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Cardiac troponin I is present in plasma of type 1 myocardial infarction patients and patients with troponin I elevations due to other etiologies as complex with little free I☆

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ABSTRACT

Background: Cardiac troponin (cTn) is a complex of three subunits (T, I, and C), with some studies reporting that ~5–10% is cytosolic and unbound ('free'). It has been hypothesized that free cTn is released before complex and before or without cell death dependent on the severity of ischemia. In this context, new generation assays that can discriminate free, binary (IC) and ternary (TIC) complex forms may aid to differentiate between type 1 myocardial infarction (MI) and cTn elevations due to different etiologies, *e.g.* demand ischemia and type 2 MI.
Methods: Serial plasma samples from six type 1 MI patients and twenty-seven patients with other cTnI elevations, *e.g.* due to demand ischemia and type 2 MI, were analyzed using high-sensitivity ET Healthcare Pylon assays for total cTnI, complex cTnI (IC and TIC), and cTnTIC. The specificity of the anti-cTnT antibody in the cTnTIC assay was such that only full-size cTnTIC is detected. *In vitro* stability of different cTnI forms was assessed by spiking free cTnI and cTnTIC in cTnI-free serum, incubating at 4 or 37 °C, and measuring different cTnI forms over 0–182 h. Presence of cytosolic free cTnI was evaluated on fixed rat cardiac tissue using an antibody against free cTnI.
Results: Pylon assays for total and complex cTnI tracked well over time with each other and gave similar results, both for type 1 MI and non-type 1 MI patients, indicating that the vast majority was complex cTnI. As a minority of complex cTnI was full-size cTnTIC, this indicated that complex cTnI mainly consisted of a degraded form of cTnTIC (low-molecular weight cTnTIC) and/or cTnIC. Full-size cTnTIC was more abundant in early compared to late samples. *In vitro* studies indicated that free cTnI and cTnTIC are not stable at 37 °C (28% and 11% recovery after 24 h, respectively) and this is also true to some extent for cTnTIC at 4 °C (60% recovery after 24 h). Free cTnI was not readily detected in rat cardiac tissue.
Conclusions: In agreement with type 1 MI, cTnI in samples of patients with cTnI elevations due to other etiologies is found predominantly as complex cTnI, of which some is full-size cTnTIC. In most cases, assays for total and complex cTnI indicated there was little free cTnI; however, its presence cannot be completely excluded, due to the inability of its direct measurement and limited stability.

Abbreviations: cTn, Cardiac troponin; MI, Myocardial infarction; PCI, Percutaneous intervention; LMW, Low-molecular weight; FBS, Fetal bovine serum; RA, All-trans-retinoic acid; AST, Aspartate aminotransferase; CK, Creatine kinase; LD, Lactate dehydrogenase; PFA, Paraformaldehyde; BSA, Bovine serum albumin; SPR, Surface plasmon resonance; LOB, Limit of blank

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1. Introduction

Cardiac troponins (cTns) are components of the sarcomere of striated muscle regulating the excitation-contraction coupling in the heart. Three subunits exist (T, I, and C), with only cTnT and cTnI as cardiac specific isoforms. Within the cell they are part of the myofibril as a ternary complex, however, it has long been thought that ~5–10% of cTnI and cTnT are found in the cytoplasm as free subunits. This is based on studies that showed low extraction solubility for cTnI and cTnT in myocardial tissue using a cold low-salt buffer [1–4]. For cTnT, this concept has been challenged by the observation that up to 80% of cTnT can be extracted from cardiac tissue using large volumes of serum at 37 °C [5]. Interestingly, kinetic experiments previously indicated that cTnI, but not cTnT, has a precursor pool of free subunits [6].

It is indisputable that the original site of release of cTn is the heart, and its presence in patient sera has always been considered a marker of cardiac myocyte death [7]. Yet the appreciation of detectable cTn in healthy individuals and the presence of short-lived increases, especially after exercise, have fueled the concept of cTn leakage from viable cardiac myocytes [8,9]. A release mechanism proposed by Hickman et al. describes a situation of ischemia in which the cytosolic compound of cTn (free subunits) is released first into circulation by the formation of membranous blebs that bud off from the cellular plasma membrane before necrosis occurs, *i.e.* from a viable cardiac myocyte [9]. As ischemia prolongs, the cell will undergo necrosis and release complex cTn forms derived from the myofibril.

The cTn constitution found in blood, *e.g.* free *vs.* complex cTn, or different proteoforms of cTn [10], may hold additional information that can assist a better diagnosis. For example, based on the theory of Hickman et al., the ratio of free *vs.* complex cTn could hold information on the age and severity of the myocardial injury. A relatively high concentration of complex cTn in blood would indicate a more severe and older injury, whereas a relatively high concentration of free cTn would indicate a younger injury in a more reversible state of ischemia. Currently with the advent of high-sensitive cTn testing in the US, an assay that can discriminate different cTn isoforms might help distinguish between patients with either persistent and more severe ischemia in type 1 myocardial infarction (MI) who benefit most from immediate percutaneous coronary intervention (PCI), or transient less severe ischemia in the presence of an oxygen supply-demand imbalance with type 2 MI not caused by atherothrombotic plaque rupture.

Previous research has shown that cTn is present in serum as a binary cTnIC complex with free cTnT, little free cTnI, and some ternary cTnTIC complex [11–15]. However, these studies have largely been performed with insensitive methods such as gel filtration and typically in large type 1 MIs. Furthermore, serum may not be the ideal matrix, as thrombin has been shown to cleave cTnT between amino acids 68 and 69 [16]. Recently, Vylegzhanina et al. [17] showed that in plasma samples of MI patients different cTnI complexes exist: (i) 'full-size' cTnTIC ternary complex, consisting of full-size 37 kDa cTnT or its 29-kDa fragment and full-size cTnI of 29 kDa or its 27-kDa fragments; (ii) 'low-molecular weight' (LMW) cTnTIC, in which cTnT is truncated to 14 kDa C-terminal fragments (amino acids > 190 to ~281) and cTnI is either full-size or slightly fragmented (21–25 and 27 kDa); and (iii) binary cTnIC complex, in which cTnI is truncated to ~14 kDa. However, as in most previous studies, only samples with cTn concentrations high enough for evaluation by gel filtration and western blot were analyzed. In the present study, we used highly sensitive immunoassays for determination of different forms of cTnI, *i.e.* total cTnI, complex cTnI (binary cTnIC and ternary cTnTIC), and cTnTIC specifically, and analyzed plasma samples from both type 1 MI patients and patients with non-type 1 MI cTnI elevations, largely demand ischemia and type 2 MI. Importantly, based on the observations by Vylegzhanina et al. [17] and our cTnT antibody specificity (amino acids 171–190), our assay for cTnTIC detects full-size cTnTIC and not LMW cTnTIC.

2. Methods

2.1. Cell culture, cardiac tissue, and immunofluorescence staining

H9c2 rat myoblast cells were acquired from the American Type Culture Collection and cultured according to supplier's instructions. Cells were differentiated by fetal bovine serum (FBS; Axenia BioLogix) reduction to 1% and addition of 50 nM all-trans-retinoic acid (RA; Sigma-Aldrich) according to Suhaeri et al. [18]. Cells were lysed in 1% Triton X-100 in PBS with protease inhibitors (Halt™ Protease Inhibitor Cocktail; ThermoFisher Scientific) and lysates were analyzed for enzymatic activity of aspartate aminotransferase (AST), creatine kinase (CK), and lactate dehydrogenase (LD) using an ADVIA XPT analyzer (Siemens Healthineers). 5× and 25× dilutions with PBS gave excellent linear regression ($R^2 > 0.999$). Enzyme activity was corrected for protein concentration as determined using a Pierce™ BCA Protein Assay Kit (ThermoFisher) according to manufacturer's instructions. For immunofluorescence staining, cells were cultured and differentiated on Lab-Tek® chamber glass slides (Sigma-Aldrich), fixed with 4% paraformaldehyde (PFA) in PBS for 10 min at room temperature (RT), permeabilized with 0.2% Triton X-100 in PBS for 10 min, blocked with 4% bovine serum albumin (BSA) in PBS, sequentially incubated with the primary and secondary (donkey anti-mouse IgG Alexa488; Invitrogen) antibodies in 1% BSA in PBS and 4′,6-diamidino-2-phenylindole (DAPI), and embedded with Prolong Diamond (ThermoFisher). The primary antibodies were mouse monoclonals from Hytest for cTnT (376: binds cTnT amino acid residues 145–164), free cTnI (247: binds cTnI amino acid residues 65–74), and complex cTnI (Tcom8: recognizes cTnIC and cTnTIC, but not free subunits).

Rat cardiac tissue was isolated from an adult athymic nude rat from Harlan and the experimental procedure was approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco (UCSF). The heart was fixed in 4% PFA at 4 °C overnight, and subsequently transferred into 10% sucrose for 1 h, 20% sucrose for 1 h, and then 30% sucrose for 2 h. The heart was then placed in a cryomold filled with OCT and frozen using dry-ice cooled 2-methylbutane. Sections of 10 μm thickness were cut using a cryostat and stained according to the procedure described above.

2.2. Specificity testing of antibody 247 for free cTnI

Specificity of antibody 247 (Hytest) was determined using several analytical techniques.

An indirect ELISA assay was performed by coating free cTnI (recombinant human cTnI; Abcam) or cTnTIC (Hytest) at a concentration of 1 μg/mL in a 0.05 M carbonate-bicarbonate buffer, pH 9.6, on a 96 well high binding polystyrene plate at 4 °C overnight. Subsequently, the plate was washed with PBS and blocked (animal-free blocker; Vector Laboratories), and sequentially incubated with antibody 247 (1 μg/mL in blocking buffer), biotinylated anti-mouse antibody (5 μg/mL in blocking buffer; Vector), and horseradish peroxidase labeled streptavidin (5 μg/mL in blocking buffer; Vector), with 3× PBS washes in between. The substrate 3,3′,5,5′-tetramethylbenzidine solution was added to the plate and after color-formation the reaction was stopped by addition of 2 M H_2SO_3. The plate was read at 450 nm on a Victor Multilabel plate reader (Perkin Elmer).

Surface plasmon resonance (SPR) was performed on a ProteOn XPR36 instrument (Bio-Rad Laboratories) using a GLC sensor chip. Antibody 247 was coupled to the chip by standard amine coupling using a 1:1 activation mixture of 133 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and 33 mM N-hydroxysulfosuccinimide. After activation, antibody 247 (12.5 μg/mL in acetate buffer) was injected over the surface. An acetate buffer with pH of 4.5–5.0 was optimal for coupling as evidenced by the immobilization level measured in response units (RU) (pH 4.5 = 3945 RU, pH 5.0 = 3970 RU, pH 5.5 = 1221 RU; no antibody: 32 RU). 1 M ethanolamine (pH = 8.5)

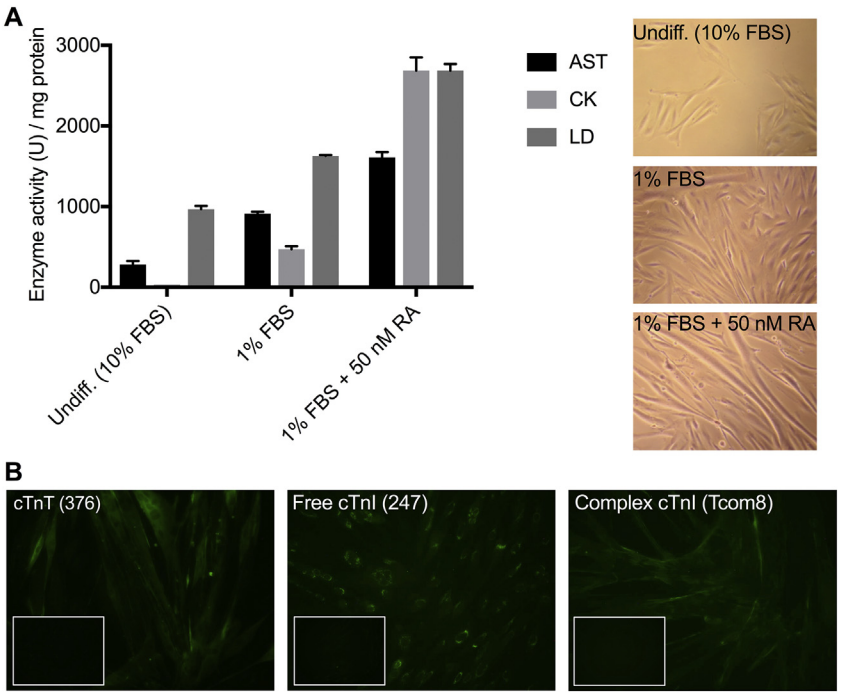

Fig. 1. A) Enzymatic activity of AST, CK, and LD (adjusted for protein content; left) and visible presence of myotubes (right) shows a robust differentiation of H9c2 rat myoblasts towards a cardiac phenotype after culturing in FBS-reduced and RA-supplemented medium. B) Staining for cTnT and cTnI confirms differentiation (inset shows staining of undifferentiated cells). Staining for free cTnI shows a Golgi/ER-like staining.

was used to deactivate the chip surface. Multiple concentrations of free cTnI or cTnTIC spiked in 10% cTnI-free human serum in running buffer (PBS/0.05% Tween20/0.5% BSA) were injected over the chip and run at 25 µL/min for 4 min.

Label-free detection using Pylon analysis (ET Healthcare) was performed by biolayer interferometry [19]. First, a streptavidin polymer was coated to the reflective layer of a pin surface. After a PBS wash, the pin was dipped in a well with biotinylated 247 antibody (20 µg/mL) or a control well without antibody, after which the pin was PBS washed and transferred to a well with or without antigen (2 µg/mL free cTnI or cTnTIC). This was followed by a PBS wash.

2.3. Immunoassay testing of patient and stability samples

Left-over venous blood heparin plasma samples from patients who presented with an increased and rising or falling cTnI concentration at Zuckerberg San Francisco General (ZSFG), San Francisco, CA were collected after all clinical tests were performed. Serial samples from 6 patients presenting with type 1 MI (age range: 52-91y, median: 72y; 5 males, 1 female) and 27 patients presenting with a non-plaque rupture cause of increased cTnI concentration (age range: 44-92y, median: 70y; 17 males, 9 females, and 1 male-to-female transgender) were collected. The majority of the patients at ZSFG with an increased cTnI concentration present with a non-plaque rupture cause, frequently noted in the chart as demand ischemia or type 2 MI. These patients have been grouped here as non-type 1 MI cTnI elevations. The specific etiologies are shown in Fig. 4 and Suppl. Figs. 1–3 in the graph heading for each patient for which MI (type 1 or 2) was not specifically mentioned in the chart. All type 1 and type 2 MIs were NSTEMIs. Samples were kept at 4 °C for 1–4 days before storage at −80 °C. The study was approved by the institutional review board of UCSF.

The Pylon assay (ET Healthcare) for hs-cTnI has been recently described [20]. This assay is performed in unitized test strips and a quartz-glass probe tip coated with capture antibody (purified goat polyclonal antibody recognizing amino acids 27–40) moves between wells [20]. Two additional assays were developed using a capture antibody specific for complex cTnI (cTnIC and cTnTIC). With the exception of the antibodies used, the parameters were the same as for the hs-cTnI Pylon assay. The first assay uses the capture antibody for complex cTnI in combination with the detection antibody for total cTnI used in the total hs-cTnI Pylon assay (a biotinylated mouse monoclonal antibody that binds cTnI amino acid residues 41–49). A second assay uses this capture antibody in combination with an antibody for cTnT (a biotinylated mouse monoclonal antibody that binds cTnT amino acid residues 171–190). Assay specificity was as expected; the first assay recognized both cTnIC and cTnTIC, whereas the second assay only recognized cTnTIC. The total hs-cTnI Pylon assay recognized free and complex cTnTIC equally. All three assays were calibrated with cTnTIC. The limit of blank (LOB) was calculated by the mean and standard deviation of blank samples, in which $LOB = mean_{blank} + 1.645 * SD_{blank}$ (SD: standard deviation). Ten replicates were used to calculate the LOB and precision.

The stability of free cTnI and cTnTIC was evaluated by spiking 100 ng/L free cTnI or ternary cTnTIC in cTnI-free human serum (Hytest). Samples were incubated at 4 or 37 °C and aliquots were taken at 0, 24, 72, 120, and 182 h, frozen at −80 °C and measured simultaneously using the Pylon assays. This experiment was performed once.

3. Results

3.1. Staining for free cTnI is observed in differentiated rat myoblast cells but not in rat cardiac tissue

To investigate the presence and localization of free cTnI in cardiomyocytes and cardiac tissue, we used an antibody specific for free cTnI (antibody 247). We first tested H9c2 rat myoblasts that were differentiated towards a cardiac phenotype by serum reduction and addition of RA. Enzyme activity of AST, CK, and LD in cell lysates, as well as the visual presence of myotubes, indicated a robust differentiation (Fig. 1A). Evaluation of cTn expression included staining using an antibody for cTnT (antibody 376), free cTnI (antibody 247), and complex cTnI (antibody Tcom8: recognizes cTnIC and cTnTIC, but not free subunits). Staining for complex cTnI (Tcom8) and cTnT (376) corroborated the differentiation towards a cardiac phenotype, whereas antibody 247 showed a markedly different staining pattern, consistent with Golgi/ER localization (Fig. 1B; the insets show the lack of staining in undifferentiated cells). Although staining for free cTnI using antibody 247 was observed in differentiated H9c2 cells, it was not readily

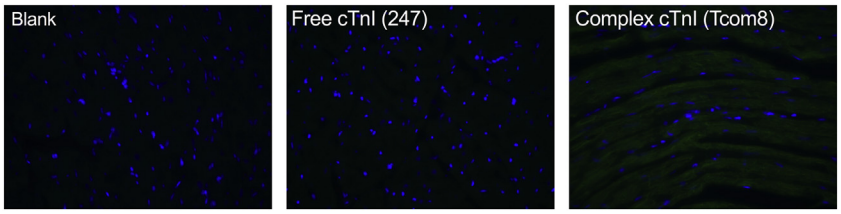

Fig. 2. Staining of rat cardiac tissue shows presence of complex cTnI (cTnIC and cTnTIC), whereas free cTnI is not readily detected. Cellular nuclei are stained in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

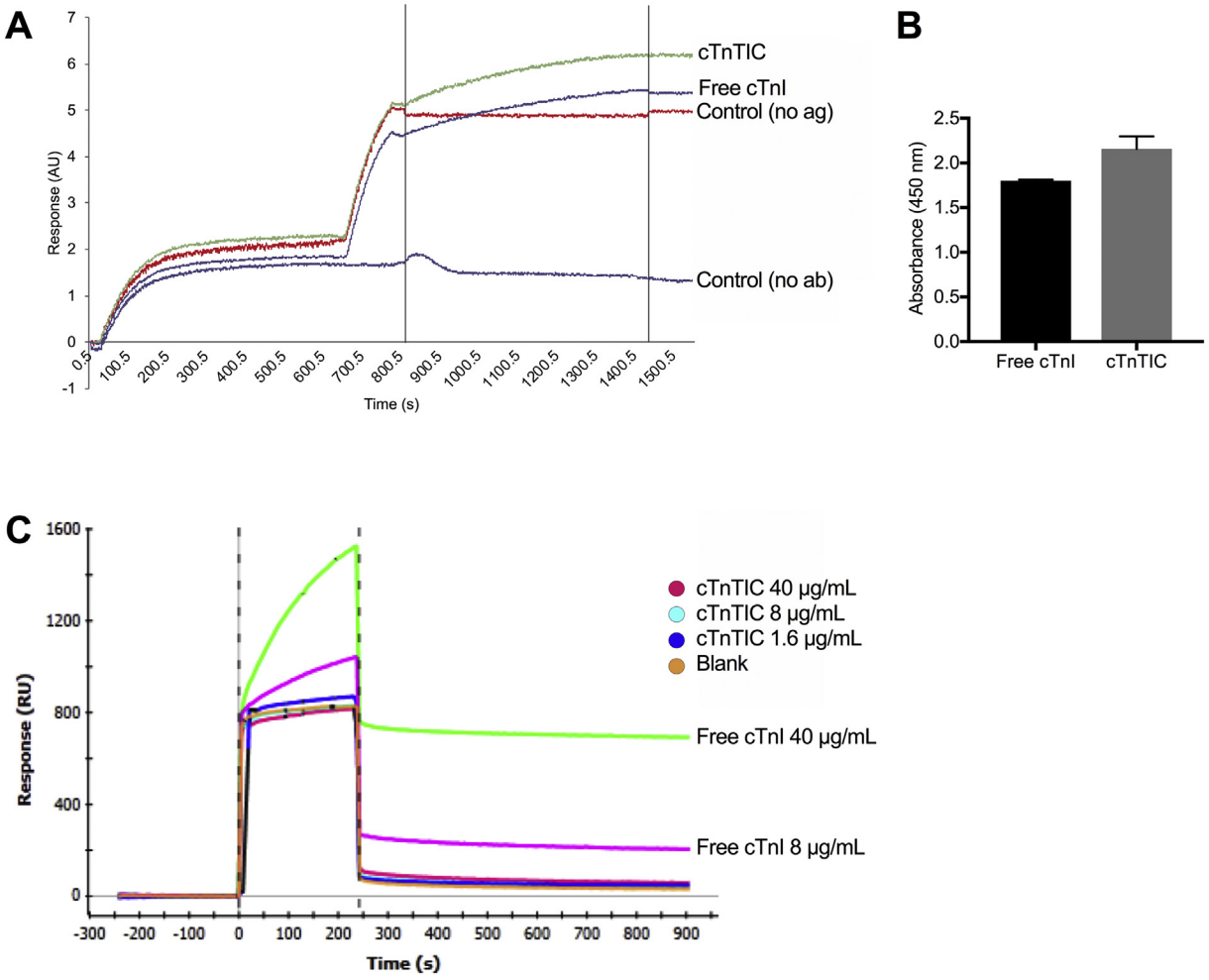

Fig. 3. A) Antibody 247 shows reactivity towards both free cTnI and cTnTIC in a label-free Pylon experiment, as shown by increasing response over time (in between vertical lines). B) Similarly, antibody 247 reacts with both free cTnI and cTnTIC in ELISA. C) In an SPR experiment in which antigen flows over an antibody-coated layer, antibody 247 is specific for free cTnI.

detected in sections of rat cardiac tissue (Fig. 2). This is in contrast to staining for complex cTnI (Tcom8), which clearly identified cTn myofilaments. It is likely that the Golgi/ER-like staining for free cTnI in H9c2 reflects the synthesis of new cTnI for myofilaments in actively differentiating cells, which is not the case in cardiac tissue.

3.2. Antibody 247 specificity testing

Immunoassays for different cTnI forms were developed using a high-sensitive Pylon platform. Notably, in a label-free Pylon analysis antibody 247 bound not only free but also complex cTnI (Fig. 3A). This lack of specificity was also observed in ELISA (Fig. 3B). However, using SPR, we found that this antibody recognizes free cTnI, but not complex cTnTIC (Fig. 3C). Please see the Discussion section for our thoughts regarding this discrepancy.

3.3. cTnI in plasma of patients with cTnI elevations is mainly present as complex cTnI

Given the lack of specificity for antibody 247 in the Pylon system, two immunoassays were developed: one assay specific for complex cTnI (cTnIC and cTnTIC), using antibodies directed against complex cTnI and total cTnI, and one assay specific for cTnTIC, using antibodies against complex cTnI and cTnT. As mentioned in the introduction, based on observations by Vylegzhanina et al. [17], our cTnTIC assay detects full-size cTnTIC and not LMW cTnTIC. An assay for total cTnI on the Pylon system has been previously described [20]. Using these assays, the difference between the results for total cTnI and complex cTnI is interpreted as free cTnI, within the limits of imprecision, and the difference between complex cTnI and cTnTIC as cTnIC together with LMW cTnTIC. The LOBs of the two new assays were similar to that of the assay for total cTnI (0.7–0.8 ng/L [20]): 1.2 and 0.9 ng/L, for the

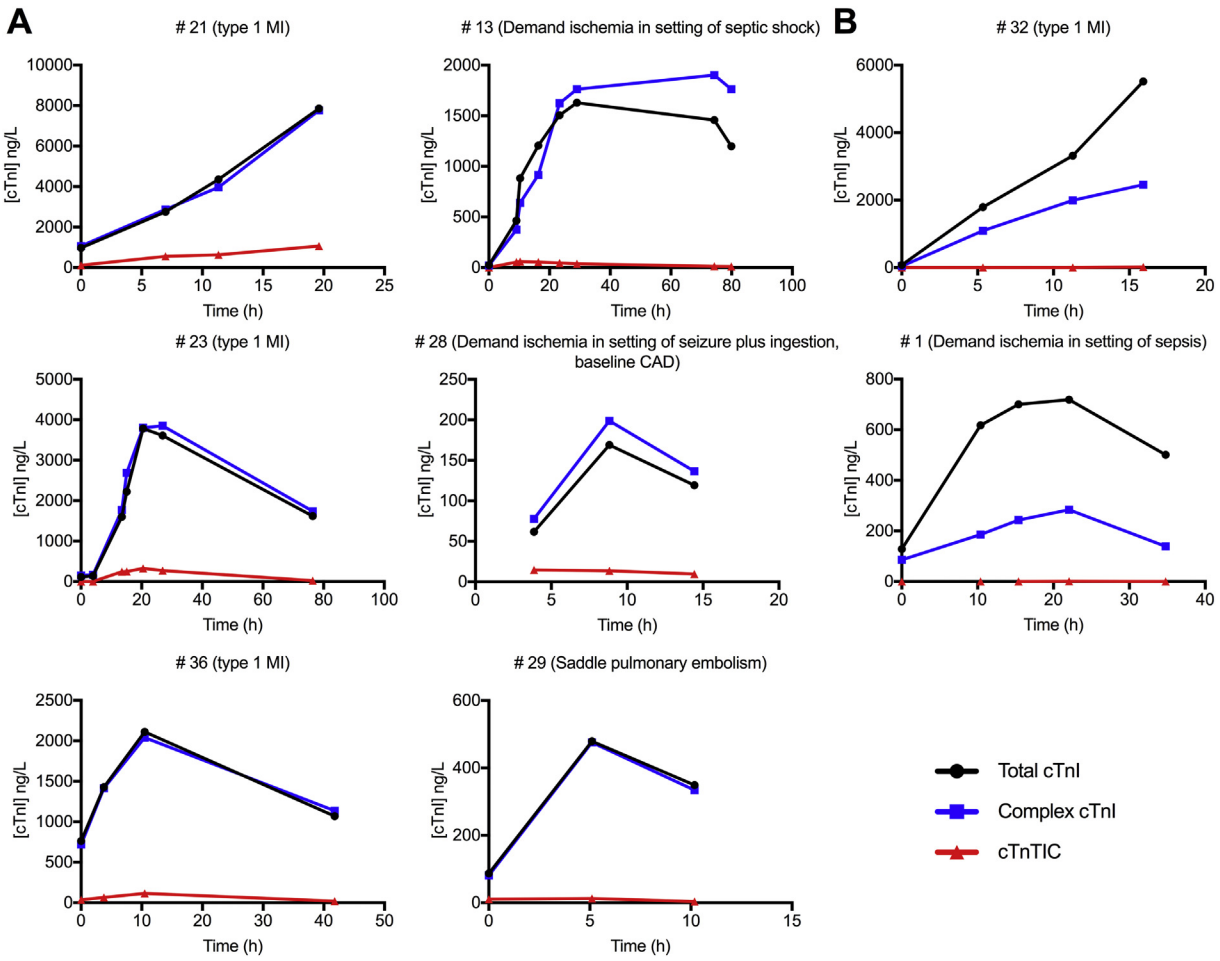

Fig. 4. A) Examples of serial samples from patients with type 1 MI or cTnI elevations due to other etiologies (each shown in the graph heading) analyzed for total cTnI, complex cTnI (cTnIC and cTnTIC), and cTnTIC using Pylon assays. Time is shown as hours (h) after first sample collection. Samples of the remaining patients are shown in Suppl. Figs. 1–3. The majority of cTnI is complex, whereas findings in only two cases (#1, #32) (shown in B) suggested the presence of significant free cTnI.

complex cTnI assay and cTnTIC assay, respectively. The recovery and imprecision at 20 ng/L were 92% and 9.0%, and 89% and 8.2%, for the complex cTnI assay and cTnTIC assay, respectively. This is in line with the recovery and imprecision profile for the total cTnI assay (recovery: 91–107% at 20 ng/L; imprecision: 10.1% CV at 24 ng/L and 9.2% at 13 ng/L, and from best-fit line: 10% CV at 10 ng/L and 20% CV at 2 ng/L [20]).

132 samples from 33 patients with cTnI elevations (6 type 1, 27 due to other etiologies) were analyzed using the Pylon assays. Assays for total and complex cTnI tracked well with each other over time and gave similar results, for both type 1 MI and other cTnI elevations (6 representative examples are given in Fig. 4A, others are shown in Suppl. Figs. 1–3). Interestingly, only 2 specimen sets, #32, a type 1 MI, and #1, a non-type 1 MI cTnI elevation, showed a difference that would indicate the presence of significant free cTnI (Fig. 4B). Not including these two sets, the results for total cTnI and complex cTnI showed good correlation (Fig. 5A; Pearson r and R^2 using linear regression are 0.983 and 0.965, respectively). Full-size ternary complex (cTnTIC) was usually detected at a low concentration and was less well correlated with total cTnI (Figs. 4A, Suppl. Figs. 1–3, and Fig. 5B; Pearson r and R^2 using linear regression are 0.903 and 0.815, respectively), possibly due to limited stability and variable dissociation. Overall, these data suggest that in samples of both type 1 MI patients and patients with other cTnI elevations, cTnI is mainly present as cTnIC and/or LMW cTnTIC. A second evaluation method that consisted of complex cTnI removal using magnetic beads confirmed in an evaluated subset of patients that the majority of cTnI is present as complex cTnI and also suggested the presence of significant free cTnI in cases #1 and #32 (Suppl. Text and Suppl. Fig. 4).

3.4. Full-size cTnTIC is more abundant in early than in late samples

To determine whether time-dependent changes were present, early *vs.* late samples were compared. An early sample was defined as the first sample with a known rising cTnI concentration, usually the second sample. A late sample was defined as the last sample with a falling cTnI concentration, usually the last sample. Thirteen patients, of which three type 1 MI, were omitted for this analysis as there was no clear pattern of rising *and* falling cTnI concentrations. Two patients (#6 and 9) were omitted for sensitivity reasons, as the late sample total cTnI concentration was < 50 ng/L (20 and 36 ng/L for #6 and 9, respectively), which requires cTnTIC to be ~4% or greater to be detected. For complex cTnI, there was no difference for early *vs.* late samples, however, early samples had proportionally more full-size cTnTIC than late samples (mean 7.0 *vs.* 3.3%) (Fig. 6A). Given that three type 1 MI patients (#21, 32, and 34) did not show a rising and falling pattern and were omitted, we analyzed both type 1 MI and non-type 1 MI etiologies together; omittance of all type 1 MI samples resulted in non-significant difference in full-size cTnTIC for early *vs.* late samples (mean 6.0 *vs.* 3.5%, $p = 0.13$).

3.5. Free cTnI and full-size cTnTIC have limited stability at 37 °C

To determine stability, free cTnI or ternary cTnTIC was spiked into

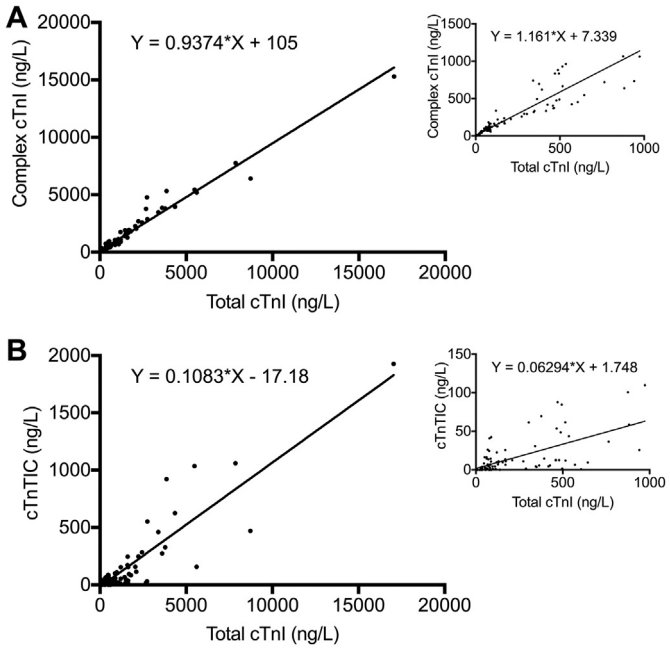

Fig. 5. Total cTnI in type 1 and 2 MI patients (excluding #1 and #32) correlates well with complex cTnI (A), but less well with cTnTIC (B). Deming regression analysis using GraphPad Prism is shown. Linear regression gave similar results: A (y = 0.922x + 120.47, Pearson $r = 0.983$, $R^2 = 0.965$), B (y = 0.108x - 16.891, Pearson $r = 0.903$, $R^2 = 0.815$). Insets show data over 0–1000 ng/L total cTnI.

commercially available cTnI-free serum and incubated at 4 or 37 °C. The cTnI composition was then measured using the Pylon assays at multiple time points (Fig. 6B). Whereas free cTnI was relatively stable refrigerated, after 24 h at 4 °C, 40% of ternary cTnTIC complex was no longer intact and had most likely degraded into LMW cTnTIC and/or binary cTnIC. At 37 °C, both free cTnI and cTnTIC were not stable.

4. Discussion

Much has been discussed on the difficulty of diagnosing patients with type 2 MI, which has raised a clinical demand for laboratory tests that may help distinguish type 1 MI from other etiologies of cTnI elevation, such as demand ischemia and type 2 MI [21]. Based upon the notion that the pathophysiological mechanisms of cTn release in patients with demand ischemia and type 2 MI are more akin to the situation of increased myocardial blood flow during extreme exercise, we hypothesized based on the theory of cytosolic cTn release by Hickman et al. that the constitution of circulating cTn might significantly differ between patients with type 1 MI and cTnI release due to other etiologies, particularly demand ischemia and type 2 MI. Despite the supposed pathophysiological difference regarding coronary flow, we found that cTnI in both type 1 MI and other etiologies of cTnI elevation was mostly complexed (binary cTnIC and/or LMW cTnTIC) with some full-size cTnTIC. Therefore, based on the cTn constitution in the circulation we could not distinguish between cTnI release due to type 1 MI or other etiologies such as demand ischemia or type 2 MI. In addition, being primarily part of complex cTn, the cTnI that we found is more likely to be myofibrillar troponin.

The Pylon assay is the first immunoassay that can distinguish cTnIC and ternary complexes from total cTnI at an LOB down to ~1 ng/L. This makes it the first high-sensitive cTn assay that can distinguish different cTnI forms, with an assay sensitivity at least ten times higher compared

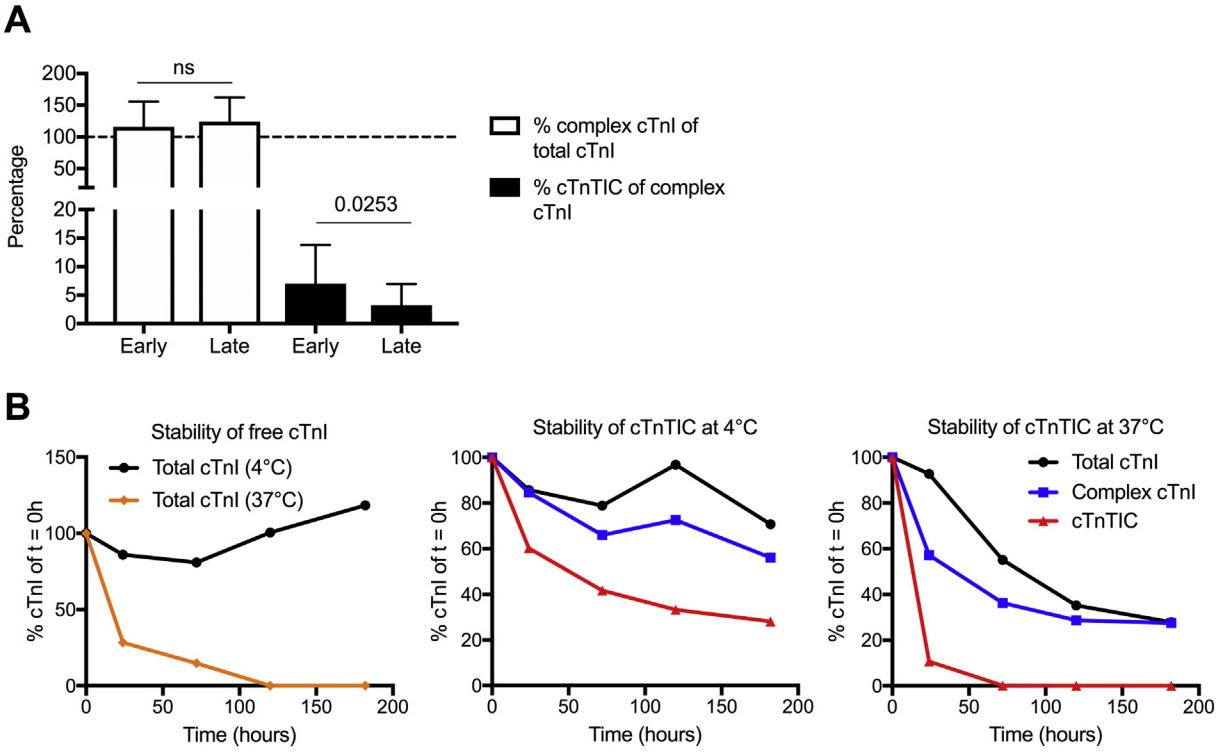

Fig. 6. A) The percentage complex cTnI (LMW cTnTIC and cTnIC) is similar for early (first sample with rising cTnI concentration) and late (last sample with falling cTnI concentration) samples, whereas the percentage full-size cTnTIC is higher in early samples compared to late samples. Patients #8, 10, 11, 14, 16, 17, 19, 21, 25, 30–32, and 34 were omitted as there was no clear rising and falling pattern of total cTnI. Patients #6 and 9 were omitted as the total cTnI concentration of the late sample was < 50 ng/L. Mean with SD is shown. Statistical analysis was performed using a paired *t*-test in GraphPad Prism ($n = 18$). B) Stability of free cTnI and cTnTIC complex spiked into commercially available cTnI-free serum at 4 °C and 37 °C. This experiment was performed once.

to previous immunoassays that can distinguish different cTnI forms [13,14]. Our results are in accordance with findings from immunoassays described by Giuliani et al. in patients with type 1 MI and unstable angina pectoris, who concluded that circulating cTnI mostly consists of cTnIC and undetectable or low concentrations of cTnTIC [13,14]. However, as they used an anti-cTnT antibody directed towards the N-terminus (mAb 7G-7 recognizing amino acids 60–71), based on recent work of Vylegzhanina et al. [17] as outlined in the introduction, the assay by Giuliani et al. detected full-size cTnTIC, and not LMW cTnTIC, similar to our assay [13]. Technically, Giuliani et al. used an IT-TIC assay that also detected IT binary complexes, however, cTnI and cTnT undergo only partial complex formation (see later in the Discussion). Bates et al. concluded that cTn was present predominantly as binary cTnIC and free cTnT, with cTnTIC as a minor constituent in only 4 of 10 MI patients [15]. Similarly, Wu et al. found that cTn was present in serum from two MI patients as cTnTIC, cTnIC, and free cTnT [12]. Bates et al. and Wu et al. used gel filtration and as shown by Vylegzhanina et al. [17] peaks of binary cTnIC and LMW cTnTIC overlap. Thus, from studies by Giuliani et al., Bates et al., and Wu et al., we can conclude that cTnI is mostly complexed, with full-size cTnTIC as a minor constituent in some MI patients. However, those studies have been performed with serum samples and thrombin has been shown to cleave cTnT at the N-terminus between amino acids 68 and 69 resulting in a 29 kDa fragment [16]. In case of the immunoassay by Giuliani et al., the use of serum almost certainly affected their ability to detect cTnTIC as their anti-cTnT antibody recognized amino acids 60–71. As our assay recognizes cTnT amino acids 171–190, use of serum should theoretically not affect the ability to detect cTnTIC. Gel filtration results by Bates et al. and Wu et al. may have been affected by using serum, however this is not as clear as full-size cTnTIC contains both 37 kDa full-size cTnT and 29 kDa cTnT [17].

Vylegzhanina et al. [17] recently found, through a combination of immunoassays specific for different cTn forms and gel filtration, a significant amount of full-size cTnTIC in plasma samples of 5 MI patients. Based on the gel filtration data for a representative patient shown in their Fig. 5B, we estimate that this is ~1/3 of total complex cTnI (*i.e.* full-size cTnTIC, LMW-cTnTIC, binary cTnIC) in an early sample (6 h). In a late sample (30*h*) of this patient, full-size cTnTIC had almost all degraded to a combination of LMW cTnTIC and binary cTnIC. They found that when looking at cTnTIC (full-size and LMW) the proportion of full-size cTnTIC decreased over time, with a consequential increase in LMW cTnTIC. This is in line with their earlier work [22] that showed that anti-cTn autoantibodies inhibit immunodetection of cTnTIC, but not of cTnIC or free cTnI, and that this inhibition by anti-cTn autoantibodies is greater in early (4-9 h after symptom onset) compared to late (22-36 h) MI samples, suggesting that cTnTIC is proportionally more abundant in samples early after MI onset.

Thus, the above-mentioned studies indicate that the cTnI in type 1 MI is predominantly present as complex cTnI, of which the majority is LMW cTnTIC and cTnIC. Furthermore, the proportion of full-size cTnTIC in type 1 MI decreases over time. This is in line with the data presented here. We noticed the biggest difference in early *vs.* late samples for type 1 MI patients (12.0 *vs.* 2.0%, $n = 3$, $p = 0.11$), although not statistically significant likely due to the low sample size, and when only non-type 1 MI patients ($n = 15$) were analyzed, the early *vs.* late difference was not significant. This may indicate that the proportional decrease in full-size cTnTIC is more pronounced in type 1 MI patients or our group of non-type 1 MI patients is too heterogenous, or both, although future studies are necessary to further explore this. Regarding group heterogeneity, analysis of patients with demand ischemia or type 2 MI only also showed no statistically significant difference in early *vs.* late samples for full-size cTnTIC (7.2 *vs.* 3.9%, $p = 0.13$, $n = 11$).

Our mean of 7.0% (range: 0–20.9%, interquartile range: 2.7–14.0%) full-size cTnTIC in early samples is somewhat lower than the representative early sample presented by Vylegzhanina et al. [17]. We consider three major possible reasons for this difference: i) our study was performed with 'left-over' clinical samples and to allow for the possibility of add-on tests, samples were kept refrigerated for the first day and in a few cases were kept refrigerated for up to 4 days. As shown in Fig. 6B, this has likely caused us to underestimate the concentration of full-size cTnTIC by a factor ~2. ii) Timing in our study was based on the time of the first cTnI sample. We do not know how fast after the time of onset the first sample was collected. iii) Whereas Vylegzhanina et al. analyzed only type 1 MI patients, we analyzed predominantly patients with cTnI elevations due to other etiologies.

It is important to highlight here that we observed substantial *in vitro* changes with regard to the different cTnI forms even within 24 h at 4 °C storage. Future studies that analyze different forms of cTnI, or cTnT, should take this into account. Immediate storage at −20 or −80 °C after collection and/or rapid analysis may be required, or alternatively protease inhibitors may be necessary to halt *in vitro* cTn changes.

In vitro studies with bovine cTn have shown that cTnI and TnC form a stable binary complex, cTnT and TnC do not interact significantly and cTnI and cTnT undergo only partial complex formation [23]. Whether myofibrillar cTn is released as full-size cTnTIC and subsequently degrades to LMW cTnTIC, cTnIC and free cTnT in the circulation, is released as LMW cTnTIC and/or cTnIC due to intracellular degradation by proteases, or both, is still to be determined. Both intracellular dissociation of the cTn complex [24] and peripheral degradation of cTnTIC to cTnIC and free cTnT have been previously suggested [25]. Our stability data presented here would suggest that at least some peripheral degradation of full-size cTnTIC occurs. Samples from MI patients are known to contain multiple cTnI proteolytic fragments [26] and cTn is known to be degraded by intracellular proteases such as caspases and calpains [27]. It was recently shown that the ratio of full-size cTnI to its major fragments does not change much over time, which was the basis of the suggestion that degradation occurs primarily within the myocardium and not in the blood [26]. Interestingly, Zahran et al. [28] recently showed that based on the extent of C-terminal cTnI degradation one could distinguish type 1 STEMIs from type 1 NSTEMIs and type 2 MIs. They hypothesized that this is because cTnI is more proteolyzed by intracellular proteases in case of a more focal, intense area of infarction.

Our findings of little free cTnI in plasma, as inferred from the Pylon and magnetic bead assays, are also in accordance with earlier studies in patients with type 1 MIs [11,12,15]. As a consequence of inherent imprecision in the assays for complex and total cTnI, we were not able to confidently detect a low (*i.e.* < ~20%) percentage of free cTnI and there may have been small differences in free cTnI between type 1 and non-type 1 MI that we were not able to directly measure. In two of our cases there was a substantial difference between total cTnI and complex cTnI that suggested the presence of free cTnI. However, as we were not able to measure free cTnI directly, it was not possible to determine whether this was indeed the case or whether other factors, *e.g.* modification or degradation of key amino acids in the antibody epitope, resulted in the reduced reactivity of the complex cTnI antibody in these cases. Another issue to consider is the limited stability of free cTnI at 37 °C. It is possible that we underestimated the amount of free cTnI, as free cTnI is not as stable as cTnI as part of the cTnTIC complex (compare 24 h stability of total cTnI for free cTnI and cTnTIC in Fig. 6B, *i.e.* 28% *vs.* 93% recovery). Finally, even though we saw a rising cTnI pattern in most patients, as we do not know the timing of the first sample collection with respect to the time of onset, it is very well possible that the first sample was already 'too late' to detect the presence of free cTnI.

Lack of specificity of antibody 247 for free cTnI in the Pylon assay prevented direct measurement of free cTnI. In contrast to the SPR system, in which new sample constantly flows over an antibody-coated chip, in the Pylon and ELISA systems the antibodies and sample are contained in a single well. We believe that complex cTnI is in equilibrium with (a small amount of) free cTnI and that in case of the Pylon

and ELISA systems, antibody 247 can compete with TnC for binding of free cTnI. If the affinity of antibody 247 for free cTnI is greater than that of TnC for free cTnI, this effectively shifts the equilibrium towards 247-cTnI complexes. As immunofluorescence was performed on fixed samples, this concept would not have applied there.

Experimental evidence for free cTn cytosolic pools is mainly based on the findings of extraction experiments with cardiac tissue from humans and animals, including rats [1–4]. In these studies, the free fraction was defined as the limited amount of cTn that could be extracted using a cold low-salt buffer. As mentioned in the introduction, Starnberg et al. already challenged the concept of a free cTnT pool by showing that the solubility of cTnT was very low in these cold low-salt buffers and up to 80% of cTnT can be extracted from cardiac tissue using large volumes of serum at 37 °C [5]. Thus, the non-irreversible bound, or diffusible, fraction is likely much larger than previously thought and probably consists of myofibrillar cTnT interacting reversibly with tropomyosin. Our findings of free cTnI staining in H9c2 cardiac differentiated myoblasts reflect a Golgi/ER-like staining pattern that could be explained by a high synthesis rate of cTn which is to be expected in actively differentiating cells. Our inability to detect free cTnI in rat cardiac tissue suggests it is either not present in terminally-differentiated cardiac tissue, or its presence cannot be easily confirmed by immunofluorescence due to low amounts. Recent studies in swine have shown that delayed cTnI release can occur in the setting of brief, 10 min, ischemia (traditionally considered as completely reversible), or increased left ventricular preload, in the absence of tissue necrosis; however, TUNEL staining demonstrated apoptosis of isolated myocytes [29,30]. In those studies, the cTnI concentration was the highest 24 h after the insult, *i.e.* brief ischemia or transient increase in preload, which is not consistent with an early release due to blebbing from an exchangeable, unbound, or free pool. These results, as well as our findings of primarily complex cTn that is likely to be of myofibrillar origin, are in direct contrast with the theory by Hickman et al., which hypothesizes that cytosolic cTnI may be released in the absence of cell death. These results suggest that cTnI may be released in the absence of detectable tissue necrosis, but not in the absence of cell death.

In conclusion, in agreement with type 1 MI, our data indicates that cTnI is present in plasma of patients with cTn elevations due to other etiologies, such as demand ischemia and type 2 MI, as complex, of which the majority is LMW cTnTIC and/or cTnIC and the minority is full-size cTnTIC complex, and in general with little free cTnI. It is likely that LMW cTnTIC and cTnIC originate from degradation of myofibrillar full-size cTnTIC, whether intracellular or in the circulation, or both. Free cTnI and cTnI immunoreactive fragments may originate from further degradation of the LMW cTnTIC and cTnIC complexes or release of free cTnI. However, the evidence for the presence and release of free cTnI in the cytosol remains limited and free cTnI was also not readily detected in rat cardiac tissue using immunofluorescence in this study. Future clinical studies that analyze samples taken closer to the site of release, *e.g.* from the coronary veins, with a larger number of patients and controlled timing may help to clarify the location of degradation (intracellular *vs.* peripheral) and may determine the clinical applicability of an assay specific for full-size cTnTIC. Studies that perform immunohistochemistry for free cTnI in human myocardium and assays that are able to directly measure free cTnI with high sensitivity may be necessary to confidently determine whether or not free cTnI is present and released from the human heart.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2019.06.012.

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