Tanja Savukoski*, Jenna Jacobino, Päivi Laitinen, Bertil Lindahl, Per Venge, Noora Ristiniemi, Saara Wittfooth and Kim Pettersson

Novel sensitive cardiac troponin I immunoassay free from troponin I-specific autoantibody interference[1)]

Abstract

Background: Cardiac troponins (cTnI and cTnT) are the recommended biomarkers of myocardial infarction. As cTn-specific autoantibodies (cTnAAb) can interfere with the cTn detection by state-of-the-art cTnI assays, our objective was to develop a sensitive cTnI immunoassay free from this analytical interference.

Methods: The assay used antibody-coated spots containing three capture Mabs/Fabs directed against the N-terminus, midfragment and C-terminus of cTnI and a europium chelate-labeled tracer Mab against the C-terminus. Following a 3-h sample incubation and washing, cTnI was quantified by time-resolved fluorometry.

Results: The limit of detection (LoD) was 2.9 ng/L and the assay was linear up to 50,000 ng/L. The total precision of 10% CV was not reached, but 20% CV was reached at 10 ng/L. Mean cTnI (10–50,000 ng/L) recoveries were 100% and 119% in three cTnAAb-positive and two cTnAAb-negative individuals, respectively, verifying the interference resistance of the antibody design used. On average, Architect hs-cTnI assay gave seven-fold higher cTnI concentrations than the new assay but the correlation between the assays was good ($r=0.958$). Of apparently healthy individuals ($n=159$), 18% had measurable cTnI values (>LoD) and 10% were cTnAAb-positive. The proportion of measurable cTnI values, however, was significantly higher in cTnAAb-positive individuals (13/16, median cTnI 8.5 ng/L) than in cTnAAb-negative individuals (15/143, median cTnI <LoD) ($p<0.001$).

Conclusions: Although the developed sensitive cTnI assay without cTnAAb interference takes too long for diagnostic purposes, it could serve as an important analytical tool for exploring the impact of cTnAAbs for cTn testing and for unraveling the etiology behind cTn-related autoimmune responses.

Keywords: antibody-coated spot; autoantibody; cardiac troponin I; immunoassay; interference; myocardial infarction.

[1)] Part of the results has been presented as a poster at the 20th IFCC-EFLM European Congress of Clinical Chemistry and Laboratory Medicine in Milan, 2013.

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Introduction

Cardiac troponin I and T (cTnI and cTnT) are components of the myofibril contractile apparatus of the heart muscle. Due to their unique cardiac specificity, the Global Task Force recommends the determination of circulating cTns along with the evaluation of patient symptoms and electrocardiographic abnormalities to be used for the diagnosis of myocardial infarction (MI) [1]. Therefore, they have a central role in clinical assessment of acute coronary syndrome (ACS).

To enable earlier ACS diagnosis and improved patient outcomes, a new generation of high-sensitivity (hs) cTn assays that can measure cTn in at least 50% of healthy individuals has been introduced. These assays allow a more accurate determination of the 99th percentile reference value with high precision (CV ≤ 10%). Hs assays are commercially available for cTnI and cTnT, and their superior performance in rapid patient triage and long-term risk prediction has been demonstrated [2–4]. Due to the use of hs assays in clinical decision-making increases, it is important that their low-end accuracy is ascertained by avoiding preanalytical and analytical problems leading to false-positive and false-negative cTn results.

Our previous epitope specificity studies [5] demonstrated that the midfragment (amino acids, aa, 30–110) of

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cTnI, which is the IFCC-recommended target for antibodies to be used in cTnI assays [6], is also most frequently targeted by cardiac troponin-specific autoantibodies (cTnAAb). As cTnAAbs that are present in approximately one in 10 chest pain patients can negatively interfere with the cTn detection by state-of-the-art cTnI assays [7–10], their effects at lower concentrations presently reached by hs assays need to be studied.

We have previously discovered that cTnAAb interference can be decidedly diminished by choosing assay antibodies deviating from the IFCC-recommendation [9]. The interference was actually avoided by using a 3+1-type antibody configuration with three capture antibodies against the N-terminus, midfragment and C-terminus (epitopes at aa 23–29, 41–49 and 190–196) and one detector antibody against the C-terminus (epitope at aa 169–178). Due to the insufficient analytical sensitivity of the assay which limited the previous cTnAAb interference studies to a relatively high cTn concentration range, the aim of this study was to develop a sensitive cTnI assay based on this assay configuration.

The analytical sensitivity of the 3+1-type immunoassay was here improved by using antibody-coated spots. Conventional surface coating provides a capturing area covering the bottom and part of the walls in microtitration wells. However, epifluorometric detection collects the signal from only a small part of the well bottom. The full potential of the assay sensitivity is therefore not utilized in the regular whole well approach. The spot-type binding surface concentrates the sandwich formation to coincide more closely with the excitation beam of the fluorometer, which results in a 5- to 7-fold increase in assay sensitivity [11–13].

Materials and methods

Antibodies and cTnI calibrators

cTnI-specific Mabs 19C7 and 8I7 (epitopes at aa 41–49 and 169–178, respectively) were purchased from HyTest Ltd (Turku, Finland) and International Point of Care Inc (Toronto, Canada), respectively. cTnI-specific Fabs 4C2 and MF4 (epitopes at aa 23–29 and 190–196, respectively) of similarly named Mabs were cloned from the hybridoma cell lines of HyTest Ltd and produced at the Department of Biotechnology, University of Turku (DBUT) (Turku, Finland) [12]. The 8I7 epitope was obtained from a recent publication [14] while all other epitope specificities were obtained from HyTest's package inserts. Human cardiac troponin complex (ITC) was provided by HyTest Ltd, and ITC standards were prepared into Tris-buffered saline with azide (TSA; 50 mmol/L Tris-HCl, pH 7.75, 150 mmol/L NaCl, 0.5 g/L NaN_3) containing 75 g/L BSA (Millipore, Purchase, NY, USA) as described previously [9].

Labeling of antibodies with biotin and lanthanide chelate

Mabs were labeled with biotin isothiocyanate (DBUT) or intrinsically fluorescent europium chelate, {2,2′,2″,2‴-{[2-(4-isothiocyanatophenyl)ethylimino]bis(methylene)bis{4-{[4-(α-galactopyranoxy)phenyl]ethynyl}pyridine-6,2-diyl}bis(methylenenitrilo)}tetrakis(acetato)}europium(III) [15] (DBUT) using procedures described earlier [16]. Fabs were site specifically biotinylated with maleimide-PEO_2-biotin (Thermo Fisher Scientific, Waltham, MA, US) as described previously [9, 17]. Labeled antibodies were stabilized with BSA (1 g/L) and stored at +4 °C.

Antibody-coated spots

Biotinylated capture antibodies, 33 mg/L of each 4C2 Fab, 19C7 Mab and MF4 Fab in TSA containing 10 g/L glycerol, were printed (150 drops of ~250 pL/spot, room temperature, 70% humidity) onto the bottom of streptavidin coated microtiter wells (Kaivogen Oy, Turku, Finland) using Nano-Plotter NP 2.1 (Gesim, Grosserkmanndorf, Germany). After 1-h incubation in the closed humidity chamber and washing, 40 μL of Insulation Layer II (Radiometer/Innotrac Diagnostics Oy, Turku, Finland) supplemented with 62.5 mmol/L Tris (pH 8.5) were added into the wells and the wells were dried overnight at +35 °C. The dry-reagent wells were stored at +4 °C with desiccant until use.

cTnI assay conducted on antibody-coated spots

Twenty μL of standard or sample and 75 ng of europium labeled 8I7 Mab in 10 μL Innotrac Aio! Buffer (Innotrac Diagnostics Oy) were added into antibody-coated spot wells. The wells were incubated for 3 h at +36 °C, 1400 rpm in a plate shaker (iEMS incubator/shaker, Thermo Electron Corporation/Labsystems, Helsinki, Finland). The washed wells were then dried and the time-resolved fluorescence was measured directly from the surface with a Victor X4 Multilabel Counter (Perkin-Elmer/Wallac, Turku, Finland).

cTnAAb assay

Human cTnAAbs were measured with a previously published cTnAAb assay [10]. Autoantibody positivity was defined as ≥100 counts above background (no ITC added) when the t-test gave a p-value <0.05.

Assay evaluations

The study protocols were approved by the Local Ethics Committees. Informed consent was obtained from all participants and the study was conducted in accordance with the Declaration of Helsinki as revised in 2006. Samples were stored at –20 °C and had been thawed maximum three times if not otherwise stated. ITC spiked samples were incubated for 1 h at +4 °C before the cTnI detection and the

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results were calculated after background correction for possible endogenous cTnI.

To study the possible sample matrix effects of the developed cTnI assay, the ITC recovery (250 ng/L cTnI) was measured from matched lithium heparin plasma and serum samples. The samples were collected from 11 apparently healthy volunteers at DBUT in 2012–2013.

The limit of blank (LoB) and limit of detection (LoD) were determined according to Classical Approach of CLSI Guideline EP17-A2 using BSA-TSA as a zero calibrator and four lithium heparin plasma pools whose cTnI concentration was 2–5×LoB. Due to the non-normal distribution of the blank sample results, a non-parametric data analysis option was used. Total precision was determined by following CLSI Guidelines EP17-A2 and EP5-A2 for long-run methods with eight plasma pools. Dilution linearity was assessed by serial dilution of four lithium heparin plasma samples and each sample was diluted up to 1/243 with analyte-free (cTnI concentration<LoB) plasma pool. Lithium heparin plasma samples were obtained from anonymous chest pain patients admitted to Central Ostrobothnia Central Hospital (Kokkola, Finland) in 2010 and from apparently healthy volunteers at DBUT in 2012–2013.

To study cTnAAb interference in the developed cTnI assay, cTnI was measured from ITC spiked serum samples (10–50,000 ng/L) in parallel with a midfragment targeting investigational assay (analytical sensitivity ~50 ng/L) [9]. This assay was found to suffer from circulating cTnAAbs in a similar way as representative state-of-the-art assays used in clinical practice, and utilizes biotinylated Mabs 228 and 560 as captures and europium labeled Mab 19C7 as a detector (epitopes at aa 26–35, 83–93 and 41–49, respectively). The serums were collected from two cTnAAb-negative and three cTnAAb-positive apparently healthy volunteers at DBUT in 2013.

The stability of endogenous cTnI was assessed by measuring the cTnI concentrations with the new assay after thawing and freezing (–20 °C) aliquoted samples for 0, 1, 3 or 5 additional time(s) and after incubating the samples at +4 °C/room temperature for 1 day or 1 week. Used lithium heparin plasma samples were obtained from 10 chest pain patients admitted to Central Ostrobothnia Central Hospital in 2010 and were thawed once before the aliquoting.

Method comparison was performed using 250 clinical serum samples (stored at –70 °C) from the non-ST-elevation ACS patients of Global Utilization of Strategies To open Occluded arteries IV (GUSTO IV) trial [18]. The GUSTO IV trial included 7800 patients from Europe and North America during 1999 and 2000. In this study, the developed cTnI assay was evaluated against Architect hs-cTnI assay (Abbott Laboratories, Abbott Park, IL, USA) performed on an Architect i2000$_{SR}$ platform. Architect hs-cTnI utilizes one capture antibody directed against cTnI epitope at aa 24–40 and one tracer antibody against aa 41–49. LoB and LoD of the assay are 0.5 and 1.0–1.2 ng/L, respectively, and the 99th percentile among healthy individuals is 14–23 ng/L [19, 20]. The autoantibody status of the samples was analyzed with the cTnAAb assay. Only cTnAAb-negative samples with analytically reliable cTnI concentrations (>LoD) with both assays (n=160) were included in the method comparison. Weighted Deming regression parameters were calculated with Analyse-it for Microsoft Excel (version 2.30, Analyse-it Software Ltd, Leeds, UK). Because of the non-normal distribution of cTnI values, the correlation between the assays was assessed with Spearman's rank correlation.

To study cTnI levels in a reference population, a cohort of 159 randomly chosen serum samples (stored at –70 °C) from apparently healthy Finnish individuals (Labquality, Helsinki, Finland) was analyzed with the new cTnI and cTnAAb assay.

Statistical analysis

The analyses were performed using IBM SPSS Statistics 21 (IBM, Armonk, NY, USA) and all p-values <0.05 were considered statistically significant. Friedman, Wilcoxon signed-rank and Fisher's exact tests were used when appropriate.

Results

As cTnI was measured both from lithium heparin plasma and serum, possible sample matrix effects were first studied in matched-pair analysis. With the new cTnI assay, median ITC recoveries (25th–75th percentiles) in 11 individuals were 96% (85%–105%) and 99% (74%–104%) for lithium heparin plasma and serum, respectively. The difference between the matrices was not statistically significant (p=0.721).

The calibration curve (y=79.28x) of the developed cTnI assay is presented in Figure 1A. The curve was linear up to 50,000 ng/L (R^2=0.992) and no high-dose hook effect was seen up to 1,000,000 ng/L. Using the CLSI criteria, LoB and LoD of the assay were 1.4 and 2.9 ng/L, respectively. Total precision was determined by running sample pools (n=8) in triplicate once a day for 20 days (Figure 1B) using two different spotting batches. The mean cTnI concentrations of the pools with different batches were 1.3–732 ng/L and 1.0–673 ng/L. The accuracy goal of 10% CV was not reached but with both batches, 20% CV was achieved at 10 ng/L. Linearity was studied with serial dilutions of samples containing 255–53,639 ng/L cTnI, and assessed with linear regression analysis by plotting the detected cTnI concentrations against the dilution factor. The assay was linear (R^2=0.980–0.996) throughout the measured cTnI range (7.0–53,693 ng/L).

The resistance to cTnAAb interference of the new assay was verified over a wide range of concentrations in comparison with the midfragment targeting cTnI assay, which is highly prone to cTnAAb disturbance (Figure 2). Increasing amounts of ITC (10–50,000 ng/L cTnI) were added into five samples, two of which were cTnAAb-negative and three cTnAAb-positive. In the two cTnAAb-negative samples, both assays showed good recoveries over the whole range measurable with the assay in question. In the three cTnAAb-positive samples measured with the midfragment assay, ITC amounts up to 1000 ng/L remained below the analytical sensitivity of the assay. Individual recoveries with each of these samples increased from the smallest to the highest detectable ITC concentration and the mean recovery was 28% while being 85% for the cTnAAb-negative samples. With the new assay, all ITC amounts added were measurable and recoveries were similar over the whole ITC range. The mean recoveries

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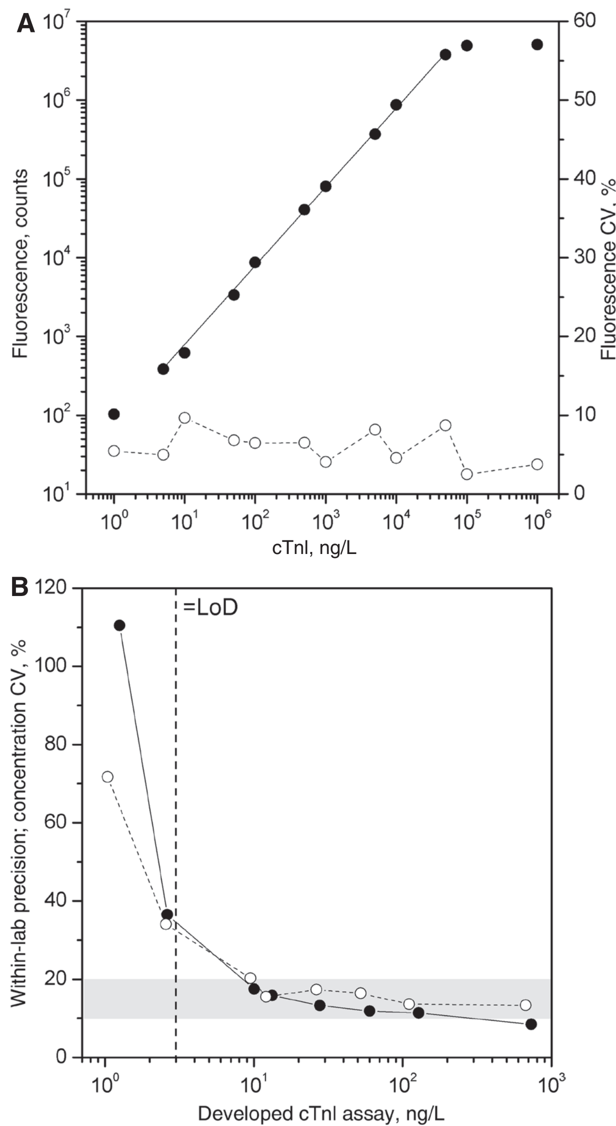

Figure 1 Calibration curve and total precision of the developed cTnI assay.
(A) Calibration curve (●) and within-assay precision profile (○) illustrate the means of six replicates. (B) Total precision profiles for two spotting batches were measured with eight low cTnI sample pools.

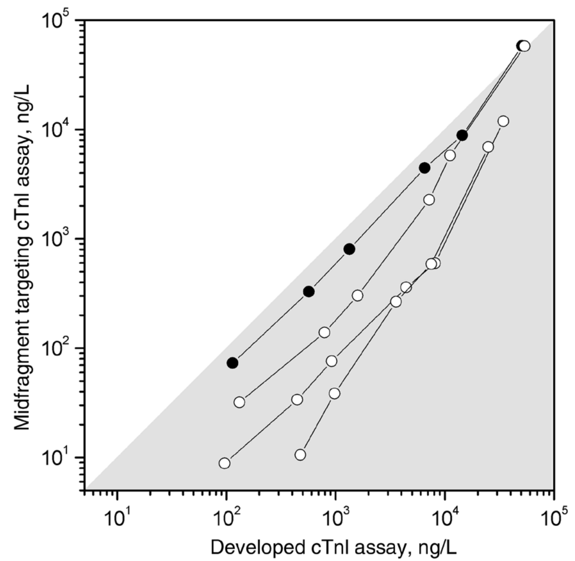

Figure 2 Means of measured cTnI values in two ITC-spiked cTnAAb-negative individuals (●) and measured cTnI values separately in three ITC-spiked cTnAAb-positive individuals (○).

were 100% and 119% for the cTnAAb-positive and cTnAAb-negative samples, respectively.

The stability of endogenous cTnI was assessed with 10 samples containing 16–26,158 ng/L cTnI. After one, three and five freeze-thaw cycle(s), the median (25th–75th percentiles) recoveries from the original cTnI values were 104% (89%–123%), 101% (65%–118%) and 107% (79%–130%), respectively. The corresponding values after incubating the samples for 1 day or 1 week at +4 °C were 103% (83%–117%) and 102% (77%–123%), and at room temperature 102% (77%–109%) and 71% (61%–98%), respectively. Differences between the studied conditions were not statistically significant (p=0.051).

Of 250 patients analyzed for the method comparison, 34 (14%) were cTnAAb-positive. As the developed assay is expected to suffer less from cTnAAb interference than the midfragment targeting Architect hs-cTnI assay, the comparison was limited to cTnAAb-negative samples with cTnI concentrations exceeding the LoDs of both assays (n=160). Although the correlation was good (Spearman's r=0.958, p<0.001), a notable difference was seen between the absolute values measured with the two assays; Architect hs-cTnI assay gave on average seven-fold higher cTnI concentrations than the new assay. Deming regression yielded a slope (95% CIs) of 0.20 (0.17–0.22) and y-intercept of 1.65 (0.78–2.52) ng/L ($S_{y|x}$=0.21 ng/L) (Figure 3A). The mean relative difference (95% limits of agreement) with Bland-Altman agreement was 134% (70%–198%) (Figure 3B). Additionally, the results from those cTnAAb-positive samples with cTnI concentrations above the LoDs of both assays (n=30) were included to the figures. Especially four of those samples stand out from the scatter of the cTnAAb-negative samples because of the higher cTnI values with the new assay.

The reference population included 159 apparently healthy individuals (50% females). The samples had been grouped so that there were 65 samples from individuals 31–50 years of age, 47 samples from individuals 51–70 years of age, and 47 samples from individuals >70 years of age. Of all individuals, 28 (18%) had the measured cTnI concentration above the LoD of the new cTnI assay and 16 (10%) were cTnAAb-positive (Figure 4). The proportion

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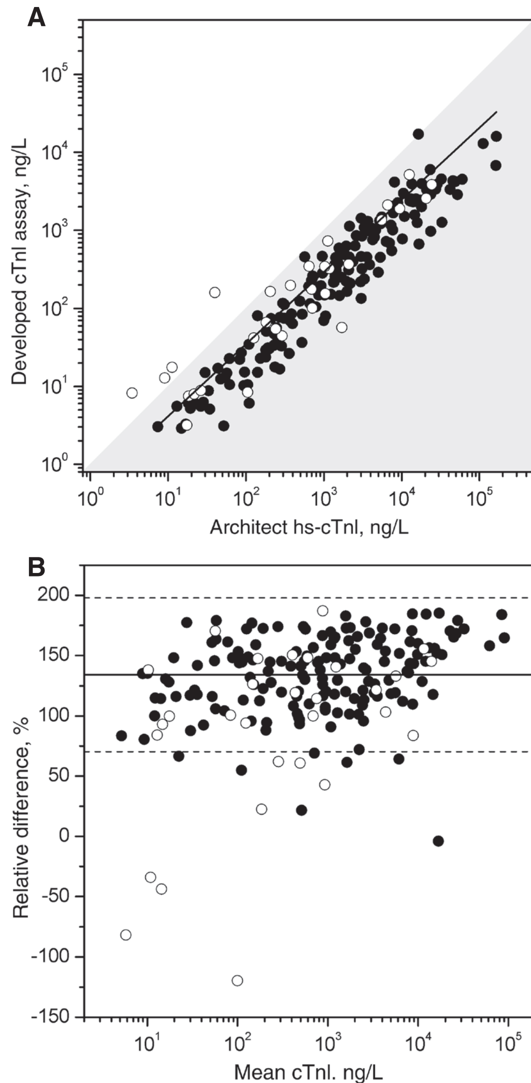

Figure 3 Method comparison with cTnAAb-negative (●) and cTnAAb-positive (○) samples.
(A) With cTnAAb-negative samples, Deming regression equation (solid line) was y=0.20x+1.65. (B) With cTnAAb-negative samples, mean difference (Architect hs-cTnI – Developed cTnI assay/Mean cTnI) was 134% (solid line) and 95% limits of agreement were 70% and 198% (dashed lines).

of measurable cTnI values, however, was significantly higher in cTnAAb-positive individuals (13/16) than in cTnAAb-negative individuals (15/143) ($p<0.001$). Median of measured cTnI concentrations (25th–75th percentiles) for the cTnAAb-positive group was 8.5 (3.4–28) ng/L and median for the cTnAAb-negative group <LoD.

Discussion

The current guidelines for MI diagnosis emphasize the need for reliable detection of minor cTn elevations [1]. This calls for effective measures to reduce false-positive or -negative effects due to preanalytical and analytical interferences in cTn assays. As circulating cTnAAbs can have a decidedly inhibiting effect on cTn detection, it is important to investigate their effect on the new generation of hs cTn assays. To enable such a study, we here developed a sensitive cTnI assay that is not affected by this analytical interference.

Although the cTnI midfragment is the most frequently targeted by cTnAAb interference, the interference extends to the flanking termini and there is remarkable individual variation at the affected sites [5]. In the 3+1-type assay configuration, there are three separate capture antibodies for the midfragment, N-terminus and C-terminus, and one tracer antibody for the C-terminus, on the epitope shown to be virtually unaffected by cTnAAbs [5]. Therefore, the configuration suffers minimally from cTnAAbs [9] and was used for the new cTnI assay. In addition, the use of two site specifically biotinylated capture Fabs enable the formation of a denser and more efficient capture surface, and may reduce false-positive and -negative signals from human anti-animal antibodies, rheumatoid factor and complement [12, 21]. As no significant difference was found between the ITC recoveries from lithium heparin plasma and serum, both sample matrices are suitable for the new cTnI assay.

The developed cTnI assay has a remarkably wide dynamic range; LoD is 2.9 ng/L and the assay gives linear response up to 50,000 ng/L. Although the ideal total precision of 10% CV was not reached, 20% CV was obtained at 10 ng/L. It has been frequently suggested that assays with imprecision up to 20% CV may be reasonably used for MI diagnosis [22–24]. The high variability of the new assay presumably originates mainly from the non-uniformity of the spots. Large scale production with optimized spotting techniques in combination with complete automation of the assay protocol is likely to result in substantially improved assay precision.

Minimal cTnAAb interference in the developed cTnI assay was ascertained with the small-scale ITC recovery study in comparison to the previously published investigational assay resembling state-of-the-art cTnI assays [9]. With the new assay, ITC recoveries (mean) were good in two cTnAAb-negative (119%) and three cTnAAb-positive (100%) samples over the whole cTnI range (10–50,000 ng/L) studied. The comparison additionally demonstrated that in cTnAAb-positive samples, cTnI levels can be substantially underestimated with conventional assays affected by cTnAAb interference to the extent that the initial cTnI release is not recognized. A repeated measurement at 3–6 h after admission may conceivably pick up the ACS-related

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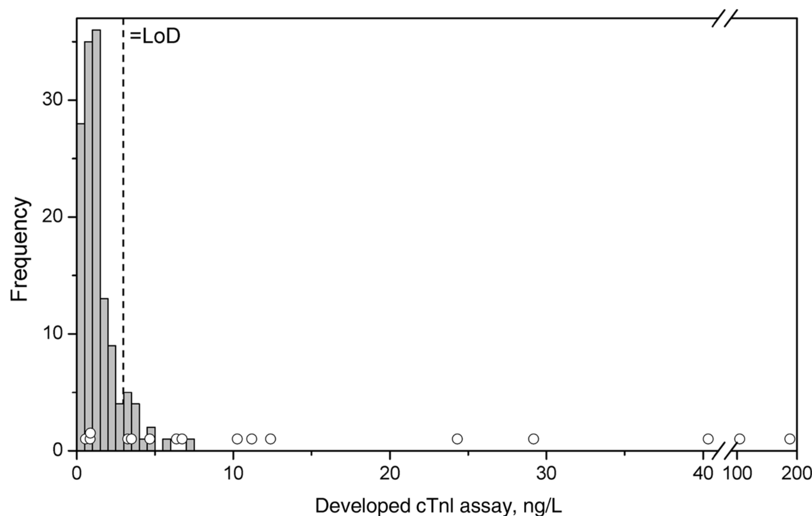

Figure 4 Distribution of measured cTnI values in apparently healthy individuals (n=159).
The circulating cTnI values were higher in cTnAAb-positive (circles) than in cTnAAb-negative (columns) samples (median 8.5 ng/L vs. <LoD).

cTnI increase but in some cTnAAb-positive samples, even high cTnI concentrations can be severely blunted.

As the tracer used binds to the C-terminus of cTnI, the more stable midfragment alone is not recognized by the new assay. Therefore, the stability of cTnI was studied. Endogenous cTnI in patient samples was found to be surprisingly stable; approximately 100% of cTnI was detected after five thaw-freeze cycles and after storage for 24 h at +4 °C and at room temperature. Although statistically significant differences were not seen between the studied conditions, longer storage at room temperature seemed to decrease the recoveries. One limitation of this stability study was that we did not have fresh samples to be included. However, when compared to freshly analyzed samples, only minor losses of immunoreactivity of clinical samples incubated for 24 h at room temperature were previously seen with 2+1-type cTnI assay (Radiometer) also unable to generate signal from the midfragment [25, 26].

The developed cTnI assay showed a considerable systemic bias when compared to Architect hs-cTnI assay (Deming regression slope of 0.20), which conceivably stems from the use of different antibodies, and/or differences in calibration and standard material used. We previously demonstrated that despite the use of the same standard material, the differences in the epitope specificities, affinities, and formats of chosen antibodies may lead to differential recognition of standard and endogenous cTnI [9]. Similar biases have been previously obtained by comparing above mentioned 2+1-type assay to the midfragment targeting AxSYM first-generation assay (Abbott Diagnostics) and the midfragment targeting AccuTnI second-generation assay (Beckman Coulter) [26] but also by comparing the midfragment targeting Liaison cTnI assay (DiaSorin) to AccuTnI second-generation assay [27]. Therefore, the observed difference merely highlights the well-known complexity of cTnI assay standardization. The relative difference in our method comparison study was reasonably constant over the whole cTnI range and the correlation between the assays was good.

Although the method comparison was limited to the cTnAAb-negative samples, the cTnAAb-positive samples were included in Figure 3. A few of these samples with low cTnI values clearly deviated from the cTnAAb-negative samples, which support our previous notion that cTnAAbs can have a decisive negative impact on troponin testing. The small number of cTnAAb-positive samples with cTnI values within the reliable ranges of both assays (n=30) prevents any final conclusions to be made. Significantly larger patient cohorts would be needed to address this question.

The developed cTnI assay detected measurable cTnI values (>LoD) from 18% of the reference population. It has been demonstrated that low but detectable cTn values in healthy individuals and cardiac patients have prognostic value, and that a more stringent selection of a reference population decreases the derived 99th percentile. Consequently, new criteria for the reference population has recently been introduced [28, 29] proposing that a minimum number of 300 individuals are needed to appropriately determine the 99th percentile and using a more thorough screening (e.g., with electrocardiogram) to detect any underlying cardiac conditions. Therefore, due to the small number of apparently healthy individuals and their poor clinical characterization, we refrained from 99th percentile calculations. As the developed assay did

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not meet the goal proportion of 50%, it cannot be classified as a hs assay. Yet, the proportion of analytically reliable concentrations with the new cTnI assay is comparable to the most sensitive contemporary and point-of-care assays; in a recent publication, the proportion was more than 6% only for one contemporary and one point-of-care assay [20].

Interestingly, cTnAAb-positive healthy individuals as a group had significantly higher cTnI levels in circulation. Of the cTnI values in the 16 cTnAAb-positive individuals, 13 were above LoD with the highest reaching up to 100–200 ng/L (median cTnI 8.5 ng/L). While in the 143 cTnAAb-negative individuals, only 15 were above LoD (median cTnI <LoD). Although animal models have associated cTnAAbs to the etiology of cardiac diseases [30–32], their impact on the patient outcome has remained controversial [10, 33–38]. In ACS patients, however, the presence of cTnAAbs has been associated with chronically elevated cTnI concentrations [38, 39] and persistent cTnI elevations with higher mortality during long-term follow-up [40]. Therefore, the high cTnI concentrations in apparently healthy cTnAAb-positive individuals raise new questions about the cTnAAb etiology. This may result from a cTnAAb-associated persistent injury or reflect a longer half-life of circulating cTnI-cTnAAbs complexes.

Conclusions

The developed cTnI assay facilitates sensitive and reliable cTnI detection in the presence of circulating cTnAAbs. In its present format the assay is not rapid enough calling for further development to enable its routine clinical use. However, it can serve as an important analytical tool for investigating the possible negative impact of cTnAAbs on troponin testing. In combination with well-characterized assays for cTnAAb testing, it may help to unravel the etiology behind cTn-related autoimmune responses and their clinical relevance.

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Conflict of interest statement

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