pared with other commercial efforts for ovarian cancer testing. We will continue to post all raw data, the exact methods, and the results of the blinded trial(s) for public scrutiny. These are very important times for biomarker discovery and validation. In keeping with the National Cancer Institute Director's 2015 challenge, effective biomarkers for early detection, risk stratification, and monitoring can save lives and reduce suffering.

Both Dr. Liotta and Dr. Petricoin are co-inventors on US Government Patent filings that cover technologies described in this reply. As such, under US Government law, the co-inventors may receive royalties on these patent filings once granted and/or licensed. However, US Government scientists are not allowed to participate in licensing negotiations and royalty rate negotiations.

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### Serum 99th Percentile Reference Cutoffs for Seven Cardiac Troponin Assays

*To the Editor:*

Several international expert panels, including the European Society of Cardiology, the American College of Cardiology, American Heart Association, the IFCC, the COURAGE trials group, the Italian Federation of Cardiology, and the American Heart Association and World Heart Federation Councils on Epidemiology and Prevention, have endorsed the concept that increased cardiac troponin I or T (cTnI or cTnT) should be defined as a measurement above the 99th percentile concentration of a reference population *(1–7)*. Furthermore, an increased cTnI or cTnT value is considered indicative of myocardial injury (cell death) and, in the clinical setting of ischemia, is considered evidence of myocardial infarction and a high-risk profile for adverse cardiac and noncardiac events.

Previously, work from our laboratory reported on heparin plasma 99th percentile reference limits for both cTnT and seven cTnI assays *(8)*. However, because heparin plasma is known to cause a negative bias compared with some, but not all cTnI and cTnT assays, reference limit determinations in serum would also be useful because serum and heparin plasma are the most commonly used specimen types in clinical practice. Preanalytical considerations suggest the use of plasma for reducing turnaround times. In addition, a recent study of imprecision for cardiac troponin assays to determine the lowest concentration that meets a 10% total imprecision (CV) was predicated on serum *(9)*, with the goal to assess the size of the gap between the 99th percentile reference limit and the lowest troponin concentration that provides a 10% CV.

Although several studies have published serum-based 99th percentile reference limits for individual assays, no study has used the same population to evaluate reference limits for numerous cardiac troponin assays. The purpose of this study was to determine (using nonparametric statistics) the 99th percentile reference limit, based on samples from 403 healthy volunteers.

After receiving Institutional Review Board approval and having participants complete a health questionnaire ruling out any past or current history of heart disease, we collected nonanticoagulated blood (Becton Dickinson Vacutainer SST Gel and Clot Activator tube), separated the serum, and froze it at −70 °C. We recruited 215 females (53%) and 188 (47%) males, of whom 168 were African American (42%), 181 were Caucasian (45%), and 54 were of other ethnicities (13%). Ages ranged from 30 to 84 years (median, 49 years). As shown in Table 1, not all assays were performed on all samples because of limited specimen volumes. To verify lack of interference by heterophile antibodies, we screened selected samples with high cardiac troponin by a commercial heterophile antibody blocking system (Scantibodies Laboratories). Shown in Table 1 are the serum 99th percentile limits for six cTnI assays and one cTnT assay for all participants, as well as a breakdown for males and females.

For cTnI, the reference limits ranged from 0.03 μg/L (Dade Dimension) to 1.1 μg/L (Abbott AxSYM), a

**Table 1. Serum 99th percentile reference limits by gender and ethnicity for Food and Drug Administration-cleared cardiac troponin assays.**

| | | 99th percentile reference limit,[a] μg/L | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | n | Abbott | Beckman | Dade | OCD | Roche[b] | Tosoh | Bayer |
| All participants | 374 | 1.1 | 0.05 | 0.03 | 0.29 | <0.010 | 0.13 (n = 403) | 0.07 (n = 373) |
| Males | 189 | 1.1 | 0.06 | 0.03 | 0.44 | 0.015 | 0.13 (n = 201) | 0.06 (n = 188) |
| Females | 185 | 0.6 | 0.03 | 0.02 | 0.15 | <0.010 | 0.13 (n = 202) | 0.08 |
| *P* (males vs females) | | 0.7 | <0.001 | 0.4 | 0.007 | 0.3 | 0.9 | 0.004 |
| Caucasians | 216 | 1.1 | 0.05 | 0.02 | 0.10 | <0.01 | 0.13 (n = 215) | 0.07 (n = 193) |
| Blacks | 154 | 1.2 | 0.05 | 0.04 | 0.44[c] | <0.01 | 0.15 (n = 162) | 0.07 |
| *P* (Caucasians vs blacks) | | 0.3 | 0.2 | 0.31 | 0.05 | 0.5 | 0.08 | 0.4 |

[a] Not all assays were performed on all samples because of insufficient sample volume. The number of samples assayed with the Tosoh and Bayer assays are shown in parentheses when they differ from the number of samples tested by the other assays.

[b] Only cTnT assay on the marketplace; all other assays are for cTnI.

[c] Significantly different ($P = 0.05$) vs Caucasians based on mean concentrations.

36-fold difference. We found significant differences between males and females for the Beckman Access, Ortho cTnI Vitros ECi, and Bayer Centaur cTnI assays. All cTnT concentrations were ≤0.015 μg/L, with the overall 99th percentile limit being <0.01 μg/L. Only the Ortho Vitros ECi cTnI showed a significant difference by ethnicity.

Our results for serum are similar to those obtained in our previous study on 99th percentile reference limits for plasma *(8)*: (*a*) results obtained with different cTnI assays vary considerably because of differences in standardization and in the antibodies used *(10)*; and (*b*) there is evidence of gender and ethnic variation. In general, the current serum reference concentrations (n ≤403 individuals) compared with our previous plasma findings in a larger population (n ≤696 individuals) were not predictably higher for all assays, as has been suggested in several Food and Drug Administration-cleared package inserts and previous literature *(4, 8, 11, 12)*. The findings also demonstrate that only one of seven assays (Beckman) gave a 99th percentile concentration (0.05 μg/L) close to the previously reported total 10% imprecision concentration (0.06 μg/L) *(9)*. Thus it is possible that some of the gender differences may be related to assay imprecision. A limitation of this study, however, was that matched serum and plasma samples were not available for analysis. Overall, our findings add to the literature to assist in setting reference limits for the cardiac troponin assays available from the major in vitro diagnostic manufacturers. Further investigations are necessary to explore gender and ethnic differences that may impact diagnostic or risk stratification decisions in patients with acute coronary syndrome *(13–17)*.

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## Rapid Detection of *UGT1A1* Gene Polymorphisms by Newly Developed Invader Assay

*To the Editor:*

Recent progress in human genome analysis has been providing tools for a new approach to disease treatment based on individual differences identified by use of genetic information. The feasibility of genotyping for DNA polymorphisms before treatment depends on the availability of rapid, accurate, and efficient genotyping methods. We previously reported that genetic polymorphisms of the UDP-glucuronosyltransferase 1A1 (*UGT1A1*) gene were significantly related to severe toxicity of irinotecan *(1)*. We now report that we have succeeded in detecting a 2-bp insertion of repeated sequence in the *UGT1A1* gene by use of Invader assay technology.

Sixty patients who had received irinotecan-containing chemotherapy from July 1994 to June 1999 were enrolled in this study. All gave informed consent in writing for their peripheral blood to be used for the research. We used the QIAamp Blood Kit (QIAGEN GmbH) to prepare genomic DNA from whole blood (100–200 μL) and genotyped three sites of DNA polymorphisms in *UGT1A1* (*UGT1A1*28*, *UGT1A1*6*, and *UGT1A1*27*) by the previously described method *(1)*. *UGT1A1*28* was distinguished from the most common allele (*UGT1A1*1*) by direct sequencing (nucleotides −147 to +106) of the 253- to 255-bp fragments produced by PCR. *UGT1A1*6* and *UGT1A1*27* were distinguished from *UGT1A1*1* by direct sequencing combined with PCR-restriction fragment length polymorphism (RFLP) analysis.

The Invader assay detects single-nucleotide polymorphisms (SNPs) by use of Cleavase enzyme and a fluorescence resonance energy transfer cassette *(2, 3)*. Two sets of probes were designed for detecting the SNPs associated with *UGT1A1*6* and **27* (see Table 1 in the Data Supplement that accompanies the online version of this letter at http://www.clinchem.org/content/vol50/issue8/). The *UGT1A1*6* target site was +211G/A, and the **27* target site was +686C/A. In addition, a set of probes was designed to differentiate between the *UGT1A1*28* polymorphism and its reference allele (*UGT1A1*1*; Table 1 in the online Data Supplement) based on the number of TA repeats; *UGT1A1*28* has seven TA repeats, and *UGT1A1*1* has six. An important portion of the probe was designed with the help of Third Wave Technologies, Inc. With these detection systems, the reference and variant alleles are indicated by the Redmond Red (Epoch Biosciences) and 6-carboxyfluorescein (FAM) fluorescent signals, respectively, which were released from the fluorescence resonance energy transfer cassette.

The 60 samples included 4 homozygous [$(TA)_7/(TA)_7$] and 11 heterozygous [$(TA)_6/(TA)_7$] for *UGT1A1*28*; the remaining 45 samples were homozygous for the reference allele [$(TA)_6/(TA)_6$]. The distribution of genotypes showed 1 homozygous and 17 heterozygous for *UGT1A1*6* and 42 homozygous for the reference allele. Two samples heterozygous for *UGT1A1*27* and 58 homozygous for the reference allele were assayed. These data were determined by direct DNA sequencing of *UGT1A1*28* and by direct DNA sequencing combined with PCR-RFLP analysis for *UGT1A1*6* and *UGT1A1*27*, and the results obtained from the Invader assay were compared with them (see Table 2 in the online Data Supplement).

Three of the 60 samples could not be assayed for *UGT1A1*28* by the Invader assay. These samples showed low fluorescence intensity because of their small genomic DNA content (DNA concentration, 15 ng/10 μL, 16 ng/10 μL, and 21 ng/10 μL, respectively). The minimum input of DNA for which *UGT1A1*28* was measured by the Invader assay was 21 ng/10 μL. The genotyping of 57 samples measured by the Invader assay agreed completely with the results obtained by direct DNA sequencing.

Six of the 60 samples could not be measured for *UGT1A1*6* by the Invader assay. These samples showed low fluorescence intensity because of the small content of genomic DNA (content, per 10 μL, of 15, 16, 21, 21, 38, and 46 ng, respectively). The minimum input of DNA for which *UGT1A1*6* was measured by the Invader assay was 29 ng/10 μL. The results for the remaining samples obtained by the Invader assay agreed well with those obtained by the PCR-RFLP assay.

Forty-seven of the 60 samples were genotyped correctly for *UGT1A1*27* by the Invader assay, and the results were consistent with those obtained by the PCR-RFLP assay. Three samples could not be measured by the Invader assay because of their low fluorescence intensity (DNA per 10 μL, 15, 16, and 21 ng, respectively). In addition, 10 samples could not be measured because of an indistinct fluorescence signal ratio (range, 2.861–4.995; median, 4.718; Table 1 in the online Data Supplement). These samples also contained small amounts of genomic DNA (range, 21–59 ng/10 μL; median, 44 ng/10 μL). Thirty-nine samples containing genomic DNA >60 ng/μL were correctly genotyped by the Invader assay. The minimum input of DNA for which *UGT1A1*27* was measured by the Invader assay was 39 ng/10 μL.