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Chimeric recombinant antibody fragments in cardiac troponin I immunoassay

Heidi Hyytiä [a,*], Taina Heikkilä [a], Eeva-Christine Brockmann [a], Henna Kekki [a], Pirjo Hedberg [b,c], Tarja Puolakanaho [b,c], Timo Lövgren [a], Kim Pettersson [a]

[a] University of Turku, Department of Biotechnology, Tykistökatu 6 A, 20520 Turku, Finland
[b] NordLab Oulu, Oulu University Hospital, Oulu, Finland
[c] Department of Clinical Chemistry, University of Oulu, Oulu, Finland

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ABSTRACT

Objectives: To introduce a novel nanoparticle-based immunoassay for cardiac troponin I (cTnI) utilizing chimeric antibody fragments and to demonstrate that removal of antibody Fc-part and antibody chimerization decrease matrix related interferences.

Design and methods: A sandwich-type immunoassay for cTnI based on recombinant chimeric (mouse variable/human constant) antigen binding (cFab) antibodies and intrinsically fluorescent nanoparticles was developed. To test whether using chimeric antibody fragments helps to avoid matrix related interferences, samples (n = 39) with known amounts of triglycerides, bilirubin, rheumatoid factor (RF) or human anti-mouse antibodies (HAMAs) were measured with the novel assay, along with a previously published nanoparticle-based research assay with the same antibody epitopes.

Results: The limit of detection (LoD) was 3.30 ng/L. Within-laboratory precision for 29 ng/L and 2819 ng/L cTnI were 13.7% and 15.9%, respectively. Regression analysis with Siemens ADVIA Centaur® yielded a slope (95% confidence intervals) of 0.18 (0.17–1.19) and a y-intercept of 1.94 (−1.28–3.91) ng/L. When compared to a previously published nanoparticle-based assay, the novel assay showed substantially reduced interference in the tested interference prone samples, 15.4 vs. 51.3%. A rheumatoid factor containing sample was decreased from 241 ng/L to <LoD.

Conclusions: Utilization of cFab-fragments enabled the development of a sensitive (LoD = 3.3 ng/L) immunoassay for the detection of cTnI and decreased matrix related interferences, thus resulting in a lower number of falsely elevated cTnI-values.

Introduction

Cardiovascular disease (CVD) is the main cause of mortality in Europe costing the economy almost €196 billion a year [1]. Due to excellent cardiac specificity, cardiac isoforms of troponins I and T (cTnI, cTnT) have become the dominating biochemical markers for the detection of myocardial infarction (MI). Measurement of either cTnI or cTnT, along with analyzing patient symptoms and electrocardiographic abnormalities, is recommended when detecting MI [2]. Recently highly sensitive cTn assays based on single-molecule counting and chemical signal enhancement have been introduced [3–5]. An assay capable of measuring cTnI-concentrations in 100% of normal study participants above the limit of detection (LoD) has also been reported [6].

The trend toward highly sensitive assays and the ability to measure cTnI-concentrations even from a healthy population have recently raised questions of how to define normality and who should be included in the normal range studies [7]. Another important concern should be whether other mechanisms besides cTnI may contribute to the recorded signal in such assays. Human anti-mouse antibodies (HAMAs), rheumatoid factor (RF) and complement are the most commonly described factors causing either negative or positive analytical interference in immunoassays [8,9]. HAMAs are described as high-affinity antibodies directed against specific immunogens from foreign origin, whereas heterophilic antibodies, including RF, are considered to have low affinity and weak binding and are produced without known exposure to animal antibodies [10]. Heterophilic antibodies have been reported to display variable specificity toward different immunoglobulin G (IgG) subclasses, especially IgG1 which is found to be interference susceptible [11]. HAMA and RF are problematic in two-site immunoassays, since they can bridge solid phase and detection antibodies even in

Abbreviations: cTnI, cardiac troponin I; cTnT, cardiac troponin T; Fab, fragment antigen binding; Fc, fragment crystallizable; HAHA, human anti-human antibody; HAMA, human anti-mouse antibody; IgG, immunoglobulin G; LiH, lithium heparin; LoD, limit of detection; Mab, monoclonal antibody; MI, myocardial infarction; RF, rheumatoid factor.

* Corresponding author. Fax: +358 2 333 8050.
E-mail address: heidi.hyytia@utu.fi (H. Hyytiä).

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the absence of an analyte, thus causing artifactually elevated analyte values. Possible interferences from HAMA and RF are problematic to predict: a recent study indicated the existence of HAMA in >10% of the samples tested [12]. However not all HAMAs cause interferences in all assays. The exact prevalence of heterophilic antibody interference in cTnI-assays is unknown, but suspected to be higher than traditionally thought [13]. As high as 3.1% false-positive rate in a certain cTnI-assay has been found in a routine population, and when a RF positive cohort was tested, up to 11.5% of the measured cTnI values were found to be falsely elevated by RF [14,15]. In our recent study we have shown that removing the Fc-region from one cTnI immunoassay solid phase antibody minimized interferences caused by factors related to the antibody Fc-part and decreased the measured cTnI signals significantly [16]. Elimination of the Fc-region from an antibody eliminates interferences from RF and complement, but only partly from HAMA, which can also be directed against antigenic determinants on the constant parts of the antibody fragments. Replacing constant parts of the light and heavy chains with corresponding human antibody sequences to provide mouse/human antibody chimera of fragment antigen binding (cFab) should decrease the possible interferences even further.

The scope of this study was to introduce a nanoparticle-based immunoassay for cTnI that utilizes chimeric antibody fragments (mouse variable/human constant), thus showing possibly minimal susceptibility to matrix related interferences. In addition, to demonstrate the hypothesis that removal of the antibody Fc-part and antibody chimerization decrease matrix related interferences significantly, samples (n = 39) with known amounts of triglycerides, bilirubin, RF or HAMA were measured with the novel assay and compared to results obtained with a previous version of the research assay utilizing the same antibody epitopes, but not recombinant chimeric antibody fragments [17].

Materials and methods

Blood samples

The procedures were in accordance with the Helsinki Declaration as revised in 2006. Informed consent was obtained from all participants and the study protocols were approved by the local ethics committee. Lithium heparin (LiH) plasma samples from apparently healthy volunteers with no cardiac symptoms (n = 64) were collected at the Department of Biotechnology, University of Turku (Turku, Finland). Prior to analysis, all the normal LiH-plasma samples were tested for cardiac troponin specific autoantibodies [18]. Normal LiH-plasma pool was composed of samples negative in the autoantibody assay and cTnI levels below the LoD of the novel assay. The clinical sample panel consisted of LiH-plasma samples (n = 265) that were collected, and the comparison cTnI level was measured, at Oulu University Hospital. The samples were thereafter shipped to the University of Turku (at −20 °C) and stored at −70 °C. Samples (24 LiH-plasma, 15 serum) containing known concentrations of triglycerides, bilirubin, RF or HAMA were purchased from ProMedDx (Norton, MA). Prior to the analysis, all frozen samples were thawed at +23 °C and mixed. The LiH-samples were also centrifuged (1 min, 2000 *g*) to remove any particulate material.

Antibodies and novel nanoparticle-based immunoassay for cTnI

The molecular forms and epitopes of the antibodies used in the novel assay have been introduced in Fig. 1. Monoclonal antibody (Mab) clone 19C7 was purchased from HyTest (Turku, Finland). Recombinant cFab-fragment of antibody 9707 (cFab-9707) was originally obtained from Medix Biochemica (Kauniainen, Finland). Recombinant cFab-fragment 11N11 (cFab-11N11) was developed at the Department of Biotechnology, University of Turku (Turku, Finland). Both cFab-9707 and cFab-11N11 were produced and purified as described previously with some minor modifications [16,19,20]. The cFab-11N11 was designed not to contain an unpaired cysteine residue in the C-terminal end of the heavy chain peptide, since no biotinylation of the fragment was performed. Both cFab-9707 and cFab-11N11 were mouse/human chimeric antibody fragments, composed of mouse variable and human constant parts.

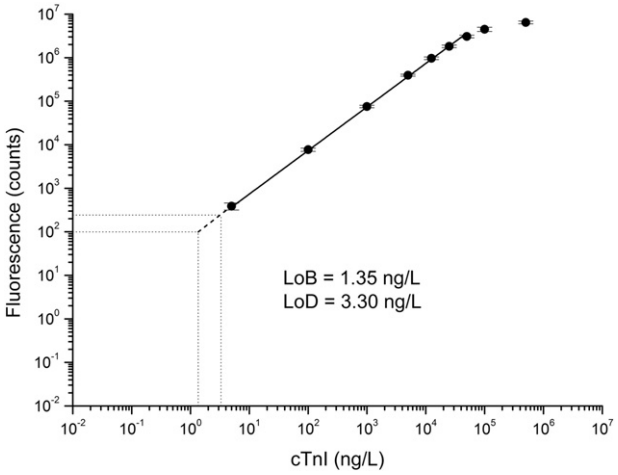

Fig. 1. Dose–response curve for the novel assay. The error bars represent the standard deviation measured from four replicate microtitration wells. y = 73.7X, R^2 = 0.993.

The novel assay was performed as described previously with some minor modifications [17]. The solid phase antibodies were used: 25 ng of 19C7 and 16.5 ng/well of 9707. The particle buffer consisted of 37.5 mmol/L Tris, pH 7.75, 500 mmol/L NaCl, 0.4 g/L NaN_3, 0.6 g/L bovine gamma globulin, 25 g/L BSA, 146 mmol/L D-trehalose, 0.8 g/L native mouse IgG, 0.05 g/L denatured mouse IgG, 2 g/L casein, 37.5 IU/mL heparin and 0.15 mmol/L biotinylated polyethylene glycol. Information on calibrators and other reagents, as well as detailed instructions for the preparation of the nanoparticle conjugates can be found in Supplemental file A1.

Assay evaluation and method comparison

Limit of blank (LoB, nonparametric option), LoD, limit of quantitation (LoQ, defined as functional sensitivity with within-laboratory precision of 20%) and assay precision were determined according to Clinical and Laboratory Standards Institute (CLSI) Guidelines EP17-A2 and EP5-A2 [21,22].

The applicability of the developed assay was studied by measuring LiH-plasma samples with the novel assay and by comparing the results to cTnI-results obtained at Oulu University Hospital with the Siemens ADVIA Centaur® TnI-Ultra™ reference assay [23]. Assay descriptives and Pearson correlation coefficient were calculated with IBM SPSS statistics 21 (SPSS Inc., Chicago, IL). Method comparison was performed by calculating Passing & Bablok regression parameters with Analyze-it software (version 2.30, Analyze-it Software Ltd., Leeds, United Kingdom).

The ability of the novel assay to avoid falsely increased cTnI values was evaluated by testing 39 LiH-plasma/serum samples containing known amounts of triglycerides, bilirubin, RF or HAMA. To determine the effect of Fc-part removal and antibody chimerization, the samples were additionally tested with a previously described old version of the research assay [17]. The antibodies, antibody molecular forms and epitopes are presented in Table 1. The interference panel was not tested with any commercial cTnI-assay prior to testing with the nanoparticle-based assays. Additional information about the interference samples is presented in Supplemental data Table A1.

Table 1
Molecular forms and antibody epitopes of the novel and old comparison assays.

	Novel assay		
	19C7 (solid phase)	9707 (solid phase)	11N11 (detection)
Antibody epitope	41–49	190–196	160–179
Antibody molecular form	Mab	cFab	cFab
	Old comparison assay		
	19C7 (solid phase)	9707 (solid phase)	8I7 (detection)
Antibody epitope	41–49	190–196	169–178
Antibody molecular form	Mab	$F(ab')_2$	Mab

Results

Kinetics

The novel assay reached equilibrium in 120 min with low and medium concentration samples (50 ng/L and 500 ng/L cTnI) and in 240 min with the high concentration sample (5000 ng/L cTnI). During the 15-minute incubation time selected, the HyTest standard material of the calibrators, as well as the endogenous cTn reached 30 to 40% of the steady state with the lower concentrations (Supplemental data Fig. A1).

Calibration curve, imprecision and assay linearity

A typical calibration curve for the novel assay is presented in Fig. 1. The calibration curve was linear up to 50,000 ng/L ($R^2 = 0.993$). No high-dose hook was observed, and the signal was still increasing at 500,000 ng/L. LoB of the assay was calculated to be 1.35 ng/L and LoD was 3.30 ng/L. Functional detection limit, defined as the lowest cTnI concentration measured with within-laboratory precision of 20%, was 17 ng/L (Fig. 2).

Within-run and within-laboratory precisions were determined by spiking negative LiH-plasma pool with endogenous cTnI and the samples were measured in triplicate twice a day for 20 days. The measured mean concentrations of cTnI were: 29 ng/L, 37 ng/L and 2819 ng/L. The within-run imprecisions were 8.5%, 8.4% and 7.5%. The within-laboratory precisions for the same samples were 13.7%, 16.4% and 15.9%.

Linearity of the novel assay was assessed by testing serial dilutions of six patient samples containing initial cTnI concentrations of 22,262 ng/L; 14,540 ng/L; 10,145 ng/L; 2327 ng/L; 979 ng/L and 718 ng/L cTnI. The samples were diluted 3- to 243-fold by using a normal LiH-plasma pool and measured in six replicates. Linear regression of the observed cTnI-concentration and dilution factor showed linearity ($R^2 = 0.929–0.993$) across the measured range (2.2 ng/L–22,261 ng/L, Fig. 3).

Analytical recovery and normal LiH-plasma panel

To study assay recoveries and cTnI levels in a normal population, 64 normal LiH-plasma samples were spiked to 500 ng/L with troponin complex and measured with the novel assay. The background levels of cTnI were measured without the addition troponin. The cTnI levels of the normal LiH-plasma samples were above the LoD (3.3 ng/L) in three specimens (4.7%). The cTnI levels measured from the three normal LiH-samples were 5.74 ng/L, 7.20 ng/L and 11.8 ng/L. Analytical recoveries were expressed as the percentage of the measured cTnI value from the expected cTnI values. Recoveries from samples tested positive in the cardiac specific autoantibody assay (n = 5) varied between 31% and 78% (average 53%, median 57%). Recoveries from samples tested negative for cardiac specific autoantibodies varied between 70% and 108% (average 83%, median 83%).

Method comparison and possibly interfering factor containing samples

Method comparison between the novel assay and the Siemens ADVIA Centaur® TnI-Ultra™ reference assay is shown in Fig. 4A. All 265 samples had a reference value >LoD reported for the reference assay. Out of the 265 samples, 17 samples gave values <LoD with the novel assay and were thus excluded from the method comparison (6–300 ng/L; median, 20 ng/L). The median concentration of cTnI (lower and upper quartiles) was 1483 ng/L (201.5 ng/L; 6635 ng/L) when measured with the reference assay and 240.8 ng/L (37.08 ng/L; 1291 ng/L) with the novel

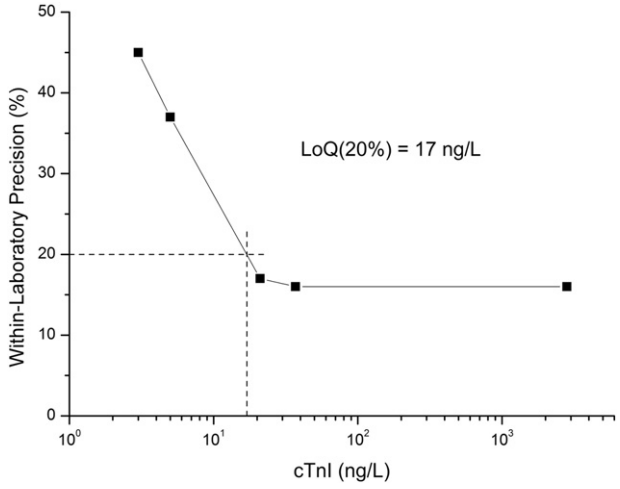

Fig. 2. Limit of quantitation defined as the lowest cTnI-concentration measured with within-laboratory precision of 20%.

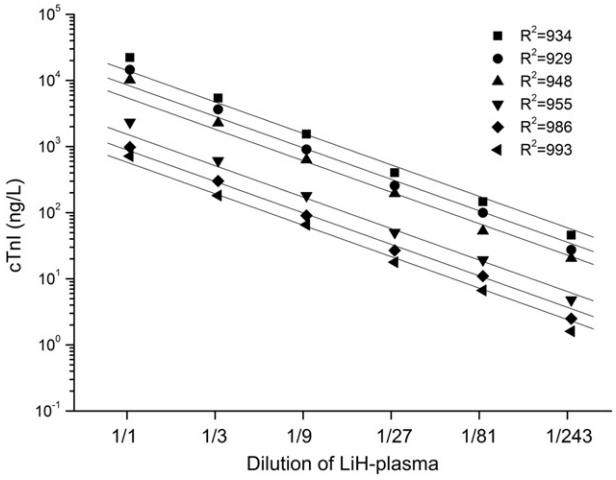

Fig. 3. Linearity of the novel assay. Six clinical LiH-plasma containing variable amounts of cTnI were serially diluted 1/1–1/243-fold with LiH-plasma pool obtained from healthy individuals with no cardiac symptoms.

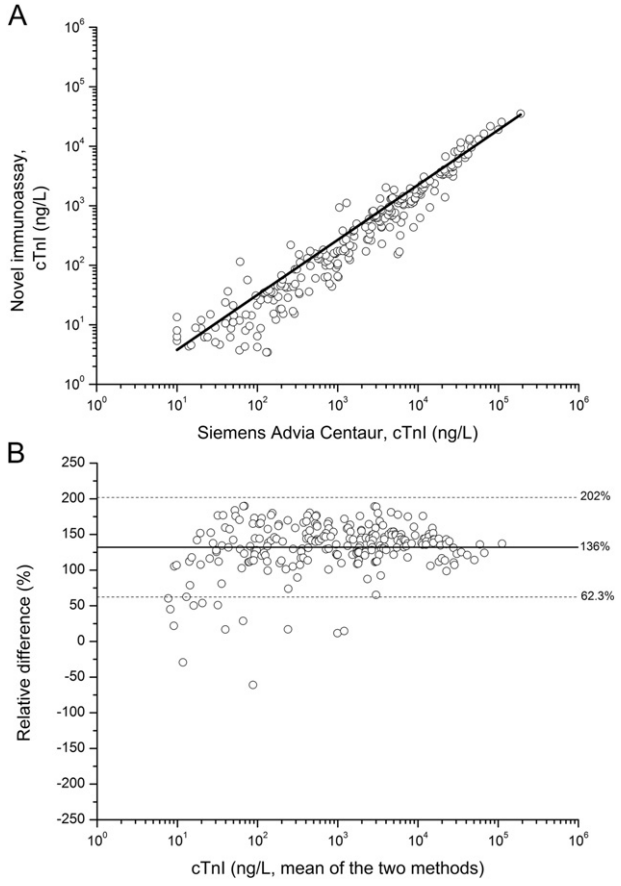

Fig. 4. Method comparison. (A) Correlation between the novel assay and the Siemens ADVIA Centaur® TnI-Ultra™ reference assay (n = 248). (B) Bland–Altman analysis of agreement between the novel assay and the reference assay. The mean difference (136%) is presented with a solid line along with dashed lines representing the 95% limits of agreement (62.3%–202%).

assay. Passing & Bablok regression analysis for the novel assay and the reference assay yielded a slope (95% confidence intervals) of 0.18 (0.17–0.19) and a y-intercept of 1.94 (−1.28–3.91) ng/L. The Spearman correlation coefficient was 0.965 ($P < 0.001$). The mean relative difference between the two methods was 136% with 95% limits of agreement ranging from 202% to 62.3% ($P < 0.001$) (Fig. 4B).

Samples (n = 39) containing known amounts of triglycerides, bilirubin, RF or HAMA were measured with the novel assay (LoD = 3.3 ng/L) and the old comparison assay (LoD = 2 ng/L). Results can be seen in Table 2. The novel assay gave detectable cTnI values (>LoD) in 15.4% of the samples (n = 6), whereas the old comparison assay detected 51.3% (n = 20). The highest cTnI-value (240.7 ng/L) with the old comparison assay was decreased to <LoD with the novel assay.

Table 2
LiH and serum samples (n = 39) containing known amounts of triglycerides, bilirubin, RF or HAMA measured with the old comparison assay and the novel assay employing cFab-fragments.

Interfering factor	Old comparison assay	Novel assay
	Measured cTnI concentration (ng/L)	Measured cTnI concentration (ng/L)
Triglycerides	<LoD	<LoD
Triglycerides	<LoD	<LoD
Triglycerides	<LoD	<LoD
Triglycerides	<LoD	<LoD
Triglycerides	4.09	4.39
Triglycerides	<LoD	<LoD
Triglycerides	<LoD	<LoD
Triglycerides	6.30	3.27
Triglycerides	<LoD	<LoD
Triglycerides	<LoD	<LoD
Triglycerides	2.23	<LoD
Triglycerides	3.31	<LoD
Bilirubin	13.6	5.34
Bilirubin	<LoD	<LoD
Bilirubin	<LoD	<LoD
Bilirubin	<LoD	<LoD
Bilirubin	20.6	10.1
Bilirubin	10.4	<LoD
Bilirubin	4.75	<LoD
Bilirubin	7.39	<LoD
Bilirubin	7.64	3.92
Bilirubin	3.64	<LoD
Bilirubin	7.01	<LoD
Bilirubin	26.7	10.7
RF	6.3	<LoD
RF	<LoD	<LoD
RF	<LoD	<LoD
RF	<LoD	<LoD
RF	3.81	<LoD
RF	2.12	<LoD
RF	7.43	<LoD
RF	<LoD	<LoD
RF	241	<LoD
RF	3.04	<LoD
RF	<LoD	<LoD
RF	2.92	<LoD
HAMA	<LoD	<LoD
HAMA	<LoD	<LoD
HAMA	<LoD	<LoD

Discussion

Most commercial immunoassays for cTnI are currently using intact monoclonal or polyclonal antibodies [24]. Contrary to expressed concerns it has been shown that employing recombinant antibody fragments does not pose threats like decreased affinity when compared to their monoclonal counterparts [25]. Employing either recombinant or enzymatically digested antibody fragments has shown decreased interference for many analytes like human kallikrein or carcinoembryonic antigen [26,27]. Decreased signal levels for normal population have also been shown for cTnI, when either enzymatically digested or recombinant Fab-fragments were used [16]. Employing recombinant antibodies can also be defended in that routine recombinant DNA techniques provide tools for a continuous and homogeneous source of the antibody as well as the possibility to modify the antibodies to contain reactive groups to facilitate for example labeling of the fragment. In our assay, the cFab-9707 contains a free sulfhydryl group, which enables direct biotinylation of the fragment. By using site-directed biotinylation, a more oriented capture surface can be created, which should ultimately enable the development of increasingly sensitive assays [28].

The novel assay was performed manually with 15-minute assay incubation. By choosing the 15-minute incubation, we measured the signal in a highly kinetic stage: the signal increased linearly to 30 min and 13–41% of the maximum signal could be measured at the selected 15-min time point (Supplemental data Fig. A1.). This resulted in hindering assay precision and the estimation of LoQ — we were not able to meet the current recommendation of within-laboratory precision of 10% [29]. Thus applying the assay to an automated platform is expected to greatly benefit assay precision, the determination of LoQ (10%) and ultimately even the determination of assay LoD.

In the method comparison, the novel assay showed good correlation with the Siemens ADVIA Centaur® TnI-Ultra™ reference assay ($r = 0.965$). The novel assay showed substantially lower cTnI values in comparison to the reference assay throughout the measuring range (mean difference: 132%). The novel assay and the reference assay differ considerably in their antibody configurations. The reference assay employs two monoclonal solid phase antibodies, which recognize epitopes in the stable midfragment (amino acids 41–49 & 87–91). The third monoclonal

detection antibody recognizes amino acids at amino acid residues 27–40 [23]. The novel assay was designed to employ cTnI-specific antibodies recognizing epitopes outside the stable midfragment, thus circumventing inhibiting effects of circulating autoantibodies [30,31]. It has been shown that antibody epitopes and affinities can lead to differences in the recognition of cTnI, even when the same standard material is used [31]. This, along with the different standard material used, may cause the assays to have differences in the measured cTnI values, thus supporting the well-known difficulties in cTnI assay standardization. Out of the 265 samples assayed, 17 were observed to be <LoD with the novel assay. The reason for this is probably the abovementioned bias. In our previous study we have demonstrated that by replacing just one monoclonal assay antibody with a corresponding antibody fragment, the measured apparent cTnI values were significantly decreased [16]. Therefore another possible explanation for the <LoD signal levels could be that by employing two cFabs we have been able to decrease matrix-related interferences, thus resulting in cTnI values <LoD. This is supported by the fact that most, but not all, of the 17 samples represented low range values with the Siemens reference assay (6–300 ng/L; median 20 ng/L).

A small number (n = 64) of normal plasma samples were run with the novel assay, so no 99th percentile value could be determined. In a recent study determining the 99th percentiles of 19 cTnI and cTnT assays, up to 32-fold variations in the assay 99th percentiles were found even when the same sample population was used [32]. Thus determining the 99th percentiles in the future will probably be increasingly stringent, more extensive and in agreement with generally accepted recommendations regarding reference populations used [33]. When normal samples were measured with the novel assay, only 4.7% (3 out of 64) had concentrations >LoD (3.3 ng/L). It must be noted that the samples were obtained from young healthy individuals presenting with low likelihood of elevated cTn values which is found more frequently in older subjects, thus possibly resulting in a low number of samples >LoD [34].

Commercially available highly fluorescent nanoparticles can be used to develop rapid and sensitive immunoassays [17,35]. Here, we have shown that even small cFab-fragments can covalently be linked to the particle surface without losing assay sensitivity. By coupling cFabs onto the particle surface, we are increasing both the binding site density and the binding area of the label, as well as amplifying the signal obtained from one binding event, when compared to a single cFab labeled with a small number of individual labels. This avidity derived signal enhancement enables the development of highly sensitive assays, but potentially also makes the assay vulnerable to falsely elevated analyte signals caused by low affinity matrix related interferences. Therefore comparing the degree of interference from the novel, potentially minimally interference susceptible assay to that from the old comparison assay should give us an evident picture of the possible decrease in interference induced by exploiting cFabs. Samples containing known amounts of possibly interfering factors (triglycerides, bilirubin, RF or HAMA) gave cTnI-concentrations >LoD with the novel assay in 15.4% of samples (6 out of 39), when the corresponding value was 51.3% (20 out of 39) for the old comparison assay. As was expected, interference from RF was eliminated by employing cFab-fragments: all RF-samples were <LoD with the novel assay, but when measured with the old comparison assay, 58.3% of samples gave detectable cTnI-signals and as high as 241 ng/L falsely elevated cTnI-signal was measured from one of the RF-samples. Bilirubin has mostly been reported to generate false negative results in cTnI-immunoassays [36,37], but explicit cTnI-signal deductions were seen with bilirubin containing interference samples. Out of the 12 bilirubin samples measured, the old comparison assay gave values >LoD in 9 (75.0%) of the samples, when only 4 (33.3%) were >LoD with the novel assay. With the 4 samples that were >LoD with both of the assays, 48.7–60.9% decrease in the measured cTnI-value was observed. Elevated bilirubin is often accompanied by the generation of free hemoglobin by hemolysis, which has been shown to result in falsely increased cTnI-values, along with the combination of increased bilirubin [38,39]. This is a very possible scenario with the bilirubin samples used in our assay, since only the bilirubin status of the samples was known.

During the recent years, humanized and chimeric antibodies have emerged especially in cancer therapeutics, and the possibility of a patient having human anti-human antibodies (HAHAs) cannot be excluded [40]. Hence, employing two fragments that consist of parts from human origin in both the capture and detection modes may cause falsely elevated signals in some individuals. No blockers against such, assumable rare, incidents have been used in the current assay setup. As the usage of therapeutic antibodies becomes more frequent, assay developers using chimeric or humanized antibodies should investigate the prevalence and severity of possible interferences caused by HAHAs.

Due to the low volume of the interference susceptible samples, no spiking with cTnI, linearity studies, and cTnI-value measurement with the Siemens reference assay were possible. The samples were also run in the matrices (LiH-plasma, serum) available to us. From the current study it can be concluded that utilizing chimeric antibody fragments enables the development of a sensitive (LoD = 3.3 ng/L) and rapid sandwich-type immunoassay for cTnI. By employing cFab-fragments, we were able to develop an assay that is minimally prone to known sources of signal elevating artifacts. According to the presented results, the exploitation of recombinant antibody fragments, and in particular using cFab-fragments or humanized antibodies, should be seriously contemplated for new generations of high sensitivity cTnI assays. This is likely to become mandatory in order to establish a reliable analytical foundation for the emerging widened clinical use of cardiac troponin determinations.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clinbiochem.2014.06.080.

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