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Plasma 99th Percentile Reference Limits for Cardiac Troponin and Creatine Kinase MB Mass for Use with European Society of Cardiology/ American College of Cardiology Consensus Recommendations

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Background: **The European Society of Cardiology/ American College of Cardiology (ESC/ACC) consensus document for definition of myocardial infarction (MI) is predicated on increased cardiac troponin or creatine kinase (CK) MB mass above the 99th percentile reference limit. The purpose of this study was to determine the plasma (heparin) 99th percentile reference limits for the leading in vitro diagnostic cardiac troponin and CKMB mass assays.**

Methods: **Blood (heparin plasma) was obtained from healthy adults (n = 696; age range, 18–84 years) stratified by gender and ethnicity. Cardiac troponin I (cTnI) and T (cTnT) and CKMB mass concentrations were measured by eight assays. Reference limits were determined by nonparametric statistical analysis.**

Results: **Two cTnI assays demonstrated at least a 1.2- to 2.5-fold higher 99th percentile for males vs females, with the mean concentrations significantly higher for males ($P < 0.05$). Two cTnI assays also demonstrated a 1.1- to 2.8-fold higher 99th percentile for blacks vs Caucasians, with the mean concentrations significantly higher for blacks ($P = 0.05$). There was a 13-fold variance between the lowest measured 99th percentile (0.06 μg/L) and the highest (0.8 μg/L). All CKMB assays demonstrated a 1.2- to 2.6-fold higher 99th percentile for males vs females, with mean concentrations significantly higher for males ($P < 0.0001$). Four CKMB assays also showed significantly higher (1.2- to 2.7-fold) mean concentrations for blacks ($P < 0.02$) vs Caucasians.**

Conclusions: **The heparin-plasma 99th percentile reference limits for cardiac troponin and CKMB mass provide an evidence base in support of the ESC, ACC, and American Heart Association guidelines for detection of myocardial injury. Selective gender and ethnic differences were demonstrated. These data allow clinicians, trialists, and epidemiologists a common point for operational use.**

The consensus document of the Joint European Society of Cardiology/American College of Cardiology (ESC/ACC)[1] Committee for the redefinition of myocardial infarction (MI) has established criteria for acute, evolving, or recent MI predicated on a typical increase in cardiac troponin in the clinical setting of myocardial ischemia *(1, 2)*. Furthermore, the ACC/American Heart Association (AHA) guidelines for management of unstable angina recommend monitoring of cardiac troponin in acute coronary syndrome (ACS) patients for differentiating unstable angina (cardiac troponin within the reference interval) and non-ST-segment-elevation MI (increased cardiac troponin) *(3, 4)*. Both documents define cardiac troponin as an indicator of myocardial necrosis when the

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[1] Nonstandard abbreviations: ESC, European Society of Cardiology; ACC, American College of Cardiology; MI, myocardial infarction; AHA, American Heart Association; ACS, acute coronary syndrome; cTnT and cTnI, cardiac troponin T and I, respectively; CKMB, creatine kinase MB; DPC, Diagnostic Products Corporation; OCD, Ortho-Clinical Diagnostics; LLD, lower limit of detection; and FDA, Food and Drug Administration.

maximum concentration of either cardiac troponin T (cTnT) or cardiac troponin I (cTnI) exceeds the decision limit (defined as the 99th percentile of a reference control group) on at least one occasion during the first 24 h after the index clinical event.

Furthermore, in the absence of cardiac troponin monitoring, a maximum creatine kinase MB (CKMB) value exceeding the 99th percentile of a reference control group on two successive samples or a maximum value exceeding twice the 99th percentile on one occasion is indicative of myocardial necrosis. Although some studies have determined 99th percentile reference limits for individual assays *(5, 6)*, no study has simultaneously determined reference limits for a large number of cardiac troponin or CKMB mass assays based on the same reference population. The purpose of the current study was to establish plasma (heparin) 99th percentile reference limits using a common reference study population for the leading in vitro diagnostic cardiac troponin and CKMB mass assays used worldwide in clinical practice and clinical trials.

Materials and Methods

We recruited 696 individuals in good health to participate. All participants were informed of the study's goals and gave informed consent to participate along institution-approved human subject guidelines. Recruitment was aimed at enrolling a diverse ethnic/racial mixture, distributed as equally as possible by sex, across the ages 18–84 years according to the NCCLS standard protocol for recruitment of healthy individuals for reference interval determinations *(7)*. Individuals were recruited from numerous locations, including hospital workers, ethnic community centers, and community wellness health clinics. By health questionnaire, no participant reported any known current or past history of coronary artery disease or cardiac-related medical condition. No other information regarding non-cardiac-related illnesses or diseases were obtained.

The participants completed a health questionnaire, and two full 5-mL tubes (Becton Dickinson Vacutainer green-top 13 × 75 mm tubes containing 72 USP units of lithium heparin; no interior coating; silicon-lubricated stopper; reorder no. 36 6485) of blood (heparinized plasma) were donated. The blood was immediately centrifuged, and the plasma was poured off and frozen at −70 °C until analysis. All specimens were recentrifuged after thawing, before analysis. Plasma cardiac troponin was measured by eight different assays and CKMB mass by seven different assays: Abbott AxSYM (cTnI, CKMB), Bayer Centaur (cTnI, CKMB), Beckman Access (second-generation cTnI, CKMB), Dade-Behring Dimension RxL (second-generation cTnI, CKMB), Diagnostic Products Corporation (DPC) Immulite 2000 (cTnI), Ortho-Clinical Diagnostics (OCD) Vitros ECi (cTnI, CKMB), Roche (third-generation cTnT, CKMB), Tosoh AIA (second-generation cTnI, CKMB). Optimal analytical characteristics defined by each manufacturer for each assay for the lower limit of detection (LLD), the lowest concentration to give a 10% total imprecision (CV) for cardiac troponin, total imprecision data for CKMB mass, and manufacturers' claims for the 99th percentile for cardiac troponin and either the 95th or 97.5th percentile for CKMB are shown in Table 1. In the current study, each method was performed according to the manufacturer's guidelines except for the Roche cTnT and DPC cTnI assays, which state that heparin-plasma should not be used because of heparin-induced decreases in cardiac troponin compared with serum *(5, 8, 9)*. To verify lack of interference by heterophile antibodies, selective high cardiac troponin samples were screened by a commercial heterophile antibody blocking system (Scantibodies Laboratory). Nonparametric analysis for determination of the 99th percentile for each assay for all partic-

Table 1. Analytical characteristics of cardiac troponin and CKMB mass assays (μg/L) as claimed by each manufacturer.[a]

	LLD		Reference limit[b]			
Manufacturer: Instrument	Cardiac troponin, μg/L	CKMB, μg/L	Cardiac troponin, μg/L (99th percentile)	CKMB, μg/L	10% CV cutoff (cardiac troponin),[c] μg/L	Precision[d] (CKMB)
Abbott: AxSYM (n)[e]	0.14	0.7	0.5	3.8 (95th)	0.8	5.2 (6.3%)
Bayer: Centaur (s)	0.02	0.18	0.1	4.8 (99th)	0.35	3.5 (3.9%)
Beckman: Access (p)	0.01	0.3	0.04	4.0 (95th)	0.06	3.2 (8.2%)
Dade-Behring: Dimension (s)	0.04	0.5	0.07	3.1 (97.5th)	0.14	7.2 (8.8%)
DPC: Immulite 2000 (s)	0.2	0.2	0.2	3.5 (97.5th)	0.6	13.9 (6.5%)
OCD: Vitros ECi (p)	0.02	0.6	0.08	3.4 (97.5th)	0.12	1.3 (7.8%)
Roche: Elecsys 2010[a] (s)	0.01	0.1	0.01	2.9 (99th women) 6.7 (99th men)	0.03	5.7 (2.3%)
Tosoh: AIA 600II (b)	0.06	0.5	<0.06	5.8 (99th)	0.06	9.9 (3.7%)

[a] Data obtained from FDA-cleared package inserts or through personal communications with manufacturers. Roche is the only cTnT assay on the market; all other assays are cTnI.

[b] 99th, 95th, and 97.5th refer to percentiles.

[c] No standardized protocol exists for determination of this value, which refers to total imprecision.

[d] The number outside the parentheses indicates the concentration (μg/L); the number in parentheses indicates %CV.

[e] Specimen types as indicated in the manufacturers' package inserts used for determination of 99th percentile reference intervals: s, serum; p, heparin plasma; b, both; n, not specified.

ipants by sex, race, and age were determined along NCCLS guidelines (SPSS Mac, Ver.10). Statistically significant differences based on mean concentrations by sex, race, and age were assumed at a probability value of $P < 0.05$. Calculations for observed differences between gender and race were based on the LLD when concentrations were below the LLD.

Results

Of the 696 individuals enrolled, 45% were male and 55% were female. The ethnic distribution was as follows: 58% Caucasian, 31% black, 2% Hispanic, 5% Asian, 2% Native American, 2% other/mixed race. Table 2 shows the 99th percentiles by sex and ethnic distribution for cardiac troponin. For all individuals for the majority of assays, the 99th percentiles (Table 2) agreed fairly closely with the concentration provided by each manufacturer, either in their Food and Drug Administration (FDA)-cleared package inserts or through personal communications when not provided in the package inserts (Table 1). Two of the cTnI assays, Ortho and Beckman, demonstrated a significant ($P < 0.05$) mean difference between males and females, with a 1.2- to 2.5-fold higher 99th percentile for males. Two assays, Beckman and Tosoh, also demonstrated a significant ($P = 0.05$) difference between the mean cTnI concentrations in blacks and Caucasians, with a 1.1- to 2.8-fold higher 99th percentile for blacks. Enrollment numbers in the Hispanic, Asian, and Native American groups were too small for additional analysis.

We found no significant differences across the ages of 30–69 years, as demonstrated by the Beckman Access cTnI assay results shown in Fig. 1. Although not displayed in Fig. 1, the mean (95% confidence interval) for ages 70–84 years (n = 24) was not significantly different: 0.012 (0.006–0.020) μg/L. For cTnI assays, the 99th percentile limits varied by as much as 13-fold between the lowest (Dade and Tosoh, 0.06 μg/L) and highest (Abbott, 0.8 μg/L) concentrations. There were no heterophile antibody interferences in 20 randomly selected high-cTnI-concentration samples, which were responsible for selective racial and gender differences in selected assays (data not shown).

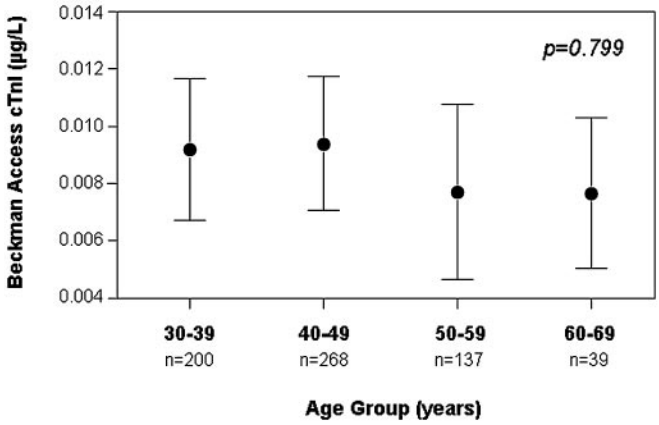

Fig. 1. Results from the Beckman Access cTnI assay demonstrating trends of cTnI concentrations (μg/L) by age.
Results are shown as the means and 95% confidence intervals (*error bars*).

The 99th percentiles by sex and race distribution for CKMB mass are shown in Table 3. All seven CKMB assays demonstrated a 1.2- to 2.6-fold higher 99th percentile for males vs females, with mean concentrations significantly higher for males ($P < 0.0001$). For all participants, the 99th percentile limits varied by as much as 2.0-fold between the lowest (Dade, 3.9 μg/L) and highest (Beckman, 7.9 μg/L) concentrations. For all participants combined, there was a significant ($P \leq 0.006$) trend for increasing mean

Table 2. Heparin-plasma 99th percentile reference limits (μg/L) by gender and race for FDA-cleared cardiac troponin assays.

	n[a]	Abbott	Beckman	Dade	OCD	Roche[b]	n[c]	Tosoh	n[d]	Bayer	n[e]	DPC
All participants	696	0.8	0.08	0.06	0.10	<0.01	473	0.07	403	0.15	281	0.21
Males	315	0.8	0.10	0.06	0.11	<0.01	223	0.07	187	0.17	115	0.21
Females	381	0.7	0.04	0.06	0.09	<0.01	250	<0.06	216	0.14	166	<0.2
P		0.739	0.034	0.985	0.017	0.534		0.521		0.441		0.219
Manufacturer's 99th percentile[f]		0.5	0.04	0.07	0.08	0.01		<0.06		0.1		0.2
Caucasians	400	0.8	0.07	0.04	0.11	<0.01	215	<0.06	193	0.17	166	0.21
Blacks	218	0.5	0.08[g]	0.03	0.10	<0.01	196	0.17[g]	156	0.17	91	<0.2
Hispanics	17	0.5	0.02	0.01	0.02	<0.01	13	<0.06	9	<0.02	8	<0.2
Asians	35	1.1	0.10	0.09	0.07	<0.01	31	0.07	30	0.09	4	<0.2
Native Americans	13	0.3	0.02	0.01	0.09	<0.01	11	<0.06	8	0.03	8	<0.2
Other	13	0.4	0.02	0.06	<0.02	<0.01	7	0.07	7	<0.02	4	<0.2

[a] Number of samples tested in the Abbott, Beckman, Dade-Behring, OCD, and Roche assays.
[b] The Roche assay is the only cTnT assay on the market; all other assays are for cTnI.
[c] Number of samples tested in the Tosoh assay.
[d] Number of samples tested in the Bayer assay.
[e] Number of samples tested in the DPC assay.
[f] Data obtained from FDA-cleared package inserts or through personal communications with manufacturers.
[g] Significantly different ($P = 0.05$) from Caucasians based on mean concentrations.

Table 3. Heparin-plasma 99th percentile reference limits (μg/L) by gender and race for FDA-cleared CKMB mass assays.

	n[a]	Abbott	Beckman	Dade	OCD	Roche	n[b]	Tosoh	n[c]	Bayer
All participants	696	7.6	7.9	3.9	4.07	6.96	473	5.7	403	3.28
Males	315	8.7	8.2	4.2	4.21	7.60	223	5.6	184	4.38
Females	381	4.8	5.6	3.1	2.95	4.66	250	3.6	217	1.68
P		0.0001	0.0001	0.0001	0.0001	0.0001		0.0001		0.0001
Caucasians	400	5.9	6.6	3.5	4.07	6.10	215	5.3	193	1.78
Blacks	218	9.6[d]	9.3[h]	4.2	3.97	7.27[e]	196	6.1[f]	156	4.75[g]
Hispanics	17	3.7	3.4	2.5	1.86	5.18	13	3.2	9	0.89
Asians	35	7.0	5.4	3.3	3.71	5.30	31	3.3	30	1.15
Native Americans	13	8.7	8.2	5.1	4.13	8.19	11	4.9	8	2.13
Other	13	4.3	4.5	3.0	2.32	3.68	7	2.3	7	0.50

[a] Number of samples tested in the Abbott, Beckman, Dade-Behring, OCD, and Roche assays.
[b] Number of samples tested in the Tosoh assay.
[c] Number of samples tested in the Bayer assay.
[d–g] Significantly different from Caucasians based on mean concentrations: [d] Abbott, $P < 0.0001$; [e] Roche, $P = 0.022$; [f] Tosoh, $P = 0.019$; [g] Bayer, $P < 0.0001$.
[h] $P = 0.06$ vs Caucasians for Beckman.

CKMB concentrations for all assays, except the Bayer Centaur, by age across decades (Table 4, available as a Data Supplement accompanying the online version of this article at http://www.clinchem.org/content/vol49/issue8/), but this was not always found for both males and females for individual assays. Four CKMB assays (Abbott, Bayer, Roche, and Tosoh) also showed significantly higher (1.2- to 2.7-fold; $P < 0.02$) mean concentrations for blacks vs Caucasians (Table 3). Enrollment numbers were too small for additional racial analysis for Hispanics, Asians, and Native Americans.

Discussion

The current study is unique in that it demonstrates the 99th percentile reference limits for plasma (heparin) cardiac troponin and CKMB mass in the same large, healthy population for several in vitro diagnostic assays. The leading cardiac troponin and CKMB mass assays in the marketplace were used to demonstrate that both gender and ethnic differences are assay- and analyte-dependent. No statistically significant differences were found across age for cardiac troponin assays but were observed for all CKMB assays. These observations provide the evidence-based appropriate heparin-plasma cutoff concentrations for all of the leading in vitro diagnostic assays as recommended by the ESC/ACC consensus document for the redefinition of MI as well as the AHA/ACC guidelines for management of ACS patients *(1–4)*. In agreement with the cardiology community, the laboratory medicine community also supports the 99th percentile as the recommended MI cutoff value for cardiac troponin and CKMB mass *(9–11)*.

The 99th percentile reference limits provides trialists, epidemiologists, and clinical practitioners a basis for establishing appropriate cutoff concentrations depending on their specific use intent. To be able to operationalize existing cardiac troponin assays, it has been suggested that cardiac troponin cutoff values be at the lowest concentration that provides a 10% CV (total imprecision shown in Table 1) until a 10% total imprecision is found at the 99th percentile limit *(10, 11)*. As shown in Table 2 for cardiac troponin, only one assay (Tosoh) currently is able to meet this goal if we rely on the manufacturer's claim of a 10% total CV at both the 99th percentile and LLD of 0.06 μg/L. The current study was not designed to evaluate the 10% CV claims of the manufacturers.

Two assays demonstrated significant differences in mean concentrations of cardiac troponin between males and females: OCD and Beckman. In addition, two assays also showed significant mean concentration differences between Caucasians and blacks (Beckman and Tosoh). These observations would suggest that gender- and ethnic-specific cutoff values should be considered for these assays. Differences could not be explained by the possibility of heterophile antibody interferences. For CKMB, all seven assays showed significantly higher mean concentrations for males vs females, with four assays (Abbott, Bayer, Roche, and Tosoh) showing higher mean concentrations for blacks vs Caucasians.

The impact of misclassification of patients in trials or for epidemiology studies becomes challenging and possibly misleading without these considerations when hospital clinical laboratories or clinical trial laboratories use biomarkers for entry and endpoint criteria. It is important to emphasize the need to get the 99th percentile reference limits right; these were determined the best possible way in the current study using heparin plasma *(7)*. A review of the literature showed that both implementing cardiac troponin (replacing CKMB) measurements and lowering the diagnostic cutoff concentration from the traditional ROC curve cutoff value to the 10% total CV value and the 99th percentile value will substantially increase the incidence of positive tests for MI *(12–14)*. Retrospective analysis of >700 ACS patients from our hospital's 1997 quality assurance database demonstrated a 37% increase in MI rate of diagnosis when a 99th percentile cTnI cutoff

was used (Dade-Behring Stratus II, 0.35 μg/L) compared with the ROC curve cutoff (0.8 μg/L) *(14)*.

Since cardiac troponin was endorsed as the preferred biomarker for detection of myocardial injury *(1–4)*, diagnosis of MI *(1–4)*, and utilization as a risk stratification biomarker in ACS to assist in therapy management *(15, 16)*, the use of troponin assays has increased substantially. For example, one survey indicated that 90% of laboratories used cardiac troponins in clinical practice, with an increase of twofold over the past 3 years *(17)*. In a 2002 College of American Pathology survey of cardiac markers, 3512 laboratories reported the use of cardiac troponin assays compared with 2633 for CKMB mass assays *(18)*. For cardiac troponin, this was a 123% increase since 1999.

Little information has been given regarding biomarker reference limit data in clinical trial studies *(19, 20)*. In the large majority of reports, studies relied on the manufacturers' claims, which often do not support the 99th percentile cutoff limits in their package inserts. Several ACS risk stratification studies now support risk assessment outcome claims based on cardiac troponin concentrations with minor increases above the 99th percentile. Lindahl et al. (FRISC II) *(19)* have shown any detectable increase in cTnT >0.01 μg/L is associated with an increased risk of reinfarction and death, and Venge et al. (FRISC II) *(20)* demonstrated that an increased Beckman Access cTnI ≥0.03 μg/L indicates a greater risk of cardiac events. Both studies acknowledge that assay imprecision at the 99th percentile may falsely categorize patients around this cutoff. However, neither of these studies nor any study reported in metaanalyses *(15, 16)* consider the influence of gender or ethnic differences for biomarker cutoff concentrations. Thus, the potential exists for misclassification based on the differences in cutoff concentration for different assays.

For CKMB, assay imprecision is not as great a challenge as for cardiac troponin. At all 99th percentile reference limits for CKMB (Table 3), total imprecision is listed as <10% by the manufacturers of all of the CKMB assays tested (Table 1). However, as shown in Table 3, gender differences are substantial and will also impact patient classifications and endpoints. To the best of our knowledge, no clinical ACS, MI, or interventional trial or epidemiology study has taken into account gender/ethnic difference when using CKMB as the discriminating biomarker. The potential for misleading interpretations based on statistical analysis of potential false-positive or false-negative CKMB results when subgroups based on gender or ethnic origin have not been broken out remains to be demonstrated. The male/female as well as the black/white differences observed for CKMB can be explained by the larger muscle mass typically found in men vs women and blacks vs Caucasians *(21)*. Because CKMB is not 100% specific for the heart but is also found in amounts up to 1–2% of total CK in skeletal muscle *(21)*, skeletal muscle turnover gives rise to higher measurable concentrations for males.

Both cTnI and cTnT are 100% specific for the heart *(21)*. Thus, it is expected that the majority of healthy individuals will have unmeasurable cardiac troponin (below than the LLD for each assay). This was in agreement with our current findings. The low concentrations measured by some assays may reflect occult heart injury not clinically observed, or they may just reflect background noise of an individual assay. The mechanisms for male/female and black/Caucasian differences, although not known, may also reflect subclinical injury detected by these very sensitive biomarkers. However, no clear mechanisms can account for the assay-specific cardiac troponin differences observed.

Limitations regarding our findings should be noted. One limitation is that it has been shown that several, but not all, cardiac troponin assays give lower results for heparin-plasma samples vs serum samples *(5, 8, 22, 23)*. Serum specimens were not measured in our study. It is possible that lower heparin-plasma cardiac troponin concentrations for some assays would be inappropriately interpreted, impacting clinical triage, diagnostic, and management decisions. Bayer, Dade, DPC, and Roche specify that serum is their preferred or required specimen type. Although plasma was at one time the recommended specimen of choice for both the cardiology and laboratory medicine communities, to assist in keeping the turnaround time as short as possible from blood draw to result reporting *(9, 24)*, the observations that many assays demonstrate a negative bias for heparin-plasma vs serum has challenged this initial recommendation *(5, 8, 23, 25)*. Currently, it is suggested that serum be the specimen of choice for cardiac troponin assays that do show lower concentrations in heparin-plasma samples compared with serum *(9)*. Additional studies examining the potential of serum/plasma differences within the reference interval need to be carried out. We also recommend that manufacturers of cardiac troponin assays more clearly identify information in their package inserts regarding this issue. The second limitation is that thorough physical examinations were not carried out in our healthy participants. Several of the cardiac troponin assays, which demonstrated higher concentrations for men and blacks, might have been truly detecting occult myocardial injury.

In conclusion, our findings demonstrate the importance of establishing sex- and ethnicity-related cardiac troponin and CKMB mass 99th percentile cutoff concentrations for diagnostic criteria as established by the ESC/ACC/AHA and laboratory medicine recommendations for redefinition of MI. Our findings also support the clinical acceptance of cTnI and cTnT monitoring for detection of nonischemic myocardial damage, for use as a risk stratification outcomes assessment tool in ACS patients, and for implementation in clinical trials and epidemiology studies.

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