnI values, as has been reinforced in several recent papers [27–29]. The difference between men and women is more evident for Singulex, but as previously detailed, for Atellica 40% of the women had cTnI concentrations below the LoD and were thus not included in the calculations. It is important to notice the relevant difference in standardization of the two analytical methods: Siemens Atellica results were on average 3 times greater than Singulex Clarity results but for some subjects (seven out of the included 66, five women and two men) the difference was more than 10 times. This behavior was constant across all the 10 samples of these particular subjects, indicating a difference in the way the two systems detect and quantify the cTnI molecule for these specific subjects. The Singulex

Table 1: Within-subject (CV_I) and between-subject (CV_G) biological variation (BV) estimates for cTnI with 95% CIs.

	Number of individuals	Total number of results	Mean number of samples/individual	Mean number of replicates/sample	Mean value, ng/L (95% CI)[a]	CV_A% (95% CI)[b]	CV_I% (95% CI)	CV_G% (95% CI)
cTnI Singulex Clarity all subjects								
All data	89	1667	9.43	1.97	0.78 (0.75–0.82)	**11.6 (11.1–12.2)**	**16.6 (15.6–17.7)**	
Men	37	682	9.22	2.00	1.06 (1.00–1.13)		15.0 (13.7–16.6)	65.3 (51.1–90.0)
Women <50 years	42	786	9.48	1.98	0.47 (0.44–0.50)		17.8 (16.0–19.5)	**40.3 (33.5–55.0)**
Women >50 years	10	199	10	1.99	0.91 (0.86–0.99)		16.7 (14.3–19.9)	36.8 (24.5–73.0)
cTnI Singulex Clarity same subjects as Siemens Atellica								
All data	66	1240	9.42	1.99	0.92 (0.88–0.97)	10.8 (10.3–11.5)	15.8 (14.7–17.0)	
Men	35	644	9.20	2.00	1.10 (1.04–1.17)		15.8 (14.4–17.4)	59.5 (46.5–81.7)
Women <50 years	23	437	9.57	1.97	0.62 (0.58–0.66)		17.1 (14.8–19.5)	42.3 (31.6–63.4)
Women >50 years	8	159	10	1.98	1.00 (0.94–1.06)		16.1 (13.4–19.6)	30.9 (19.7–68.3)
cTnI Siemens Atellica								
All data	66	1135	9.00	1.84	2.85 (2.78–2.92)	**10.7 (10.1–11.4)**	**13.9 (12.7–15.0)**	
Men	35	602	8.94	1.92	3.06 (2.96–3.16)		14.7 (13.1–16.3)	36.5 (29.7–52.4)
Women <50 years	23	386	8.91	1.79	2.76 (2.59–2.94)		14.6 (12.5–16.7)	**36.3 (26.9–54.2)**
Women >50 years	8	149	9.63	1.94	2.93 (2.79–3.08)		12.8 (9.7–16.2)	28.8 (19.8–68.7)

[a]After elimination of outliers. [b]Analytical variation (CV_A) estimates were based on CV-ANOVA of duplicate analysis of all study samples. The numbers in bold are those used for the calculation of APS reported in Table 2.

Clarity cTnI assay uses a 2×2 pair of monoclonal antibodies recognizing epitopes in the central region and at both ends of the cTnI molecule; the capture antibodies recognize the amino acid sequences 41–49 and 24–40 and the detection antibodies recognize the sequences 190–196 and 86–90. Siemens Atellica uses two capture monoclonal antibodies that recognize epitopes in the C- and N-terminal part of the cTnI molecule and a third detection antibody recognizes the N-terminal region of the molecule. This different setting of the Atellica method that has the capture antibodies at the extreme of the cTnI molecule (while Singulex captures the central more stable part of the molecule) could be more sensitive to intact cTnI deriving from recent myocardial damage possibly present in subjects with higher cTnI values [30, 31].

These EuBIVAS-derived CV_I estimates are in line with those published by Wu et al. [13] where a research version of the Singulex analytical system was applied and by Simpson et al. [32], but higher than estimates based on short-term sampling intervals (within 24 h) [16].

For all but two subjects, all cTnI results were well within the 99th percentile for both methods, including those subjects who were excluded from the CV_G calculations.

The CV_G estimates obtained with the Atellica system (36.5% and 36.3% for men and women, respectively) as well as the Singulex CV_G for the female group (40.3%) were lower than the previously published data. The Singulex CV_G estimate for men (65.3%) was in line with those published by Vasile et al. [33], Wu et al. [13] and van der Linden et al. [16] but lower than some other published data. For the Singulex method, the only excluded subject from CV_G calculation was a 47-year-old lady practicing about 17 h per week with strenuous physical exercise (running and cycling) [30]. This subject was an outlier also for Atellica. As evident in Figures 1 and 2, her cTnI results were in the range of the male subjects. The lower Atellica-derived CV_G estimate for men is likely explained by the exclusion of study subjects classified as outliers, who were not excluded for Singulex. In fact, with the Atellica method, four subjects had significantly higher concentrations than the others (see Figure 2) and were excluded according to the Dixon algorithm for outlier detection. The same subjects had higher cTnI concentrations when measured by the Singulex method than the rest of the group, but not to such a high level that they were classified as outliers. This may be explained by the different epitope mappings of the two analytical systems.

The distribution of the homeostatic points of cTnI of the individuals is skewed, and this is reflected by the high CV_G values (>33%) (Table 1), indicating the need to be

Table 2: Analytical performance specification (APS) for imprecision (CV_{APS}) and bias (B_{APS}), reference change values (RCVs), index of individuality and number (No.) of samples needed to reach the homeostatic set point based on the biological variation estimates as reported in Table 1.

	CV_{APS} (%)[a]	B_{APS} (%)[b]	RCV (%)[c]decrease; increase	Index of individuality	No. of samples needed to reach the homeostatic set point ±10%
Singulex Clarity	8.3	10.9	−37.4/59.7	Men 0.23 Women 0.44	Men 14 Women 17
Siemens Atellica	6.9	9.7	−33.4/50.1	Men 0.40 Women 0.40	Men 13 Women 13

[a]$CV_{APS} = ½\ CV_I$; [b]$B_{APS} = 0.25(CV_I^2 + CV_G^2)^{0.5}$; [c]RCVs were calculated as described in the text delivering asymmetric values for rise and fall at the probability level of 95% for significant unidirectional change, applying CV_A estimates based on duplicate measurement of all study samples.

cautious when applying such estimates for further calculations, especially APS for bias.

The low II reduces the relevance of reference intervals and increases the importance of applying RCV when interpreting serial results. The EuBIVAS-derived asymmetrical RCVs were similar for the two analytical systems: about 35% for decrease and between 50% and 60% for increase. These RCVs are thus quite similar to those summarized by Nordenskjöld et al. [12] and Simpson et al. [32] and confirm the amount of variation expected to be caused by analytical variation and BV when monitoring a patient with cTnI within the 99th percentile. Recently, cTnI has been proposed as a prognostic marker and its rapid increase (within the 99th percentile) may indicate an accelerated myocardial damage [10], so knowledge on the natural BV in the long-term setting is of importance. Furthermore, in patients undergoing therapies with potentially cardiotoxic drugs, the obtained data would be highly relevant. Unfortunately, at these low concentration levels, 13–17 samples are needed to identify the homeostatic set point ±10%, while 3–4 samples are sufficient to estimate the set point within ±20%.

The limitations of the study are as follows: long sample storage before analysis (3 years), but the samples were continuously stored at −80 °C and thawed only prior to analysis; the study was not targeted to cTn so participants did not undergo specific cardiological evaluation and there is, in particular, a low prevalence of male elderly subjects. Furthermore, this study establishes BV estimates based on weekly sampling and the obtained results thus cannot be directly applied to AMI diagnosis and would therefore also not be appropriate to establish APS in an emergency context.

Conclusions

This is the first study able to estimate CV_I and CV_G based on weekly samplings applying two different recently available high-sensitivity cTnI methods on a large cohort of well-characterized healthy individuals, thus delivering APS and RCV allowing for objectively defined quality requirements for analytical methods and for detecting likely significant variations when performing serial measurements of cTnI within the 99th percentile.

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