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Sex-Specific 99th Percentile Upper Reference Limits for High Sensitivity Cardiac Troponin Assays Derived Using a Universal Sample Bank

Fred S. Apple,[a,*] Alan H. B. Wu,[b] Yader Sandoval,[c] Anne Sexter,[d] Sara A. Love,[a] Gary Myers,[e] Karen Schulz,[f] Show-Hong Duh,[g] and Robert H. Christenson[g]

BACKGROUND: How to select healthy reference subjects in deriving 99th percentiles for cardiac troponin assays still needs to be clarified. To assist with global implementation of high sensitivity (hs)-cardiac troponin (cTn) I and hs-cTnT assays in clinical practice, we determined overall and sex-specific 99th percentiles in 9 hs-cTnI and 3 hs-cTnT assays using a universal sample bank (USB).

METHODS: The Universal Sample Bank (USB) comprised healthy subjects, 426 men and 417 women, screened using a health questionnaire. Hemoglobin A1c (>URL 6.5%), NT-proBNP (>URL 125 ng/L) and eGFR (<60 mL/min), were used as surrogate biomarker exclusion criteria along with statin use. 99th percentiles were determined by nonparametric, Harrell–Davis bootstrap, and robust methods.

RESULTS: Subjects were ages 19 to 91 years, Caucasian 58%, African American 27%, Pacific Islander/Asian 11%, other 4%, Hispanic 8%, and non-Hispanic 92%. The overall and sex-specific 99th percentiles for all assays, before and after exclusions (n = 694), were influenced by the statistical method used, with substantial differences noted between and within both hs-cTnI and hs-cTnT assays. Men had higher 99th percentiles (ng/L) than women. The Roche cTnT and Beckman and Abbott cTnI assays (after exclusions) did not measure cTn values at ≥ the limit of detection in ≥50% women.

CONCLUSIONS: Our findings have important clinical implications in that sex-specific 99th percentiles varied according to the statistical method and hs-cTn assay used, not all assays provided a high enough percentage of measurable concentrations in women to qualify as a hs-assay, and the surrogate exclusion criteria used to define normality tended to lower the 99th percentiles.

Cardiac troponin (cTn) I and T are the preferred biomarkers for detection of myocardial injury and support the diagnosis of acute myocardial infarction (MI) *(1)*. The Fourth Universal Definition of Myocardial Infarction (2018) recommended that the 99th percentile upper reference limit (URL) should not be used in isolation as a dichotomous test for MI diagnosis, but emphasized evaluating results in the context of the clinical presentation. Before high sensitivity (hs) assays were introduced, diagnostic thresholds for contemporary cTn assays were often based on a receiver operator characteristic (ROC) curve or the 10% CV (coefficient of variation) cTn concentrations, in part because of poor analytical performance at the 99th percentile *(2, 3)*. Improvements in assay analytics have allowed for the validation of hs-cTn assays, resulting in a lowering of 99th percentile URLs *(4, 5)*, along with elimination of analytical noise (CVs <10%) and a decline in false positives.

99th percentile URLs are influenced by age, sex, and presence of comorbidities, with a lack of uniform criteria in defining patient enrollment characteristics and analysis methodologies. The AACC Academy and the International Federation of Clinical Chemistry (IFCC) Committee on the Clinical Application of

[a] Department of Laboratory Medicine & Pathology, Hennepin Healthcare/Hennepin Country Medical Center, Hennepin Healthcare Research Institute and University of Minnesota, Minneapolis, MN; [b] Department of Laboratory Medicine, University of California, San Francisco, CA; [c] Department of Cardiovascular Medicine, Mayo Clinic, Rochester, MN; [d] Chronic Disease Research Group, Hennepin Healthcare Research Institute, Minneapolis, MN; [e] Myers Consulting, Smyrna, GA; [f] Hennepin Healthcare Research Institute, Hennepin County Medical Center, Minneapolis, MN; [g] Department of Pathology, University of Maryland Medical Center, Baltimore, MD

*address correspondence to: Department of Laboratory Medicine & Pathology, Hennepin County Medical Center, 701 Park Ave, Clinical Laboratories P4, Minneapolis MN 55415. Fax: 612 9044229; e-mail: apple004@umn.edu.

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Cardiac Bio-Markers (C-CB) provided guidance to define reference populations, including the need for clinical and surrogate biomarker screening for better identification of apparently normal individuals, as well as to specify what statistical methods were used to calculate 99th percentile URLs *(3–6)*. From examination of hs-cTn assays, defined by the Academy/C-CB as assays able to measure cTn at or above an assay's limit of detection (LoD) in ≥50% of healthy men and ≥50% of healthy women, it has become evident that hs-assays vary in analytical sensitivity. Differences in the proportion of measurable values and 99th percentile URLs across sexes have been reported. With numerous hs-cTnI and hs-cTnT assays available and the varying reference populations used to determine the URLs for each assay, clinicians and laboratorians have been unable to appropriately compare cTn assays in different studies.

Following the United States Food and Drug Administration (FDA) recent 510k clearances of several hs-cTn assays and ongoing use of hs-cTn assays globally, a direct comparison between hs-assays is timely. Global confusion has been evident on how to implement 99th percentile URLs *(7, 8)*. For the Roche hs-cTnT assay outside the US, laboratories predominately use an overall 14 ng/L 99th percentile URL, while early adopters of the same assay in US hospitals are using sex-specific URLs *(9–12)*. Our study determines and compares the sex-specific 99th percentile URLs for 9 hs-cTnI and 3 hs-cTnT assays using a 'Universal Sample Bank' (USB) *(13)* of apparently healthy individuals as defined by a health questionnaire, medication use, and surrogate biomarkers including NT-proBNP, HbA1C, and eGFR.

Methods

SUBJECTS

Specimens from apparently healthy subjects were obtained from the AACC Universal Sample Bank (USB) *(13)*. Briefly, international participants, men and women, responded to the AACC's advertisement for participation in blood donation for establishing a USB at the 2015 national meeting in Atlanta GA, USA. Plasma (EDTA, lithium heparin) and serum were collected from each respondent and the specimen type used for each assay conformed with the manufacturers recommended, acceptable specimen types, per package inserts. The samples used in this study were donated by the AACC and the AACC had no oversight of any part of the study design. Participants included 426 men and 417 women who were screened using a health questionnaire (Supplemental Document 1) *(13)*. We did not enroll or have knowledge of any transgender individuals. Participants provided consent, were over 18 years of age, without any prior history of hypertension, renal failure, diabetes, congestive heart failure, heart disease, cancer, deep vein thrombosis/pulmonary embolism, warfarin use, or treatment with cardiovascular medications for known disease, and were symptom free.

CTN ASSAYS

Reagents for each cTn assay were donated by manufacturers. The manufacturers did not review the article and had no input on data analyses prior to the manuscript submission. Analytical characteristics, as defined by each manufacturer, for the 12 assays studied, have been previously published *(14)* and can be found on the IFCC C-CB website *(15)*. At the time of the study all assays were CE Marked, USA FDA cleared, and or China FDA cleared for use in clinical practice. cTn measurements were made in two core laboratories (FSA, RHC) on instruments that were in their respective laboratories, and were not placed in either laboratory specifically for this study. Specimens were only thawed once and analyzed by the respective instruments during the same thaw cycle. hs-cTnI concentrations were determined on the following instruments: Abbott Architect i2000 (FSA), Beckman Coulter Access 2 (FSA), ET Healthcare Pylon (FSA), Mitsubishi Pathfast (RHC), Ortho-Clinical Diagnostics VITROS 3600 (RHC), Siemens Healthineers Attelica (RHC), Siemens Healthineers ADVIA Centaur XP (RHC), Siemens Healthineers Dimension Vista 1500 (RHC), and Singulex Clarity (FSA). hs-cTnT measurements were determined on: the Roche Diagnostics cobas e601 (using Gen 5 reagents, RCH) and e602 (using hs-reagents, FSA) and the ET Healthcare Pylon (FSA). Hemoglobin A1c (>URL 6.5%), NT-proBNP (>URL 125 ng/L) and eGFR (<60 mL/min), along with statin use, assisted in verifying subject normality, as defined by two expert biomarker groups from the Academy and C-CB (comprised of laboratory medicine, cardiology, and emergency medicine experts), and used as surrogate biomarker health exclusion criteria *(6)*.

STATISTICAL METHODS

99th percentile URLs were determined by 3 different statistical approaches: nonparametric (NP), Harrell–Davis bootstrap (HDB), and robust (R) methods. Nonparametric analysis was conducted using SAS version 9.4 and utilizing distribution-free 95% confidence intervals *(16)*. The Harrell–Davis quantile estimator analysis was conducted using RStudio version 3.3.2 (package Hmisc) utilizing jack-knifed standard errors for 95% confidence interval calculation *(17)*. Robust analysis was conducted using RStudio version 3.3.2 (package reference intervals version 1.1.1) using bootstrapped 95% confidence intervals *(16)*. All analyses were done for the overall group and for each sex. Subgroup analysis was performed on those meeting our stricter definition of "normal/healthy" subjects following

exclusions. The limits of detection (LoD) used in the current study were taken from the IFCC C–CB high-sensitivity cTn table *(14, 15)* as supplied by each manufacturer in their package inserts. It should be noted that following completion of this study, Singulex was no longer in business.

Results

Subject demographics are shown in Table 1. Surrogate biomarker exclusion screening removed 149 individuals, overall, of which 75 were men and 74 were women, as shown in Fig. 1. Subjects excluded were: n = 89 by NT-proBNP (range 125–5000 ng/L); n = 15 by eGFR (range 34–59 mL/min/1.73 m^2); n = 19 by HbA1C (6.6–16.3%); n = 38 by statin use; with 12 subjects having more than one reason for exclusion.

CTNI

Table 2 shows the percentages of measurable cTn concentrations and 99th percentile URLs for the 9 hs-cTnI assays after subject exclusions, by sex and by statistical methods used. Pre-exclusion data are shown in Supplemental Table 1. For all subjects Fig. 2 shows the percentages of measurable concentrations (≥LoD) along with 99th percentile URLs determined by the nonparametric method on the post-exclusion subjects for: A) overall data, B) for men, and C) for women. 99th percentile URLs before (Supplemental Fig. 1A–C) and after exclusions differed only slightly, by 1 to 2 ng/L. Given that the patterns of results were similar before and after health exclusions, we focused only on the post-exclusion concentrations that we would recommend for use in clinical practice. Supplemental Fig. 2 displays on the same graph the post-exclusion data for all subjects, and for men and for women to highlight

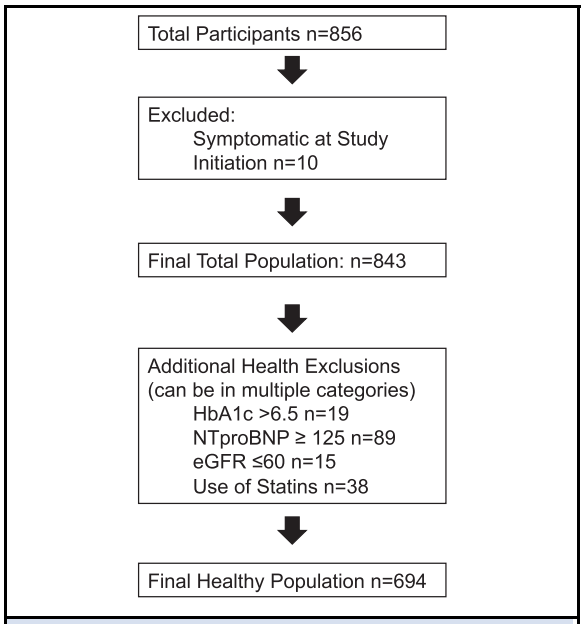

Fig. 1. Flow diagram showing the initial Universal Sample Bank population and secondary exclusions by surrogate biomarkers and statins.

Table 1. Universal Sample Bank subject demographics.

	Pre-exclusions			Post-exclusions			
	All	Men	Women	All	Men	Women	Exclude
N	843	426	417	694	354	340	149
Age mean (SD)	41 (13)	42 (13)	41 (12)	40 (12)	39 (12)	40 (12)	50 (13)
Age range	19-91	20-74	19-91	19-74	20-74	19-71	21-91
Race n (%)							
Caucasian	488 (57)	207 (48)	281 (67)	396 (57)	171 (48)	225 (66)	92 (61)
African American	228 (27)	151 (35)	77 (18)	185 (26)	123 (34)	62 (18)	43 (28)
Pacific mean (SD) Islander/ Asian mean (SD)	91 (10)	50 (11)	41 (9)	84 (12)	44 (12)	40 (11)	7 (4)
Other mean (SD)	29 (4)	12 (2)	17 (4)	23 (3)	11 (3)	12 (3)	6 (4)
Hispanic n (%)	68 (8)	37 (8)	31 (7)	56 (8)	32 (9)	24 (7)	12 (8)
HbA1C mean (SD) %	5.6 (0.6)	5.6 (0.8)	5.5 (0.4)	5.5 (0.3)	5.5 (0.3)	5.5 (0.3)	5.9 (1.3)
NT pro-BNP mean (SD) ng/L	71 (202)	65 (271)	77 (87)	43 (28)	31 (24)	54 (28)	204 (453)
Creatinine mean (SD) mg/dL	9 (0.2)	1.0 (0.2)	0.8 (0.1)	0.88 (0.2)	0.99 (0.1)	0.75 (0.1)	0.90 (0.3)
Smoker n	198	132	66	167	106	61	31

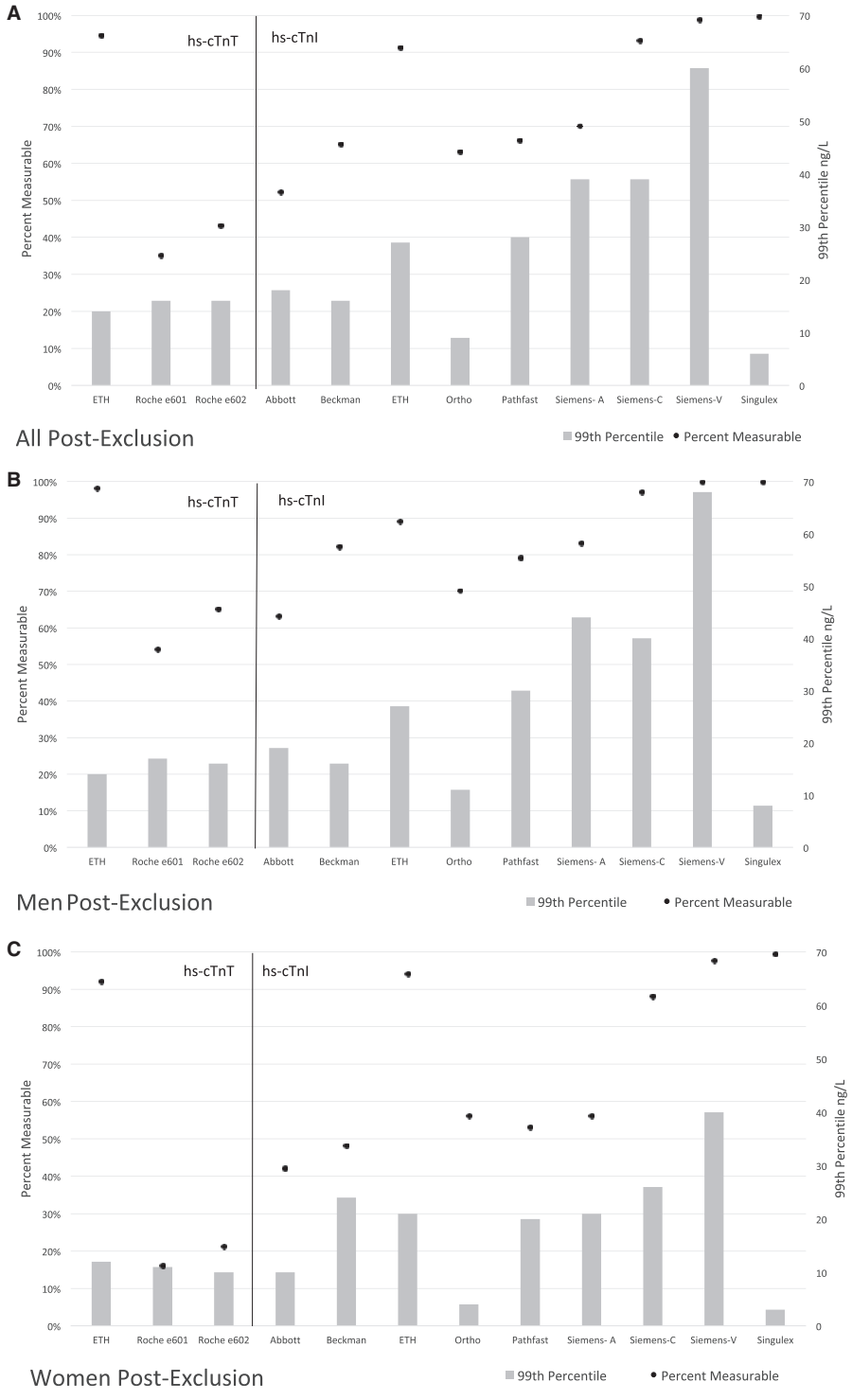

Fig. 2. Schematic representation of the hs-cTnI and hs-cTnT assays percentages of measurable concentrations ($\geq$ LoD) with 99th percentile URLs based on the post exclusion nonparametric statistic for: a) overall subjects, b) men, c) women (ETH, ET Healthcare; Siemens: A, Atellica; C, Centaur: V, Vista).

differences by cTnI assay, with Supplemental Fig. 3 displaying pre-exclusion data on same graph. The Siemens Vista and Singulex Clarity assays were consistently the top two for percent measurable cTn concentrations, and consistently showed the highest and lowest URL concentrations, respectively. The percentages of measurable cTn concentrations ranged from 52% to 99.5% overall, from 63% to 99.7% in men, and from 42% to 99.4% in women. The Beckman Coulter Access 2 (48%) and the Abbott Architect (42%) assays fell slightly short of the 50% measurable threshold for women. The URLs varied both between assays and by statistical method, with the robust method consistently returning much lower URLs. The nonparametric URLs ranged from 6 to 60 ng/L overall (men: 8 to 68 ng/L, women: 3 to 40 ng/L). The Harrell-Davis bootstrap had similar URLs ranging from 7 to 58 ng/L overall (men: 10 to 70 ng/L, women: 4 to 45 ng/L). The robust method could not be used to calculate the women's URL for the Abbott, Beckman, and Ortho assays due to low measurable quantities. Given those exclusions, the range of URLs was 4 to 26 ng/L overall (men: 5 to 31 ng/L, women: 3 to 21 ng/L).

CTNT

Table 3 shows the percentages of measurable concentrations and 99th percentile URLs after subject exclusions, by sex, and by statistical methods used for the 3 hs-cTnT assays. Pre-exclusion data are shown in Supplemental Table 2. For all subjects, Fig. 1 shows the percentages of measurable concentrations ($\geq$ LoD) along with 99th percentile URLs determined by the nonparametric method on the post-exclusion subjects for: A) overall data, B) men, and C) women. Supplemental Figs. 4 and 5 display the data, post and pre, respectively, for all subjects and for men and women on the same plot to highlight differences by cTnT assay. The Roche assay measured on 2 different instruments demonstrated <50% measurable concentrations overall before (46%, 37%) and after (43%, 35%) exclusions. The ET Healthcare assay, however, measured 94% before and after exclusions. All three assays performed better in men than women (after exclusions: ET: 96% vs. 92%, Roche e601: 65% vs. 21%, Roche e602: 54% vs. 16%). 99th percentile URLs before and after exclusions differed only slightly, therefore we focused again only on the after-exclusion results. The Roche assays were unable to generate a 99th percentile URL using the robust statistical method. The e601 and e602 assays had the same URL using the nonparametric method of 16 ng/L. The Harrell-Davis bootstrap method produced URLs of 17 ng/L and 16 ng/L, respectively. ET Healthcare's Pylon assay had URLs of 14 ng/L, 14 ng/L, and 8 ng/L by the nonparametric, Harrell-Davis bootstrap, and robust methods, respectively. These URLs were higher in men (NP: 14 ng/L, HDB: 17 ng/L, R: 10 ng/L) than in women (NP: 12 ng/L, HDB: 13 ng/L, R: 7 ng/L).

HISTOGRAMS

Histograms for all 10 hs-assays, shown in Supplemental Fig. 6A–J for overall data, for some assays demonstrated near Gaussian distributions, while for other assays did not because of those assays' lower analytical sensitivity to detect women at low concentrations that are below the LoD. Table 4 which shows 99th percentiles when stratified by age tertiles for each assay, using all subjects, did not demonstrate a consistent trend towards higher concentrations with increasing age.

Discussion

This study is novel in several aspects and will have important educational impacts on clinicians and laboratorians as they decide how to appropriately implement hs-cTnI and hs-cTnT 99th percentile URLs in clinical practice and research.

First, we demonstrated that sex-specific 99th percentiles vary across multiple hs-cTnI and hs-cTnT assays used in practice; both overall and by sex (post-exclusion: Tables 2 and 3, Fig. 1; pre-exclusion: Supplemental Tables 1 and 2; Supplemental Figures 1–6). Concentration differences between assays are substantial and underline the lack of standardization and harmonization *(3, 16, 18, 19)*. Assays are designed with different capture and detection antibodies, which may or may not detect the multiple forms of cTnI and cTnT that are formed in the myocardium and released into the circulation during and after the myocardial injury. The differences in each assay's ability to detect the multiple circulating forms are related to each manufacturer's choice of antibodies used in their assay and how well they can detect the multiple forms circulating, as recently showed by Katrukha et al. *(20)*. This is also true regarding each assay's ability to measure circulating cTn in normal individuals, independent of the mechanism of release from the myocardium. Men typically have substantially higher concentrations than women, and these sex differences support the guideline for sex-specific URL use *(1)*. What is most obvious is that the 99th percentile URLs observed from our USB bank population are substantially different than concentrations published in manufacturers' regulatory package inserts *(3, 12, 14, 15)*. The lower URLs from our study likely reflect our rigor in excluding subjects who were thought to be apparently healthy subjects per criteria defined by the joint Academy/C-CB expert opinion *(6)*. Studies have shown the more rigorous one is in eliminating potential comorbidities, the lower the URL value becomes *(4, 21)*. Based on surrogate biomarker (eGFR, hemoglobin

Table 2. Percentages of measurable cTn concentrations and 99th percentile URLs (with 95% confidence intervals) for the 9 hs-cTnI assays after subject exclusions, by sex and by statistical methods used.

	Abbott Architect i2000	Beckman Coulter Access 2	ET Heathcare Pylon	Ortho Clinical Diagnostics Vitros	Medience Pathfast	Siemens Atellica	Siemens Centaur	Siemens Vista	Singulex Clarity
LOD	1.9 ng/L	1.4 ng/L	1.2 ng/L	1.0 ng/L	2.3 ng/L	1.6 ng/L	1.19 ng/L	2.00 ng/L	0.08 ng/L
% Measurable	54%	67%	91%	65%	68%	71%	93%	98.8%	99.3%
Nonparametric	19 (16, 30)	18 (16, 34)	28 (19, 35)	15 (9, 26)	28 (22, 30)	38 (27, 45)	39 (35, 46)	60 (42, 80)	8 (5, 15)
Harrell-Davis	20 (15, 24)	20 (14, 25)	29 (20, 37)	15 (10, 20)	27 (20, 35)	37 (30, 44)	39 (33, 45)	59 (43, 75)	9 (5, 12)
Robust	NA	10 (8, 11)	13 (11, 15)	7 (5, 8)	15 (13, 17)	18 (15, 20)	19 (17, 21)	27 (23, 31)	4 (3, 5)
Men Only									
% Measurable	65%	84%	89%	73%	81%	84%	98%	99.8%	99.4%
Nonparametric	20 (18, 35)	18 (15, 34)	31 (17, 44)	16 (11, 29)	30 (24, 56)	44 (36, 55)	40 (36, 46)	68 (42, 91)	11 (7, 15)
Harrell-Davis	22 (17, 28)	19 (14, 25)	29 (19, 39)	17 (13, 21)	30 (22, 39)	43 (37, 49)	41 (36 , 47)	68 (52, 84)	11 (6, 16)
Robust	NA	10 (9, 13)	14 (11, 17)	8 (6, 10)	18 (15, 21)	21 (18, 25)	21 (18, 25)	31 (18, 27)	5 (4, 6)
Women Only									
% Measurable	42%	48%	93%	56%	54%	59%	88%	97.8%	99.2%
Nonparametric	13 (10, 33)	20 (9, 39)	28 (15, 39)	5 (3, 26)	22 (16, 31)	26 (19, 40)	26 (22, 56)	44 (33, 69)	4 (3, 16)
Harrell-Davis	15 (8, 22)	21 (11, 31)	28 (17, 40)	9 (0, 18)	23 (11, 34)	26 (19, 33)	33 (19, 47)	48 (33, 63)	5 (2, 9)
Robust	NA	NA	13 (10, 16)	NA	12 (10, 13)	12 (10, 15)	16 (12, 20)	22 (18, 27)	3 (2, 4)

Table 3. Percentages of measurable concentrations and 99th percentile URLs (with 95% confidence intervals) after subject exclusions, by sex, and by statistical methods used for 3 hs-cTnT assays.

	ET Healthcare Pylon	Roche Cobas e602	Roche Cobase601
LOD	0.8 ng/L	3.0 ng/L	3.0 ng/L
After health exclusions			
% Measurable	94%	43%	35%
Nonparametric	14 (11, 24)	16 (14, 27)	16 (13, 27)
Harrell-Davis	14 (11, 17)	17 (13, 20)	16 (13, 19)
Robust	8 (7, 10)	NA	NA
Men only			
% Measurable	96%	65%	54%
Nonparametric	14 (13, 23)	16 (15, 27)	17 (16, 27)
Harrell-Davis	17 (11, 24)	19 (12, 25)	19 (13, 25)
Robust	10 (8, 12)	11 (10, 13)	NA
Women only			
% Measurable	92%	21%	16%
Nonparametric	12 (9, 24)	10 (9, 32)	11 (9, 31)
Harrell-Davis	13 (8, 17)	14 (6, 23)	12 (8, 16)
Robust	7 (5, 9)	NA	NA

Table 4. Cardiac troponin 99th percentiles (ng/L) when stratified by age tertiles for each assay, using all subjects

Company assay	Tertile 1, <33y N = 241	Tertile 2, 33–45y N = 269	Tertile 3, 46+y N = 333
ET Healthcare-Pylon	11	14	15
Roche-e602	11	15	18
Roche-e601	10	16	18
Abbott-ARCHITECT i2000	23	16	20
Beckman Coulter-Access 2	24	18	18
Ortho Clinical Diagnostics-Vitros	6	9	20
Medience-Pathfast	29	19	24
Siemens-Attelica	43	38	30
Siemens-Centaur	43	35	38
Siemens-Vista	63	47	55
Singulex Clarity	5	7	15

A1C, natriuretic peptide), medication use, and health question screen *(6)*, we confirmed previous observations using better defined criteria for excluding silent underlying pathologies. Ultimately, use of imaging studies (cardiac magnetic resonance imaging) should be included as a routine part of reference-participant vetting, recognizing at present the financial burden of imaging and the challenge for clinical laboratories and companies to obtain imaging *(3)*. Every study should strive to have sex-diverse reference individuals who are representative of the patient population observed in their geographic area for patients who present with symptoms suggestive of MI. We do not currently recommend specific URLs by age/decade or by ethnicity, as appropriately-powered studies are not available. While studies have suggested that African-Americans may have higher 99th

percentiles than Caucasians *(22)*, additional studies are necessary to determine if racial/ethnic cTn differences are important in clinical practice. Beyond the joint Academy/C-CB expert opinion regarding the minimum number of subjects (300 men, 300 women) and how healthy subjects should be identified, no definitive international consensus across different medical subspecialties has been agreed upon. The literature is growing in support of using sex-specific 99th percentiles in clinical practice, but this has not been definitively proven *(22–28)*. Relative to an overall 99th percentile for a combined population of men and women, a lower URL for women increases clinical sensitivity at the expense of clinical specificity, and the reverse is true for men, with a substantial increase in the rates of MI diagnoses in women *(25)*. For risk stratification, studies have shown that using cutoff values lower than either the female or overall 99th percentile concentration improved prognostication; leading to the suggestion of making risk assessments across the continuum of hs-cTn values within the reference interval *(26)*.

Second, we demonstrated that the statistical method used to calculate the 99th percentile will impact the URL *(11, 29)*. We recommend the nonparametric statistical method, as it is simple to use, and provides very similar results to the more complicated Harrell-Davis bootstrap. The robust method appears to be the least desirable method, especially for women, due to its reliance on the presence of variation around the median, which is hampered by the low-end analytical insensitivity of the Roche hs-cTnT and the Abbott, Beckman, and Ortho assays. The robust statistical methodology employed here was designed for computation of a central 95% reference interval *(30)*. Despite this, it has been common practice throughout *Clinical Chemistry* to report 99th percentiles with this methodology using the tuning constant Horn derived for central 95 percentile determination (upper reference limit of 97.5). This extrapolation beyond that for which the methodology was developed will lead to underestimation of the upper reference limit (Paul S. Horn, personal communication). Therefore, while results estimated by this method are included in our results and discussion, we would never recommend using those results. They have been reported to illustrate the consequences of inappropriate use of this statistical methodology. As seen here, the robust results were consistently much lower than their Harrell–Davis and nonparametric counterparts. Relying on those data in clinical practice would cause misclassification of many patients. We recommend that manufacturers be transparent in their package inserts with both the criteria for inclusion and exclusion of the healthy reference subjects and the statistical method used. Regulatory agencies should provide consistent guidance that would be a positive enhancement in the biomarker field for determining URLs *(7, 8)*.

Third, not all assays marketed by manufacturers as 'high sensitivity' meet the joint Academy/C-CB guideline criteria *(6)*, which includes having measurable concentrations $\geq$ LoD in >50% of individuals, especially for women *(3)*. Both the Roche hs-cTnT and Gen 5 cTnT assays measured <30% of women's concentrations in the current study and thereby do not meet the hs guideline criteria, confirming previous studies *(12)*; the Beckman Coulter and Abbott hs-cTnI assays also reported slightly less than 50% of concentrations for women. The LoD used will also influence the percentage of measurable concentrations, and assay LoDs do vary. The ability of an assay to measure low concentrations at and above the LoD will allow clinicians to better follow serial changes in a patient presenting very early to an emergency department after an acute injury or MI, thereby assisting early rule out based on a single presentation sample *(31, 32)* and early rule in and rule out algorithms that require 2 samplings within 1 to 3 h after presentation *(33–36)*.

Standardized approaches to establishing sex-specific 99th percentile URLs are necessary to improve comparisons across data sets and to allow consistency in diagnostic accuracy, outcomes risk assessment, and research studies. Our unique study, utilizing a USB, helps to fill some gaps in understanding implementation of hs-cTn 99th percentile URLs in practice. Too many multi-assay studies compare cohort data based on 99th percentiles provided by manufacturers that were determined on populations substantially different than their reported study cohorts, and potentially introduced inherent bias into their findings. Improved analytics of hs-cTn assays for detection of myocardial injury have led emergency medicine physicians to rule out MI based on a single hs-cTn result below the LoD *(31, 32)*. As nonischemic medical conditions increase cTn in the absence of MI, serial cTn testing remains relevant, with the ability to measure cTn concentrations within the reference intervals for both men and women.

Several clinical implications are noted. First, 99th percentile URLs should be derived from reference populations and limited to those without prior conditions and those with no biomarker evidence of subclinical disease. In patients presenting with symptoms suggestive of ischemia to rule in or rule out MI, subclinical heart failure, renal disease, and diabetes will be common. Excluding those subjects with undiagnosed disease for the reference population truly allows the determination of normality, and while it no longer is a true reflection of society or the population that are evaluated in clinical practice, it provides the opportunity for appropriate clinical management by identifying exactly those patients with underlying disease or injury that would

have been missed by other tools used (ECG, history, imaging). Second, as recently addressed for the CHARIOT study *(37)*, how a message is portrayed about misdiagnosis using inappropriately defined URLs confuses readers and diminishes the importance of cTn increases above the 99th percentile URL. Studies have consistently demonstrated the prognostic impact of cTn increased above the 99th percentile URL *(20)* and within the reference interval *(24–26)*. As hs-cTn increases, whether within the reference interval or above the URL, there is a continuum of increased risk for adverse events *(26)*. The advocacy of some clinicians to use higher cTn thresholds ignores at-risk patients. The CHARIOT authors suggest that it would be preferable to tailor URLs to the patients' baseline characteristics and comorbidities. This would result in numerous cut-offs by subgroups that would vary by each study, and would have the potential to impact patient care negatively. This practice is not endorsed *(1, 4–6)*.

Third, we continue to promote that laboratories report separate 99th percentile URLs for men and for women and work with their clinical colleagues to better understand how sex-specific URLs may affect clinical care. We understand that resources do not allow all laboratories to define their own reference population. Thus, laboratories need to decide collectively whether they wish to rely on the manufacturers recommendations or on data reported in studies, such as the current study. We do not recommend reporting confidence intervals around 99th percentile URLs, as these estimates may confuse the definitive nature of the URL as defined by the Universal Definition. However, knowledge of confidence intervals can educate clinicians to better understand the fragility of any measure, and that quantitative measures cannot replace clinical judgment and patient symptoms and history.

Fourth, if the USB population included a more diverse age distribution greater than 60 years, the 99th percentiles for both men and women for each assay would likely have been higher than reported in our study. Previous studies have documented trends for higher cTn values at higher ages ranges for both sexes *(14, 22)*, but our analysis of cTn 99th percentiles when stratified by age tertiles in the current study did not show this effect (Table 4). Thus, each laboratory needs to be cognizant of the fact that actionable 99th percentiles may vary based on different populations served.

Potential limitations are noted. First, while the surrogate biomarker cutoffs were a consensus recommendation by laboratory medicine, cardiology, and emergency medicine experts, we acknowledge other experts may consider our cutoff values as higher than ideal. Second, the LoDs used in the current study were based on manufacturers' regulatory package inserts *(14, 15)* and were not independently determined. Third, the plasma specimens used in this study were frozen within 1 h of sample collection at −70 °C and stored at -70 °C until thawed for cTn measurements *(13)*. No stability study was performed, as both cTnI and cTnI are known to be stable for over 5 years frozen at −70 °C *(38)*. Fourth, the average age of our reference population volunteers was younger than those often tested in clinical practice (41 [SD13] years versus 61 [SD17] years) *(39)*. While this does not impact on the comparisons between groups reported in the current study, we acknowledge that it may preclude the direct application of these thresholds in clinical practices that have shown higher 99th percentile in normal subjects >60 years for men and women *(4, 21, 28)*. Fifth, we recognize that volunteers, as used in the USB, may be healthier and different from a population based on apparently normal individuals in the general population who would meet inclusion/exclusion criteria. Sixth, at least in our data set, statin use did not have a statistical effect on the 99th percentiles calculated (data not shown). However, our population had a limited number of individuals taking statins.

In conclusion, our novel study has important clinical practice implications, in that sex-specific 99th percentile URLs vary according to the hs-assay used to measure cTn and the statistical method used to calculate the 99th percentile. We do not recommend the use of the robust statistical methodology in determination of 99th percentile calculations. Not all assays marketed as high sensitivity meet guideline criteria to provide measurable concentrations $\geq$ LoD in >50% for women, and surrogate exclusion criteria used to define normality in apparently healthy subjects tend to lower 99th percentile URLs, providing a better representation of a normal population. How a clinical or research laboratory chooses to define 'normality' will influence both diagnostic and risk assessment decisions.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

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