

Influence of Population Selection on the 99th Percentile Reference Value for Cardiac Troponin Assays

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OBJECTIVE: We sought to determine the effect of patient selection on the 99th reference percentile of 2 sensitive and 1 high-sensitivity (hs) cardiac troponin assays in a well-defined reference population.

METHODS: Individuals >45 years old were randomly selected from 7 representative local community practices. Detailed information regarding the participants was collected via questionnaires. The healthy reference population was defined as individuals who had no history of vascular disease, hypertension, or heavy alcohol intake; were not receiving cardiac medication; and had blood pressure <140/90 mmHg, fasting blood glucose <110 mg/dL (approximately 6 mmol/L), estimated creatinine clearance >60 mL · min⁻¹ · (1.73 m²)⁻¹, and normal cardiac function according to results of echocardiography. Samples were stored at -70 °C until analysis for cardiac troponin I (cTnI) and cardiac troponin T (cTnT) and N-terminal pro-B-type natriuretic peptide.

RESULTS: Application of progressively more stringent population selection strategies to the initial baseline population of 545 participants until the only individuals who remained were completely healthy according to the study criteria reduced the number of outliers seen and led to a progressive decrease in the 99th-percentile value obtained for the Roche hs-cTnT assay and the sensitive Beckman cTnI assay but not for the sensitive Siemens Ultra cTnI assay. Furthermore, a sex difference found in the baseline population for the hs-cTnT ($P = 0.0018$) and Beckman cTnI assays ($P < 0.0001$) progressively decreased with more stringent population selection criteria.

CONCLUSIONS: The reference population selection strategy significantly influenced the 99th percentile refer-

ence values determined for troponin assays and the observed sex differences in troponin concentrations.

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The 99th percentile of troponin in a reference population has been established as the decision value for the confirmation or exclusion of myocardial infarction by the joint European Society of Cardiology/American College of Cardiology/American Heart Association/World Heart Federation Task Force for the Redefinition of Myocardial Infarction. In addition, as an analytical goal, it is recommended that the imprecision of the assay should be 10% or less at a value equal to or below the 99th percentile. However, assays with the 99th percentile value within 10% to 20% imprecision are perfectly usable (1). Recently, a scoring system for assays that classifies them according to their ability to reach imprecision goals and measure a healthy population has been proposed (2). Studies have also documented that low but detectable cardiac troponin values obtained in apparently healthy individuals and patients with cardiac disease have prognostic value (3). Under these circumstances the selection of a reference population requires more exacting criteria than have been used to date. In addition to exclusion of individuals with risk factors of cardiovascular disease and those receiving cardioactive drugs, normal results for electrocardiogram (ECG)⁶ and cardiac imaging may be required to demonstrate that individuals in the reference population have normal cardiac function. To explore potential confounders of reference population assessment, we determined the reference intervals for 2 sensitive cardiac troponin I (cTnI) and 1 high-sensitivity (hs) cardiac troponin T (cTnT) assays using a fully characterized population of individuals who had undergone assessment of risk fac-

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⁶ Nonstandard abbreviations: ECG, electrocardiogram; cTnI, cardiac troponin I; hs, high sensitivity; cTnT, cardiac troponin T; FEV₁, forced expiratory volume in 1 s; FVC, FEV₁%, FEV₁/forced vital capacity ratio; LVEF, left ventricular ejection fraction; LVSD, LV systolic dysfunction; LVH, LV hypertrophy; eGFR, estimated glomerular filtration rate; NT-proBNP, N-terminal pro-B-type natriuretic peptide.

Table 1. Demographics of the study population.

Age, median (interquartile range)	58 (51–67)
Male, n (%)	259 (47.5)
Smoking, n (%)	65 (13.3)
Hypertension, n (%)	137 (25.1)
Diabetes, n (%)	34 (6.2)
Previous history of ischemic heart disease, n (%)	55 (10.1)
Previous history of cerebrovascular disease, n (%)	20 (3.7)
Previous history of peripheral vascular disease, n (%)	6 (1.1)
Cardioactive drug therapy, n (%)	153 (28.1)
Ethnicity	
White, n (%)	380 (69.7)
Asian, n (%)	142 (26.1)
Afro-Caribbean, n (%)	11 (2.0)
Other, n (%)	12 (2.2)
Systolic blood pressure, median (interquartile range), mmHg	133 (120–143)
Diastolic blood pressure, median (interquartile range), mmHg	84 (80–90)
Body mass index, median (interquartile range), kg/m ²	25.8 (23.4–28.5)

tors, biochemical testing, ECG, pulmonary function evaluation, and noninvasive cardiac imaging.

Methods

POPULATION SAMPLE

Individuals >45 years old were randomly selected from the practice lists of 7 representative local community practices in Harrow, North London (Stanmore Medical Centre, Belmont Health Centre, Headstone Road Medical Centre, Lanfranc Medical Centre, GP Direct, Enderley Road Medical Centre, and Pinn Medical Centre). Permission for the study was obtained from the local research ethics committee in accordance with the Declaration of Helsinki.

Of the 1392 individuals from the general population invited by a letter to participate, 734 (52.7%) responded and therefore enrolled in the study. Detailed information regarding the participants was collected by a questionnaire. Demographic data regarding the study population are shown in Table 1. Heart rate and blood pressure measurement (the mean of 2 readings), spirometry, ECG, and echocardiography were performed. Participants underwent venesection for fasting plasma glucose, creatinine, and cardiac troponin measurements. Samples were taken into vacutainer serum separator tubes for routine analysis or into vacutainer tubes containing fluoride oxalate as anticoagulant for blood glucose analysis (Becton Dickinson). Serum and plasma aliquots that were not analyzed immediately were initially frozen at -20°C , then transferred for long-term storage at -70°C . Frozen samples

were thawed to room temperature, mixed, and then centrifuged before analysis.

Values assessed on spirometry included forced expiratory volume in 1 s (FEV_1), forced vital capacity, and their ratio ($\text{FEV}_1\%$). Spirometry results were defined as abnormal if $\text{FEV}_1\%$ was $<60\%$ (obstructive defect), if $\text{FEV}_1\%$ was $<70\%$ with $\text{FEV}_1 < 80\%$ of the predicted value (obstructive defect), and if $\text{FEV}_1\%$ was $>70\%$ with both FEV_1 and forced vital capacity $<80\%$ of the predicted value (restrictive defect).

Two-dimensional echocardiography was performed with a SONOS 4500 machine (Philips) with second harmonic imaging. We calculated left ventricular ejection fraction (LVEF) quantitatively using Simpson's apical biplane method and taking the mean of 3 readings (4). Borderline or worse LV systolic dysfunction (LVSD) was defined as $\text{LVEF} < 50\%$. We calculated LV mass using the Devereux-modified American Society of Echocardiography equation, with LV hypertrophy (LVH) defined as LV mass index $>134\text{ g/m}^2$ for men and $>110\text{ g/m}^2$ for women (5). Valvular regurgitation was assessed qualitatively on a 5-point scale (nil or trivial, mild, mild-to-moderate, moderate, and severe). Valvular stenosis was assessed by peak pressure gradient and estimated valve area, and again ascribed the same 5-point scale. Significant valve disease was taken as mild-to-moderate or worse. Diastolic parameters assessed included isovolumic relaxation time, mitral inflow peak E-wave velocity, peak A-wave velocity, the ratio of E-wave to A-wave velocity, and E-wave deceleration time. Diastolic heart failure was defined according to the European Study Group on

Diastolic Heart Failure guidelines. All echocardiograms were read by a cardiologist who was blinded to the biochemical data.

ANALYTICAL METHODS

All measurements were performed at a single center. Glucose (coupled hexokinase method) and creatinine (Jaffe alkaline picrate) were measured with a Beckman LX20 as recommended by the manufacturer. Both assays had CVs of <5% across the analytical range. Estimated glomerular filtration rate (eGFR) was then calculated by the corrected Modification of Diet in Renal Disease equation (6). All samples were analyzed for N-terminal pro-B-type natriuretic peptide (NT-proBNP) using an Elecsys 2010 (Roche Diagnostics). The interassay %CV was 5.0 at 380 ng/L, 4.4 at 8700 ng/L, and 5.0 at 13000 ng/L, with a detection limit of 20 ng/L and upper measuring limit of 25 000 ng/L.

The following cardiac troponin assays were evaluated: The sensitive cTnI Ultra (Siemens Medical Solutions Diagnostics), the sensitive cTnI Beckman Accu enhanced (Beckman Coulter Diagnostics), and the hs-cTnT (Roche Diagnostics). The cTnI Ultra measurements were performed by using an ADVIA Centaur analyzer. The detection limit of the latter assay is 6 ng/L with a claimed 10% CV at 30 ng/L and a 99th percentile of 40 ng/L. The cTnI Accu measurements were performed by using an Access II analyzer. The detection limit of the assay is 1 ng/L with a claimed 10% CV at 30 ng/L and a 99th percentile of 40 ng/L. The hs-cTnT measurements were performed by using an Elecsys 2010 analyzer. The detection limit of the assay is 3 ng/L with a claimed 10% CV at 13 ng/L and a 99th percentile of 14 ng/L. Assay imprecision was verified in house and corresponded to the manufacturers' claims.

POPULATION SELECTION AND STATISTICAL METHODS

All obtained data were transferred to a relational database to allow data filtering and population subgrouping in preparation for analysis by use of the following procedure. The baseline population comprised all the enrolled participants who had complete sets of data including troponin concentrations as measured by the 3 methods. A first subgroup of individuals was then selected from the above population on the basis of responses to the questionnaire, excluding those with any previous history of vascular disease or diabetes mellitus and those taking any cardioactive drugs (aspirin, β blockers, diuretics, angiotensin-converting-enzyme inhibitors or angiotensin-2 blockers, α blockers, statins, calcium- or potassium-channel blockers, anti-dysrhythmic drugs, or digoxin). The selection criteria for this first group were the least stringent. Further subgroups of participants were then defined according to more stringent criteria on the basis of responses to the

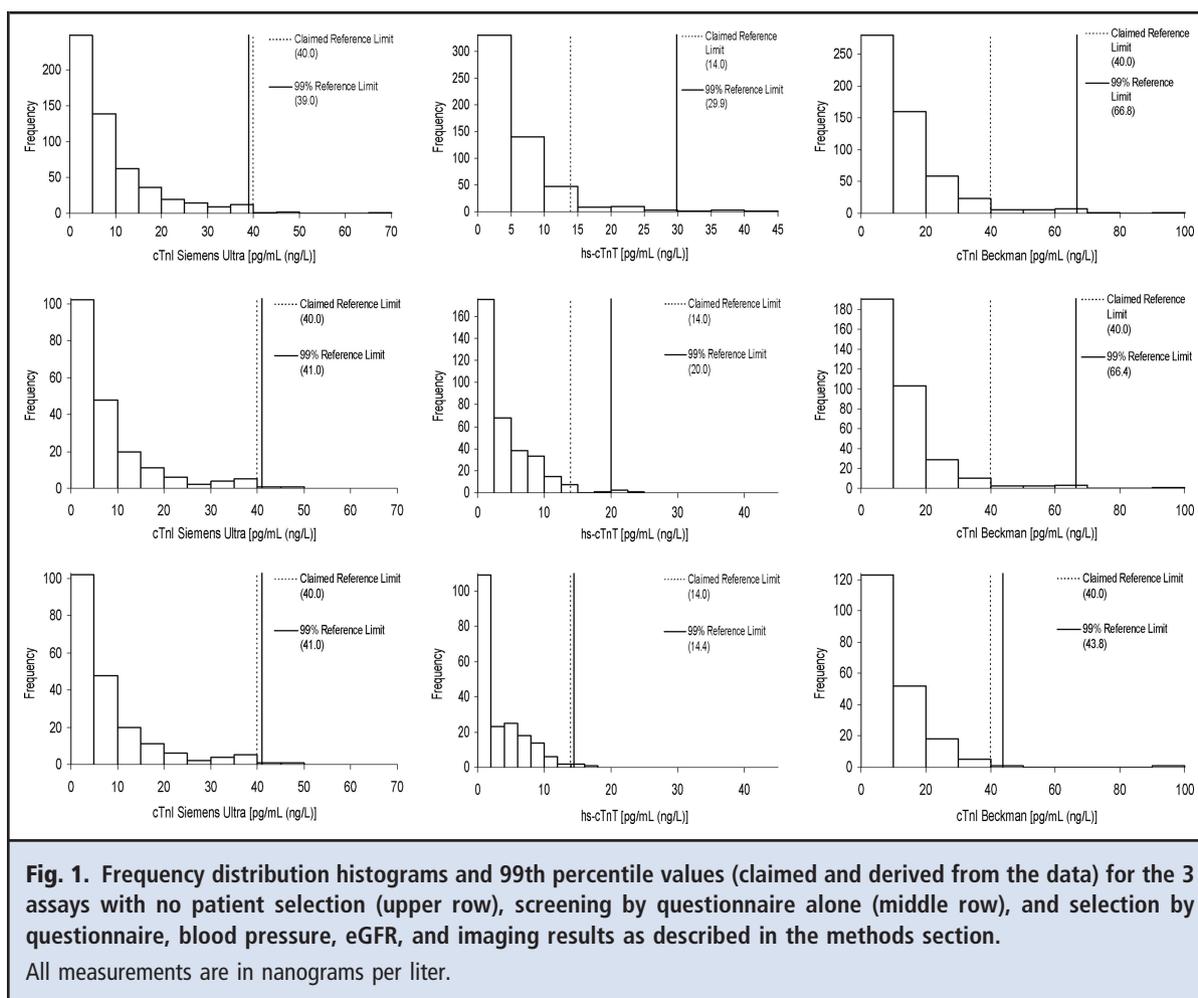
questionnaire as above, but also with the incorporation of an additional selection criterion of 1 of the following: blood pressure $\leq 140/90$ or NT-proBNP $< 250 \mu\text{g/L}$ ($\mu\text{g/L}$) (7) or LVEF $> 50\%$, because systolic dysfunction is associated with troponin elevation (8) or entirely normal ECG results or and eGFR $> 60 \text{ mL} \cdot \text{min}^{-1} \cdot (1.73 \text{ m}^2)^{-1}$.

The final and most stringently selected reference group (normals) was defined as those individuals who had no history of vascular or cardiovascular disease, diabetes mellitus, hypertension, or heavy alcohol intake and who were receiving no cardiac medication and had blood pressure $\leq 140/90$ mmHg; fasting glucose $< 110 \text{ mg/dL}$ (approximately 6 mmol/L); eGFR $> 60 \text{ mL} \cdot \text{min}^{-1} \cdot (1.73 \text{ m}^2)^{-1}$; LVEF $> 50\%$; normal lung function; and no significant valvular heart disease, LVH, diastolic heart failure, or regional wall-motion abnormalities on echocardiography. In the reference population median and interquartile ranges for blood pressure were systolic 125 mmHg (range 90–133 mmHg) and diastolic 80 mmHg (range 76–85 mmHg) and for body mass index were 25 (22.9–26.9).

All statistical analyses were performed with Analyse-it for Microsoft Excel (version 2.12) (www.analyse-it.com) by nonparametric methods with the exception of multivariate analysis. The 99th percentile was calculated as the absolute single upper 99th percentile value (1-sided 99% reference interval). We compared populations with the Mann-Whitney test. Spearman rank analysis was used for correlation. We performed multivariate analysis using SPSS v 16 (IBM SPSS) following an initial univariate analysis of the same variables and discriminants used for the patient selection. Only variables found to be statistically significant in the initial univariate analysis were used in the multivariate analysis. Probability values were 2 tailed and values of < 0.05 were considered significant.

Results

Owing to a combination of sample insufficiency and inadequate data collection, complete information was available for only 545 of the 734 individuals enrolled (74.3%, 259 male). The median age was 58 years (range 45–89 years; lower quartile 51, upper quartile 67 years). Overall, troponin was detectable by the Siemens Ultra assay in 265 participants (48.6%) by the Roche hs-cTnT assay in 315 participants (57.8%), and by the Beckman cTnI assay in 533 participants (97.8%). The frequency distributions of cardiac troponin for the 3 assays are summarized for the various population subgroups selected according to different stringency criteria in Fig. 1. Use of increasingly stringent criteria for selection of the reference population subgroups reduced the number of outliers at the high end of the



distributions and reduced the values of the 99th percentile to values that were close to those claimed by the manufacturers. There was no significant difference in age or sex distribution across all of the studied subgroups. Overall, troponin concentrations showed a positive correlation with age for the hs-cTnT ($r = 0.44$; $P = <0.0001$) and Beckman cTnI ($r = 0.22$; $P = <0.0001$) methods but not for the Siemens cTnI method ($r = 0.03$; $P = 0.5$) in the overall original sample of 545 individuals. With use of increasingly stringent criteria to define the reference population, however, this correlation was found only for hs-cTnT in the final and most stringently selected “normals” group ($r = 0.24$; $P = 0.0005$).

The reference intervals obtained for each of the populations are summarized in Table 2. The Siemens cTnI assay showed no sex effects in any of the groups studied. By contrast, the Roche hs-cTnT and Beckman cTnI assays showed a significant difference between men and women in all studied subgroups. The inclusion of $eGFR \geq 60 \text{ mL} \cdot \text{min}^{-1} \cdot (1.73 \text{ m}^2)^{-1}$ after the

initial questionnaire to the model had the most effect on the 99th percentile for cTnT but relatively less effect on the 99th percentile for cTnI. Although an NT-proBNP value of 250 ng/L had previously been shown to achieve reasonable exclusion of an LVEF $<40\%$ (7), a more recent study had shown that a lower value of 100 ng/L is more appropriate for use in younger individuals, so we reanalyzed the data using this value (9). The more aggressive NT-proBNP cutoff of 100 ng/L (data not shown, $n = 279$) reduced the 99th percentile to 37.2 ng/L for the Siemens cTnI assay, to 15.7 ng/L for the cTnT assay, and to 52 ng/L for the Beckman cTnI assay, comparable values to those obtained after full adjustment of the covariates. Univariate and multivariate analyses were not useful in predicting troponin increases above the 99th percentile.

Discussion

In the 3 assays examined in this study, progressive patient selection to exclude comorbidities resulted in a

Table 2. One-sided 99th percentiles for populations studied.^a

Population	Assay														
	cTnI Siemens Ultra						hs-cTnT Roche						cTnI Beckman Accul		
	99th Percentile all	99th Percentile male	99th Percentile female	P, male vs female	99th Percentile all, n	99th Percentile male	99th Percentile female	P, male vs female	99th Percentile all	99th Percentile male	99th Percentile female	P, male vs female			
All (n = 545, 258 male)	39.0	38.0	47.0	0.59	29.9	37.2	27.7	0.0018	66.8	80.9	62.2	<0.0001			
Questionnaire screened [n = 340 (62.4%) (157 male)]	38.6	37.4	42.0	0.49	20.0	23.2	13.6	0.001	66.4	79.0	63.4	<0.0003			
Questionnaire screened plus BP ≤140/90 [n = 266 (48.8%) (120 male)]	39.9	36.4	44.2	0.093	19.1	23.8	13.3	0.0021	59.0	80.2	62.1	0.0041			
Questionnaire screened plus normal ECG [n = 291 (53.3%) (132 male)]	39.2	37.7	43.4	0.25	21.6	23.7	13.9	0.0203	69.4	84.5	65.1	0.0041			
Questionnaire screened plus NT-proBNP within reference interval [n = 330 (62.9%) (163 male)]	38.7	37.5	42.3	0.39	16.7	22.8	12.8	0.0011	67.2	79.9	63.8	0.0006			
Questionnaire screened plus normal LVEF [n = 318 (58.3%) (143 male)]	38.8	37.6	42.4	0.45	17.3	21.9	13.7	0.0031	60.9	82.1	56.8	0.0004			
Questionnaire screened plus eGFR >60 [n = 314 (57.6%) (144 male)]	38.9	37.6	42.7	0.55	14.4	21.5	13.8	0.0069	68.3	81.9	64.3	0.0011			
Normals [n = 200 (36.7%)]	41.0			0.76	14.4			0.0295	43.8			0.0430			
Manufacturers claim ^b	40.0				14.0				40						

^a All measurements are in ng/L.^b Manufacturers' claimed 99th percentile limit taken from the manufacturers' kit datasheets. No data were available in the kit datasheets regarding male and female differences.

reduction in the number of outliers and, with the exception of the Siemens cTnI assay, a progressive reduction in the 99th percentile. There was a clear difference between men and women in the examined subgroups for the Roche hs-cTnT and the Beckman cTnI assay. This difference was reduced progressively as the patients became more highly selected but persisted even in the completely healthy reference group.

Because myocardial cell turnover has been shown to occur (10), cardiac troponin would be expected to be detectable in serum by using the new sensitive assays. Because male hearts are on average bigger than female hearts (11), a sex difference in distribution of troponin values would be expected, given that underlying occult disease is not present, which we have excluded here to the greatest extent possible with current technology. Our study showed that a normal ECG had the greatest impact in reducing sex differences in troponin values, as shown by the smallest *P* value for male/female difference in Table 2. This result is not surprising, because an entirely normal ECG would be unlikely in the presence of significant underlying preexisting myocardial damage (12) and because ischemic heart disease is more common in males. Interestingly, neither a normal ejection fraction nor increased NT-proBNP concentrations had important effects, and these variables excluded only a small percentage of the values (37.1% and 37.7%, respectively). The use of an NT-proBNP cutoff of 100 ng/L (9) resulted in a more effective filter. Screening for renal function had the most effect on reducing the 99th percentile for cTnT, as would be expected because cTnT is more sensitive than cTnI to changes in eGFR (13–15).

There was a difference between the examined assays in their ability to detect sex-related distribution of variations in troponin values. Reports in the literature regarding this issue have been contradictory. For the Siemens cTnI Ultra assay, a sex difference was initially reported but at a higher 99th percentile and with both sex and age dependence (16). A recently published study also showed a sex difference, but the values were for young healthy donors who were not screened by cardiac imaging (17). Three previous studies have shown a sex difference for the hs-cTnT assay (18–20). Data for the Beckman method are more variable. In a large-scale study of healthy elderly individuals, stringent patient selection reduced the sex differences; thus no statistically significant differences were observed in the 99th percentiles of the 2 groups, but between-sex differences were observed in the 90th and 75th percen-

tiles. Two recent studies of prototype hs-cTnI assays revealed no sex differences, although there was a trend toward higher values in males (21, 22).

Whether differences in assay sensitivity between methods are due to patient selection, assay configuration, or both remains unclear. It would appear that the patient selection criteria greatly affect determination of the 99th percentile value as well as the distribution of troponin values in men and women; there also may be ethnic differences. It therefore would seem prudent to opt for a highly selected population group when performing reference interval studies. Selection of study populations for such studies should include a health questionnaire, eGFR measurement, and the use of an appropriate BNP cutoff value.

The limitations of this study deserve mention. Owing to the progressive selection scheme used, there was a progressive reduction in the number of remaining participants as the selection stringency increased. Nevertheless, the study outcome indicates what factors were important in reference population selection. There were insufficient numbers of participants to examine the effect of ethnicity. Finally, none of the examined troponin methods was able to measure detectable concentrations of troponin across the entire population examined. This observation may have contributed to some of the interassay differences seen. However, the data reported here reflect the performance of troponin assays in current clinical use.

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