Clinical Chemistry 51:5
848–855 (2005)

Proteomics and
Protein Markers

Comparison of Cardiac Troponin I Immunoassays Variably Affected by Circulating Autoantibodies

Susann Eriksson,[1*] Tuomo Ilva,[2] Charlotte Becker,[3] Juha Lund,[2] Pekka Porela,[2] Kari Pulkki,[4] Liisa-Maria Voipio-Pulkki,[5] and Kim Pettersson[1]

Background: **We recently provided evidence that circulating autoantibodies against cardiac troponin I (cTnI) or the troponin complex cause negative interference in cTnI immunoassays. By comparing three cTnI immunoassays, we further explored the phenomenon of circulating autoantibodies and their consequences in patient samples.**

Methods: **We developed a cTnI immunoassay with a novel assay design using three antibodies, two of which bind epitopes outside the stable, central part of cTnI. Samples from 541 chest pain patients were measured with the new cTnI assay and with a first-generation cTnI assay (Innotrac Aio cTnI) using a conventional midfragment assay design. Using another sample cohort, we also compared the new assay with a second-generation cTnI assay (Access AccuTnI).**

Results: **The analytical detection limit of the new cTnI assay was 0.012 μg/L, and the lowest concentration giving a total imprecision (CV) of 10% was 0.060 μg/L. The mean difference (95% limits of agreement) between the new cTnI and Aio cTnI assays was larger in admission samples (21.0%; −107.8% to 149.7%) than in samples taken 6–12 h (12.8%; −61.5% to 87.2%) and 24 h after admission (3.0%; −71.3% to 77.4%; $P < 0.001$). With the lowest concentrations giving 10% CV (0.22 μg/L for Aio cTnI) used as cutoffs, 14.3% (n = 76) of admission samples were positive only with the new assay, whereas 13.5% (n = 72) were positive with both assays. Of samples taken at 6–12 and 24 h, 10.2% (n = 31) and 8.3% (n = 29) were positive only with the new assay. ROC curve analysis of admission samples showed a significantly higher area under the curve for the new cTnI assay (0.940) than for the Aio cTnI assay (0.846; $P < 0.001$). The new cTnI assay gave generally lower results than the AccuTnI assay; the mean (95% limits of agreement) differences were −58.9% (−151.8% to 34.0%) in admission samples. In samples with severe interference from autoantibodies, median ratios between the new assay and AccuTnI were higher than in samples with no apparent troponin autoantibodies (0.875 vs 0.481; $P < 0.001$).**

Conclusions: **The new cTnI assay, which is based on a novel antibody combination different from the conventional midfragment antibody approach, offers improved detection of cTnI in samples containing troponin autoantibodies.**

The first report of the use of cardiac troponin I (cTnI)[6] for diagnosis of myocardial infarction (MI) was published in 1987 by Cummins et al. *(1)*. Since then, both cTnI and cardiac troponin T (cTnT) have been shown to be sensitive and specific biochemical markers of myocardial necrosis. Consequently, scientific committees have advised that the troponins be the preferred biomarkers for detection of myocardial injury *(2, 3)*. Today, more than 10 manufacturers produce cTnI assays, some producing several assays on different platforms *(4, 5)*, whereas only 1 manufacturer produces cTnT assays (Roche Diagnostics).

Despite the widespread use of the troponins as cardiac markers, the molecular nature of cardiac troponins in the bloodstream has not been studied extensively or systematically. It is known that cTnI is susceptible to various modifications, either in the myocardium before release or in the bloodstream after release. These modifications include proteolysis *(6–8)*, phosphorylation *(8, 9)*, and

Departments of [1] Biotechnology and [2] Medicine, University of Turku, Turku, Finland.

[3] Department of Laboratory Medicine, Division of Clinical Chemistry, Malmö University Hospital, Malmö, Sweden.

Departments of [4] Laboratory Diagnostics and [5] Medicine, Helsinki University Central Hospital, Helsinki, Finland.

*Address correspondence to this author at: Department of Biotechnology, University of Turku, Tykistökatu 6A, FIN-20520 Turku, Finland. Fax 358-2-333-8050; e-mail susann.eriksson@utu.fi.

Received July 13, 2004; accepted February 1, 2005.

Previously published online at DOI: 10.1373/clinchem.2004.040089

[6] Nonstandard abbreviations: cTnI and cTnT, cardiac troponin I and T, respectively; MI, myocardial infarction; TnC, troponin C; CI, confidence interval; and AUC, area under the curve.

oxidation *(10, 11)*. The main part of cTnI is found in complex with the other troponins, mainly troponin C (TnC), but also with cTnT *(11–13)*. For optimal performance, cTnI assays should be capable of detecting all circulating cTnI forms and at the same time be analytically very sensitive.

We have previously reported the existence of an interfering factor that can give false-negative results if antibodies against certain midfragment epitopes are used in two-site immunoassays *(14, 15)*. The existence of this interfering component and the clinical importance of its negative effect on cTnI measurements have been questioned *(16)*. We have now provided evidence that the observed interference is attributable to circulating autoantibodies against cTnI or the troponin complex *(17)*. These autoantibodies seem to principally block the binding of monoclonal or polyclonal anti-cTnI antibodies to amino acid residues 41–91 *(15)*, which constitute a substantial part of the stable midfragment region (amino acid residues 30–110) that has been recommended as the target for cTnI assays *(18)*. On the basis of our previous results, we developed a new cTnI assay that uses antibodies that are unaffected or affected to a lesser extent by the presence of troponin autoantibodies. Because we did not find it feasible to construct such an assay using only antibodies toward the most stable central region of cTnI, we were obliged to also select antibodies outside this region.

In this report, we describe the analytical characteristics of the new assay and compare its clinical performance with a first-generation cTnI assay (Innotrac Aio) based on a conventional design with antibodies against two midfragment epitopes. For this analysis, we assayed samples from 541 consecutive patients presenting with chest pain. Using a separate sample cohort, we also compared the new assay with a second-generation cTnI assay (Access AccuTnI; Beckman Coulter).

Materials and Methods

ASSAY DEVELOPMENT

Immunoassay design. The new cTnI assay was designed to be minimally affected by the presence of autoantibodies to troponin *(14, 15)*. Central antibody selection criteria were a low influence of troponin autoantibodies, retained detection of the major cTnI degradation products, a low analytical detection limit, and preferably, only one detection antibody. Two cTnI antibodies (HyTest Ltd.) were used for capture: one detects an epitope at amino acids 41–49 and the other an epitope after amino acid residue 110 in the C-terminal region. The detection antibody (Spectral Diagnostics Inc.) recognizes an epitope after amino acid 110 in the C-terminal region of cTnI. All antibodies are specific for cTnI, with no cross-reactivity with cTnT or TnC. The assay is a noncompetitive, one-step immunofluorometric sandwich assay based on the all-in-one dry-chemistry concept *(19)*.

Labeling of antibodies. The intrinsically fluorescent europium chelate, {2,2′,2″,2‴-{[2-(4-isothiocyanatophenyl)ethylimino]bis(methylene)bis{4-{[4-(α-glucopyranoxy)phenyl] ethynyl}pyridine-6,2-diyl}bis(methylenenitrilo)}tetrakis (acetato)}europium(III) *(20)*, used for labeling of the detection antibody, was kindly provided by Jaana Rosenberg (Department of Biotechnology, University of Turku, Turku, Finland). The antibody was labeled overnight (16–20 h) at room temperature with a 35-fold molar excess of chelate in 50 mmol/L sodium carbonate buffer (pH 9.8) containing 250 mL/L ethylene glycol. Purification was performed as described previously *(14)*.

Capture antibodies were biotinylated as described previously *(14)*, except that a 10-fold molar excess of biotin-isothiocyanate was used.

Preparation of dry reagent wells. Biotinylated capture antibodies [150 ng each of two different antibodies in 50 μL of coating buffer *(20)*] were attached to streptavidin-coated single microtiter wells (Innotrac Diagnostics Oy) by incubation overnight at room temperature without shaking. The wells were washed, and 40 μL of a protective solution *(21)* was added to the aspirated wells. The wells were dried overnight in a dry-condition cabinet at 35 °C with 5% relative air humidity. The labeled detection antibody (50 ng) was added on top of the protective layer in a 1-μL drop of an optimized buffer *(20)* and immediately dried with warm air. The dry reagent wells were stored at 4 °C in sealed containers with desiccant until use.

Immunoassay protocol. The cTnI immunoassay was performed on the fully automated Innotrac Aio Immunoanalyzer (Innotrac Diagnostics Oy). The assay started with the addition of 20 μL of sample and 10 μL of a combined assay/wash buffer *(21)* to the dry reagent well. The well was incubated for 15 min with slow shaking at 36 °C, washed six times with assay/wash buffer, and dried with hot air. The time-resolved fluorescence from the europium chelate was measured by the Aio Immunoanalyzer directly from the solid phase, using the default measurement settings.

ANALYTICAL AND CLINICAL CHARACTERIZATION

Calibration curve and detection limit. Human cardiac troponin complex (native, tissue-derived cTnI-cTnT-TnC complex) from HyTest Ltd., prepared as described previously *(14)*, was used for assay calibration. The mean counts from all measurements were used to construct a master calibration curve, from which the cTnI concentrations of patient samples were calculated. The mean (SD) background was calculated from the counts of calibrator buffer without added cTnI measured twice a day for 29 days ($n = 58$) on a single instrument.

Recognition of different forms of cTnI. A diversity reagent set containing four pairs of antigen preparations (free cTnI/binary cTnI-TnC complex, native/phosphorylated cTnI in

ternary complex, native/proteolytically degraded ternary troponin complex, cTnI in normal human serum/in the presence of 10 IU/mL heparin) was kindly provided by HyTest Ltd. (cat. no. K01) and used for testing the reactivity of the assay against different cTnI forms.

Analyte stability. Seven matched EDTA- and heparin-plasma samples containing various concentrations of endogenous cTnI (cTnI range, 0.056–6.222 μg/L) were measured directly with the new cTnI assay or after storage for 24 h at room temperature or 4 °C.

Reference samples. We determined the upper reference limit by testing 144 serum samples from patients without cardiac-related symptoms from Turku University Central Hospital *(14)*. We also measured multiple matched samples from 20 healthy volunteers from our personnel (serum, heparin-anticoagulated plasma and whole blood, and EDTA-anticoagulated plasma and whole blood). Three serum pools were prepared by pooling serum samples from MI patients *(14)*. The pools were divided into aliquots and stored frozen at −20 °C until use. The serum pools were analyzed daily, and the results were used for calculation of between-run imprecision.

Patient samples. We obtained samples from 541 consecutive patients who presented to Turku University Central Hospital for evaluation of suspected MI between May 2000 and July 2001. Of these patients, 175 were discharged and 366 were hospitalized after emergency room evaluation. On the basis of the criteria of the European Society of Cardiology/American College of Cardiology Joint Committee consensus document *(2)*, 199 patients were diagnosed with MI. The patients were treated according to the routine clinical protocols of the hospital. The study protocol was in accordance with the Helsinki Declaration of 1975 as revised in 1996 and was accepted by the Ethics Committee of Turku University Central Hospital. All participants gave written informed consent to participate in the study. EDTA-plasma samples were taken at the time of presentation from all patients and 6–12 and 24 h later from patients admitted to hospital. The samples were frozen immediately and stored at −70 °C until analysis by the new cTnI assay and the first-generation Innotrac Aio cTnI assay at the same time.

Lithium-heparin-plasma samples from a separate set of 52 MI patients were collected at the Department of Laboratory Medicine, Malmö University Hospital (Malmö, Sweden). The admission samples and samples taken 6 h after admission were immediately assayed by the Access AccuTnI (Beckman Coulter), and then were frozen within 8 h and stored at −20/−70 °C until analysis by the new cTnI assay. The samples were collected as part of the routine sampling procedure at Malmö University Hospital, and all identifying labels were removed at the hospital to retain the anonymity of the patients.

OTHER IMMUNOASSAYS

The first-generation Innotrac Aio cTnI assay (Innotrac Diagnostics Oy) is a rapid one-step immunofluorometric sandwich assay *(22)* that uses antibodies against the stable midfragment region of cTnI (with epitopes at amino acid residues 41–49 and 87–91). The assay is calibrated against the human ternary cardiac troponin complex (HyTest Ltd.). This assay, which is no longer commercially available, has been replaced by a more sensitive second-generation Aio cTnI assay *(23)*, which in essence is identical to the investigational assay described in this report. The Innotrac Aio Immunoanalyzer was used to analyze the EDTA-plasma samples according to the manufacturer's instructions.

The second-generation Access AccuTnI assay (Beckman Coulter) uses two monoclonal antibodies directed to amino acid residues 24–40 and 41–49 *(24)*. Heparin-plasma samples were assayed according to the manufacturer's instructions.

RECOVERY OF cTnI

We evaluated the effect of troponin autoantibodies on the new cTnI assay compared with the Aio cTnI and Access AccuTnI assays by measuring the recovery of cTnI. Ternary troponin complex corresponding to 30 μg/L cTnI (final concentration) was added to three serum pools that were prepared by pooling samples with known low (<10%), medium (~50%), or normal (~100%) recovery of cTnI as determined previously by the Mab 1/Mab 2 assay *(14)*. The pools were also measured without addition of troponin complex (only the same amount of buffer was added), and this concentration was subtracted from the concentration in the pool with added troponin to correct for any original cTnI. The recovery of cTnI in the pool with normal recovery was assumed to be 100%, and the recoveries in the low and medium recovery pools were compared with this value. All measurements were performed in duplicate.

DETECTION OF TROPONIN AUTOANTIBODIES

We screened the admission samples from the set of 52 MI patients for the presence of troponin autoantibodies by measuring the recovery of cTnI with a capture antibody binding to amino acid residues 41–49 and a detection antibody binding to amino acid residues 87–91. The recovery was measured as described previously *(14)* except that ternary troponin complex corresponding to a final cTnI concentration of 3 μg/L was added to the samples. Low recovery of cTnI with this antibody pair indicates the presence of troponin autoantibodies.

STATISTICAL ANALYSIS

Statistical analyses were performed with the statistical software SPSS for Windows, Ver. 11.0 (SPSS Inc.), except for ROC curve analysis and Bland–Altman difference calculations, which were performed with GraphPad Prism for Windows, Ver. 4.02 (GraphPad Software). The

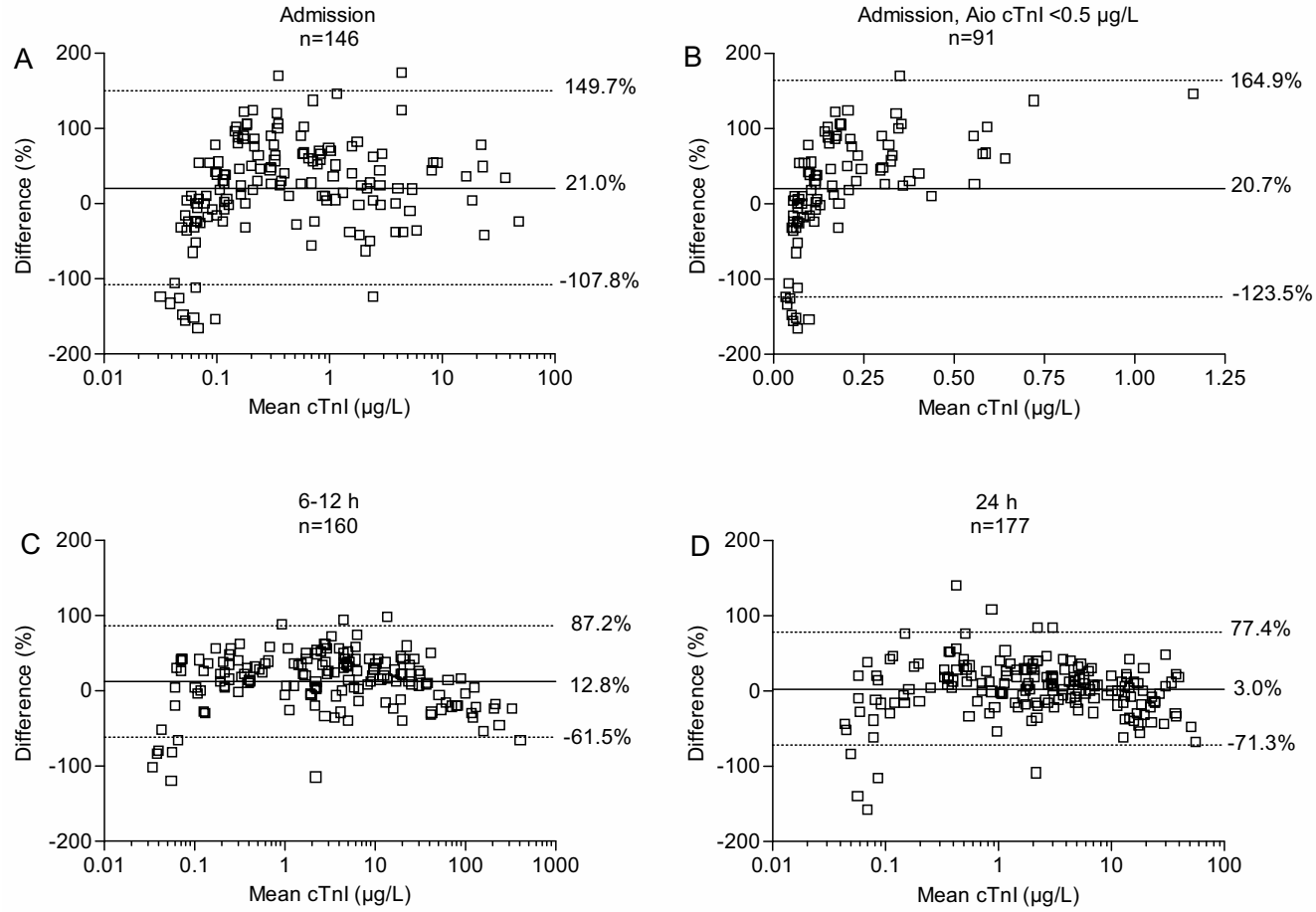

Fig. 1. Difference plots comparing the new cTnI and Aio cTnI assays in samples taken at admission and 6–12 or 24 h after admission with detectable concentrations in both assays.

The *x axis* shows the mean of the two assays, and the *y axis* shows the percentage difference [100 × (new cTnI assay − Aio cTnI)/mean of the two assays]. *Solid line*, mean difference; *dotted lines*, upper and lower 95% limits of agreement.

difference between methods was evaluated by the Bland–Altman method *(25)*, as modified by Pollock et al. *(26)*. The MultiCalc™ software (PerkinElmer Life and Analytical Sciences, Wallac Oy) was used for calculation of imprecision profiles.

Results

CALIBRATION CURVE AND DETECTION LIMITS

The master calibration curve for the new cTnI assay was constructed from the mean counts of calibrators measured during this study (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue5/). The calibration curve was linear up to 100 μg/L, and we observed no high-dose hook effect with concentrations up to 1000 μg/L. The analytical detection limit, calculated as the mean background signal + 2 SD, was 0.012 μg/L.

The detection limits of the first-generation Innotrac Aio cTnI assay and Access AccuTnI were reported by the manufacturers to be 0.05 and ≤0.01 μg/L, respectively. Thus, the detection limit of the new assay was lower than that of the Aio cTnI assay and was comparable to that of the AccuTnI assay.

IMPRECISION

We determined the precision of the cTnI assay with seven calibrators and three serum pools. The between-run CVs for calibrators (n = 20) were 6.6–11% (Fig. 1 in the online Data Supplement). For serum pools run in duplicate on 20 separate days, the between-run CVs were 5.0–10% (mean cTnI concentrations of 5.391, 0.970, and 0.073 μg/L). We calculated the imprecision profile based on 12 replicates of each calibrator and duplicates of consecutively measured samples (n >400). For concentrations ≥0.06 μg/L, the total imprecision (CV) was ≤10% (Fig. 2 in the online Data Supplement), which places the new assay approximately in the range of the Access AccuTnI assay *(24)*.

RECOGNITION OF DIFFERENT FORMS OF cTnI

The assay signal for cTnI in binary complex with TnC was 1.62-fold higher than that for free cTnI. In vitro-phosphorylated cTnI gave signals 1.22-fold higher than native cTnI.

The signal for cTnI extensively cleaved by proteases was 9.46% of the signal for intact cTnI. The addition of heparin (10 IU/mL) increased the signal 1.24-fold compared with sample without heparin.

ANALYTE STABILITY

The mean (SD) concentration measured in EDTA-plasma samples (n = 7) after incubation for 24 h at room temperature was 83 (15)% of the original concentration and after 24 h at 4 °C was 91 (12)% of the original concentration. In heparin-plasma samples (n = 7), the corresponding values were 93 (9)% and 100 (9)%. The mean concentration was significantly different from the original concentration in EDTA-plasma samples incubated at room temperature ($P = 0.020$, one-sample *t*-test), but not for the other samples ($P = 0.093$, 0.083, and 0.646, respectively).

UPPER REFERENCE LIMIT AND SAMPLE TYPE

The upper reference limit, defined as the 99th percentile value, was 0.015 μg/L. In the 20 matched samples from healthy individuals, the mean concentrations in all sample types (serum, EDTA plasma and whole blood, and heparin plasma and whole blood) were below the detection limit of the assay.

RECOVERY OF cTnI

The new cTnI assay had considerably improved recovery of cTnI in the low recovery pool (28.1%) compared with both the Aio cTnI (0.2%) and Access AccuTnI (5.7%) assays. The recoveries in the medium recovery pool were 108.0% (new cTnI assay), 73.0% (Aio cTnI), and 87.9% (Access AccuTnI).

COMPARISON WITH Aio cTnI ASSAY

Comparisons of the new assay with the Innotrac Aio cTnI assay are shown in Fig. 1. The difference between the assays was larger ($P < 0.001$, one-way ANOVA post test for linear trend), and the 95% limits of agreement were wider in admission samples (mean difference, 21.0%; 95% limits of agreement, −107.8% to 149.7%) than in samples taken 6–12 h [12.8% (−61.5% to 87.2%)] and 24 h after admission [3.0% (−71.3% to 77.4%)].

We then used the lowest concentrations corresponding to a 10% CV (0.06 μg/L for the new cTnI assay and 0.22 μg/L for the Aio cTnI assay) as cutoff values. The new assay was positive in considerably more samples than the Aio cTnI assay at all time points (Table 1A). On the other hand, none of the samples positive in the Aio cTnI assay was negative in the new cTnI assay with these criteria (Table 1A).

To investigate whether the increased number of positive samples with the new cTnI assay was a result only of the lower detection limit, we used a cutoff of 0.12 μg/L, at which the Aio cTnI assay gave CVs of 12–13%, for both assays. The concordance and discordance between the new cTnI assay and Aio cTnI assay at the same cutoff is shown in Table 1B. Of the 27 patients with admission samples >0.12 μg/L with the new assay, 20 patients had at least one follow-up sample that was >0.22 μg/L with the Aio cTnI assay and thus were classified as positive in later samples if the 10% CV cutoff was used.

Table 1. Concordance/discordance between cTnI assays at different cutoff values.

A. Concordance with lowest concentrations with 10% CV in respective cTnI assays used as cutoff values

		New cTnI assay	
Samples	**Aio cTnI**	**<0.06 μg/L**	**≥0.06 μg/L**
Admission samples	<0.22 μg/L	385 (72.2%)	76 (14.3%)
(n = 533)	≥0.22 μg/L	0	72 (13.5%)
6–12 h samples	<0.22 μg/L	144 (47.5%)	31 (10.2%)
(n = 303)	≥0.22 μg/L	0	128 (42.2%)
24 h samples	<0.22 μg/L	172 (49.1%)	29 (8.3%)
(n = 350)	≥0.22 μg/L	0	149 (42.6%)

B. Concordance with cutoff value of 0.12 μg/L for both assays

		New cTnI assay	
Samples	**Aio cTnI**	**<0.12 μg/L**	**≥0.12 μg/L**
Admission samples	<0.12 μg/L	412 (77.3%)	27 (5.1%)
(n = 533)	≥0.12 μg/L	4 (0.7%)	90 (16.9%)
6–12 h samples	<0.12 μg/L	155 (51.2%)	6 (2.0%)
(n = 303)	≥0.12 μg/L	3 (1.0%)	139 (45.9%)
24 h samples	<0.12 μg/L	184 (52.6%)	5 (1.4%)
(n = 350)	≥0.12 μg/L	5 (1.4%)	156 (44.6%)

The cTnI concentrations measured by the new assay in samples that gave results below the detection limit (0.05 μg/L) of the Aio cTnI assay are shown in Fig. 2. The number of positive (above the 10% CV concentration of the new assay) samples decreased from 22 samples at admission to 6 samples at 6–12 h and 9 samples at 24 h after admission, respectively. On the other hand, none of

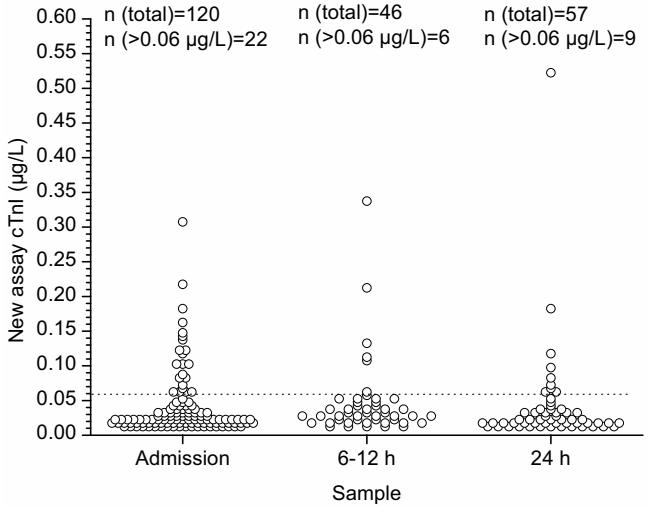

Fig. 2. cTnI concentrations measured by the new assay in samples that were below the detection limit of the Aio cTnI assay (0.05 μg/L). The *horizontal line* indicates the concentration giving a total CV of 10%.

the 28 samples with detectable cTnI by the Aio cTnI assay but below the detection limit of the new assay was above the 10% CV concentration of 0.22 μg/L of the Aio cTnI assay at any time point.

We evaluated the diagnostic accuracy of the cTnI assays by performing ROC curve analysis. For admission samples, the areas under the curves (AUC) were 0.940 [95% confidence interval (CI), 0.916–0.964] for the new cTnI assay and 0.846 (95% CI, 0.808–0.884) for the Aio cTnI assay. For 6–12 h specimens, the AUC (95% CI) were 0.985 (0.968–1.001) and 0.969 (0.951–0.988), and for 24 h specimens, the AUC were 0.991 (0.983–1.000) and 0.976 (0.961–0.991) for the new and Aio cTnI assays, respectively. The AUC was significantly larger for the new cTnI assay than for the Aio cTnI assay in admission samples ($P < 0.001$).

We examined cTnI concentrations in samples from individual MI patients to illustrate the presumed variable effects of troponin autoantibodies on cTnI measurements and found three main types of MI cases (Table 2). The presumed presence of autoantibodies negatively affected the Aio cTnI assay in samples from patients 1, 2, and 3, giving cTnI concentrations below the 10% CV concentration in the admission samples. The admission samples were all positive with the new cTnI assay, and the later samples were also positive with the Aio cTnI assay. The admission sample from patient 4 gave a 15-fold higher measured concentration with the new cTnI assay than with the Aio cTnI assay, although all concentrations were above the Aio cTnI cutoff. Finally, the samples from patients 5 and 6 showed good agreement between the two assays, illustrating the conformity between assays in the absence of autoantibodies.

Table 2. cTnI concentrations in samples from MI patients showing the discrepancy or concordance between the new cTnI assay and the Aio cTnI assay.

		cTnI assay result,[a] μg/L	
Patient	Time after admission, h	New	Aio
1	0	**0.306**	<0.05
	6–12	**0.335**	<0.05
	24	**1.337**	**0.397**
2	0	**0.182**	<0.05
	6–12	**1.369**	**0.768**
	24	**0.477**	**0.277**
3	0	**0.334**	0.078
	6–12	**20.43**	**6.915**
	24	**37.78**	**22.93**
4	0	**8.263**	**0.546**
	6–12	**179.5**	**228.2**
	24	**41.86**	**33.65**
5	0	0.047	<0.05
	6–12	**0.434**	**0.377**
	24	**1.601**	**1.706**
6	0	<0.012	0.056
	6–12	**0.972**	**1.256**
	24	**0.458**	**0.641**

[a] Values in bold indicate cTnI results above the cutoff concentrations (total CV ≤10%), which were 0.06 μg/L for the new assay and 0.22 μg/L for the Aio cTnI assay.

comparison with Access AccuTnI

Using a separate set of samples obtained early in the course of MI, we compared the new assay with the Access AccuTnI assay. For the new assay, the mean (95% limits of agreement) difference was −58.9% (−151.8% to 34.0%) in admission samples and −71.6% (−132.3% to −10.8%) in samples taken 6 h after admission (Fig. 3 in the online Data Supplement; only samples with cTnI concentrations detectable by both assays were included).

The mean (median) cTnI recovery at 3.0 μg/L was 61.7 (65.0)% in the 52 admission samples with an assay that is affected by the presence of autoantibodies *(14)*. Five patients had analytical recoveries of cTnI <40% in admission samples (see Fig. 3 in the online Data Supplement). The patients were then grouped according to the recovery of cTnI in the admission sample (recovery <40% or >40%), and the concentration ratios of the new assay to Access AccuTnI were calculated for all samples (Fig. 4 in the online Data Supplement), with the exclusion of 15 admission samples and 4 follow-up samples (recovery >40%) with cTnI concentrations below the detection limit of the new assay. The median (mean) ratio was 0.875 (1.038) in samples with recoveries <40% (n = 10) and 0.481 (0.477) in samples with recoveries >40% (n = 75; $P < 0.001$, Mann–Whitney *U*-test). The 25th and 75th percentile ratios were 0.690 and 1.402 for samples with recoveries <40% and 0.350 and 0.549 for samples with recoveries >40%. Consequently, there are some differences in the detection of cTnI between the new cTnI assay and Access AccuTnI in individual samples containing troponin autoantibodies.

Discussion

We evaluated the performance of a new investigational cTnI assay designed to circumvent the inhibiting effect of circulating troponin autoantibodies in serum and plasma. Because the interference can be related to the use of antibodies against the midfragment region of the cTnI molecule *(15)*, the novel assay uses antibodies both within and outside the stable midfragment portion of cTnI. We assayed a large panel of samples from patients with chest pain and suspected MI with the new assay and with a first-generation cTnI assay that uses antibodies against amino acid residues 41–49 and 87–91, the latter of which is severely affected by troponin autoantibodies *(14, 15)*. We used a separate panel of samples from patients in the early phases of MI for a preliminary evaluation of the new assay in comparison with a sensitive second-generation cTnI assay, Access AccuTnI, which uses antibodies against amino acid residues 24–40 and 41–49 *(24)*.

The detection limit of the new assay was approximately fivefold lower than the detection limit of the

Innotrac Aio cTnI assay, which is based on the same all-in-one dry-chemistry concept *(19)* as the new cTnI assay. The lower detection limit was mainly a result of changes in antibody selection, because both assays were calibrated against the same cardiac troponin complex preparation. The lowest cTnI concentration that gave a total CV of 10% was 0.06 μg/L, which is the same as has been reported for the Access AccuTnI assay *(24)*. The 99th percentile reference limit was 0.015 μg/L, meaning that the requirement of a CV of 10% at the 99th percentile of a reference population *(2)* was still not achieved. Only a few commercial cTnI assays have been reported to fulfill this requirement *(27, 28)*.

The selection of antibodies against epitopes outside the stable, central part of cTnI is a novel approach to improve the sensitivity and performance of cTnI assays by avoiding the inhibiting effect of troponin autoantibodies. Because the influence of the saturable autoantibodies is greatest when the amount of cTnI is low, the largest discrepancy between assay results is expected to occur early after myocardial injury. ROC curve analysis showed a higher AUC for the new cTnI assay than for the Aio cTnI assay in admission samples, whereas the difference in AUC values decreased in later samples. Other examples of this are shown in Table 2 for patients 1–4, whose admission samples gave very discrepant results with the two assays. Difference analysis showed that the mean difference between the new cTnI and Aio cTnI assays was largest in the admission samples and decreased in later samples.

The concentrations at which a 10% CV could be achieved differed substantially between the new assay and the Aio cTnI as a result of the poorer analytical performance of the Aio cTnI assay. Thus, although ROC curve analysis showed the improved clinical performance of the new assay in admission samples, it could have mainly been a result of its improved functional sensitivity. For this reason, for both assays we also classified samples as positive or negative based on the same cutoff concentration of 0.12 μg/L, at which the imprecision of the Aio cTnI was still quite acceptable; we found that the new assay detected more admission samples with concentrations above this cutoff than the Aio cTnI assay (Table 1B). Thus, the main advantage of the new cTnI assay is gained in early samples.

In the Access AccuTnI assay, the selected antibodies react with epitopes between amino acid residues 24 and 49; consequently, on the basis of our previous results *(15)*, the negative effect of troponin autoantibodies would be expected to be less pronounced than for the Aio cTnI assay. As we have pointed out previously *(14)*, the negative interference of autoantibodies can be reduced if the cTnI assay is analytically very sensitive. The antibody selection and the functional sensitivity of the Access AccuTnI may make the assay less prone to interference by autoantibodies, a possibility that deserves further study in larger patient cohorts. However, many of the samples with discrepant results in the new cTnI assay and the AccuTnI were from patients whose samples had given falsely low cTnI values in an assay known to be affected by troponin autoantibodies. These results, together with the results from the low recovery pool, indicate that our new cTnI assay and the AccuTnI assay detect cTnI differently as a result of the differences in antibody selection.

One current recommendation is that antibodies with epitopes in the midfragment region of cTnI be used because that region is more stable to proteolytic degradation than the N- and C-terminal regions *(6)*. Two of the antibodies used in our new cTnI assay are not directed toward the stable region, meaning that samples with extensive cTnI fragmentation may not be fully detected. This was observed with the in vitro proteolytically cleaved troponin complex, of which only ~9.5% was detected by the new assay. However, endogenous cTnI in patient samples was found to be considerably more stable, with >80% detected after storage for 24 h at room temperature. Therefore, cTnI extensively cleaved in vitro is obviously not similar to cTnI in patient samples. Furthermore, cTnI degradation did not seem to be a problem for the new assay in the samples of the large panel, which were measured at the same time with both the new and Aio cTnI assays: no samples gave results that were above the 10% CV concentration of 0.22 μg/L in the Aio cTnI assay and <0.06 μg/L in the new assay.

In conclusion, our study provides further insight into the significance of the variable impact of troponin autoantibodies on the clinical performance of three differently designed immunoassays. To thoroughly evaluate the clinical importance of avoiding the effects of troponin autoantibodies, the new cTnI assay should be investigated in a prospective study with particular attention paid to its improved clinical sensitivity as an early marker of cardiac injury.

This study was supported in part by the National Technology Agency in Finland (TEKES) and by grants from the Paulo Foundation and the Aarne and Aili Turunen Foundation. We thank Miia Junikka, Pirjo Laaksonen, Heidi Halenius, Katri Lehtonen, Tiina Kokko, Pia Niemi, and Karolina Karlson for excellent technical assistance. We gratefully acknowledge HyTest Ltd. and Innotrac Diagnostics for supplying immunoreagents and instrumentation, respectively.

References

1. Cummins B, Auckland ML, Cummins P. Cardiac-specific troponin-I radioimmunoassay in the diagnosis of acute myocardial infarction. Am Heart J 1987;113:1333–44.
2. Alpert JS, Thygesen K, Antman E, Bassand JP. Myocardial infarction redefined—a consensus document of the Joint European Society of Cardiology/American College of Cardiology Committee

for the redefinition of myocardial infarction. J Am Coll Cardiol 2000;36:959–69.
3. Wu AH, Apple FS, Gibler WB, Jesse RL, Warshaw MM, Valdes R Jr. National Academy of Clinical Biochemistry Standards of Laboratory Practice: recommendations for the use of cardiac markers in coronary artery diseases. Clin Chem 1999;45:1104–21.
4. Collinson PO, Boa FG, Gaze DC. Measurement of cardiac troponins [Review]. Ann Clin Biochem 2001;38:423–49.
5. Panteghini M, Pagani F, Yeo KT, Apple FS, Christenson RH, Dati F, et al. Evaluation of imprecision for cardiac troponin assays at low-range concentrations. Clin Chem 2004;50:327–32.
6. Katrukha AG, Bereznikova AV, Filatov VL, Esakova TV, Kolosova OV, Pettersson K, et al. Degradation of cardiac troponin I: implication for reliable immunodetection. Clin Chem 1998;44:2433–40.
7. Morjana NA. Degradation of human cardiac troponin I after myocardial infarction. Biotechnol Appl Biochem 1998;28:105–11.
8. Labugger R, Organ L, Collier C, Atar D, Van Eyk JE. Extensive troponin I and T modification detected in serum from patients with acute myocardial infarction. Circulation 2000;102:1221–6.
9. Bereznikova A, Katrukha A, Filatov V, Pettersson K, Esakova T, Bulargina T, et al. Monoclonal antibodies affected by cardiac troponin I phosphorylation: is part of troponin in the blood of myocardial infarction patients phosphorylated? [Abstract]. Clin Chem Lab Med 1999;37:S448.
10. Ingraham RH, Hodges RS. Effects of Ca^{2+} and subunit interactions on surface accessibility of cysteine residues in cardiac troponin. Biochemistry 1988;27:5891–8.
11. Wu AHB, Feng Y-J, Moore R, Apple FS, McPherson PH, Buechler KF, et al. Characterization of cardiac troponin subunit release into serum after acute myocardial infarction and comparison of assays for troponin T and I. Clin Chem 1998;44:1198–208.
12. Katrukha AG, Bereznikova AV, Esakova TV, Pettersson K, Lövgren T, Severina ME, et al. Troponin I is released in bloodstream of patients with acute myocardial infarction not in free form but as complex. Clin Chem 1997;43:1379–85.
13. Giuliani I, Bertinchant JP, Granier C, Laprade M, Chocron S, Toubin G, et al. Determination of cardiac troponin I forms in the blood of patients with acute myocardial infarction and patients receiving crystalloid or cold blood cardioplegia. Clin Chem 1999;45:213–22.
14. Eriksson S, Junikka M, Laitinen P, Majamaa-Voltti K, Alfthan H, Pettersson K. Negative interference in cardiac troponin I immunoassays from a frequently occurring serum and plasma component. Clin Chem 2003;49:1095–104.
15. Eriksson S, Junikka M, Pettersson K. An interfering component in cardiac troponin I immunoassays—its nature and inhibiting effect on the binding of antibodies against different epitopes. Clin Biochem 2004;37:472–80.
16. Panteghini M. The interfering component in cardiac troponin I immunoassays: need for further experimental evidence [Letter]. Clin Chem 2004;50:676–7.
17. Eriksson S, Halenius H, Pulkki K, Hellman J, Pettersson K. Negative interference in cardiac troponin I immunoassays by circulating troponin autoantibodies. Clin Chem 2005;51:839–47.
18. Panteghini M, Gerhardt W, Apple FS, Dati F, Ravkilde J, Wu AH. Quality specifications for cardiac troponin assays. Clin Chem Lab Med 2001;39:175–9.
19. Lövgren T, Meriö L, Mitrunen K, Mäkinen ML, Mäkelä M, Blomberg K, et al. One-step all-in-one dry reagent immunoassays with fluorescent europium chelate label and time-resolved fluorometry. Clin Chem 1996;42:1196–201.
20. von Lode P, Rosenberg J, Pettersson K, Takalo H. A europium chelate for quantitative point-of-care immunoassays using direct surface measurement. Anal Chem 2003;75:3193–201.
21. Pettersson K, von Lode P, Eriksson S, Lövgren J, Takalo H. Multi-assay point-of-care platform: highly sensitive time-resolved fluorometric detection in combination with a universal "all-in-one" assay format. Point of Care 2003;2:225–32.
22. Pettersson K, Katajamäki T, Irjala K, Leppänen V, Majamaa-Voltti K, Laitinen P. Time-resolved fluorometry (TRF)-based immunoassay concept for rapid and quantitative determination of biochemical myocardial infarction markers from whole blood, serum and plasma. Luminescence 2000;15:399–407.
23. Pagani F, Stefini F, Panteghini M. Innotrac Aio! Second-generation cardiac troponin I assay: imprecision profile and other key characteristics for clinical use. Clin Chem 2004;50:1271–2.
24. Uettwiller-Geiger D, Wu AH, Apple FS, Jevans AW, Venge P, Olson MD, et al. Multicenter evaluation of an automated assay for troponin I. Clin Chem 2002;48:869–76.
25. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986;i:307–10.
26. Pollock MA, Jefferson SG, Kane JW, Lomax K, MacKinnon G, Winnard CB. Method comparison—a different approach. Ann Clin Biochem 1992;29:556–60.
27. Apple FS, Quist HE, Doyle PJ, Otto AP, Murakami MM. 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. Clin Chem 2003;49:1331–6.
28. Venge P, Johnston N, Lagerqvist B, Wallentin L, Lindahl B. Clinical and analytical performance of the Liaison cardiac troponin I assay in unstable coronary artery disease, and the impact of age on the definition of reference limits. A FRISC-II substudy. Clin Chem 2003;49:880–6.