Review

Pathophysiology of Cardiac Troponin Release and Circulating Cardiac Troponin Forms – Potential Clinical Implications

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Abstract: Due to the high analytical sensitivity of current routine high-sensitivity cardiac troponin assays, unexpectedly elevated test results without an obvious clinical correlate are increasingly frequent in daily clinical practice. In these patients, myocardial injury may sometimes go undetected by imaging, including cardiac magnetic resonance imaging. This has led to an increased interest in the pathophysiology of cardiac troponin release, in particular on the fact, whether troponin can be released from reversibly injured myocardium and thereby resulting in an increase of cardiac troponin in the systemic circulation. On the other hand, a variety of circulating cardiac troponin forms were described in human blood samples using different analytical methods, raising the question whether the cause of myocardial injury can be reliably determined by measurement of specific circulating cardiac troponin forms. This review aims to provide an up-to-date overview of this current cardiac troponin research topics.

Keywords: cardiac troponin; myocardial injury; pathophysiology; release; circulating forms; myocardial infarction

1. Background

Alongside the electrocardiogram (ECG), laboratory parameters for the detection of myocardial injury have been a cornerstone in the clinical assessment of patients with suspected acute myocardial infarction (AMI) since the 1950s [1–3]. The landscape of cardiac biomarkers has evolved dramatically since then. For the routine laboratory diagnosis of myocardial injury, cardiac troponin I (cTnI) and cardiac troponin T (cTnT) emerged as the laboratory parameters of first choice, because they are currently the most sensitive and cardiac-specific laboratory parameters available for routine laboratories [1–6]. However, due to the high analytical sensitivity of current high-sensitivity (hs) cardiac troponin (cTn) assays, the positive predictive value for AMI of hs-cTn is markedly lower compared with previous cTn assay generations, and even unexpectedly elevated cTn test results without an obvious clinical correlate are increasing in daily clinical practice [4–6]. In the vast majority of these patients, these cTn elevations are caused by acute or chronic myocardial injury due to a variety of cardiac or primarily non-cardiac pathologies with cardiac involvement unrelated to an AMI [3–6]. In some of these patients, myocardial injury may even go undetected by imaging because of the higher sensitivity of hs-cTn assays, which can detect cTn even in the femtomolar range in blood samples [3–6]. The huge variety of possible mechanisms underlying myocardial injury has been extensively reviewed [1–7]. The most frequent causes are summarised in Table 1. They include, myocardial ischaemia caused by an acute coronary syndrome (ACS; i.e. coronary plaque rupture or erosion with intracoronary thrombus formation; type 1 AMI), myocardial ischaemia or hypoxia unrelated to an ACS (type 2 AMI), and myocardial injury unrelated to myocardial ischaemia or hypoxia, such as inflammation (e.g. in myocarditis), increased myocardial wall stress (e.g. in heart failure), toxic myocardial injury and trauma (e.g. cardiac contusion) [4,5]. Analytically false positive test results are very rare [6,8,9].

Table 1. Differential diagnosis of cardiac troponin increases in peripheral blood samples - cardiac diseases and primarily non-cardiac diseases leading to myocardial injury.

However, many aspects of intramyocardial cTn degradation, tissue release, and degradation within and clearance from the human circulation are still incompletely understood. The aim of this review is to provide an update on what is known about cTn degradation and circulating forms of human cTnI and cTnT. Gaps in current knowledge and potential future applications of the measurement of different circulating cTn forms for the differential diagnosis of underlying diseases leading to myocardial injury are highlightened.

2. Pathophysiology of Cardiac Troponin Release from Injured Myocardium

The cTn complex is part of the thin filaments of the myocardium, and its biochemistry and pathophysiology have been extensively reviewed previously [10–12]. In summary, the cTn complex plays a central role in calcium dependent regulation of actin thin filament function, it is essential for control of striated muscle contraction. Only troponin I (TnI), the actomyosin adenosine triphosphatase inhibitory subunit, which confers calcium-sensitivity to striated muscle contraction (about 24 kDa), and troponin T (TnT), the tropomyosin binding subunit (about 35-36 kDa depending on the addional cTnT isoform variation, which is a result of alternative splicing), but not troponin C (TnC), the calcium binding subunit, which initiates the sequence of conformational changes on the thin filament, (about 18kDa), are encoded as cardiac-specific isoforms (cTnI and cTnT) in humans (see Table 2) [13–17]. cTnT and cTnI have a cardiac specific N-terminal extension.

Table 2. Biochemistry of cardiac troponin isoforms in humans.

Isoform	Gene, location	Expression	Aminoacids	Molecular mass
cTnC	TNNC1, 3p21.1	Heart, slow-twitch skeletal muslce	161	18.4 kDa
cTnI	TNNI3, 19q13.42	Heart	210	24 kDa
cTnT	TNNT2, 1q32.1	Heart, skeletal muscle (fetal period, chronic injury)	287-298*	about 34-36 kDa

See references [13–17]. *There are several cTnT isoforms produced by alternative splicing, the major isoform in normal human hearts is isoform 6 (cTnT3), 34.6 kDa [17]. Abbreviations: cardiac troponin C (cTnC), cardiac troponin I (cTnI), cardiac troponin T (cTnT), kilo Dalton (kDa).

Initially, small (approximately 5% of the total content) "cytosolic" pools of cTnI and cTnT were reported in human myocardium [18–20]. However, considering the preparation protocols used by the investigators and the poor solubility of cTnI and cTnT in the hydrophilic sarcoplasma, a more appropriate term may be "rapidly degraded, loosely bound, early releasable pool". When not incorporated into myofilaments, e.g. cTnT is rapidly degraded (e.g. by caspase or μ-calpain) within cardiomyocytes to avoid toxic effects [21–24]. However, later data suggest that the fraction of cTnT that can rapidly dissociate from myofibrils in vivo is substantially higher than the 5-10% previously reported [25]. Thus, all cTn released is likely to be of myofibrillar origin.

A variety of factors (e.g. molecular mass, concentration gradient from cardiomyocytes to interstitial space, local blood and lymphatic flow) influence the onset of release of cardiac markers from injured myocardium and the subsequent increase in the systemic circulation. The most important factor appears to be intracellular compartmentation [20,26,27]. Myocardial necrosis is

preceded by a substantial reversible pre-lethal phase [28]. In contrast to cytosolic proteins, the release of structurally bound molecules, such as cTns, requires both a leaky plasma membrane and dissociation from or degradation of cellular structures, such as the contractile filaments, and this is a slower process. In addition, the susceptibility of a structural protein to early intracellular degradation by cytosolic proteases, such as the cytoplasmic calpains or caspases, which are rapidly activated during myocardial injury [21–24], is of great importance. In contrast, lysosomes are stable within the first 3-4 hours after the onset of cell injury, and therefore, lysosomal enzymes are not involved in the early degradation of structurally bound proteins.

Both cTnI and cTnT are substrates of the cytoplasmic enzyme μ-calpain [21–24], which is activated by increased cytoplasmic calcium, an important and early feature of cell injury. This occurs through increased influx and redistribution of calcium from intracellular compartments (e.g. endoplasmic reticulum, mitochondria). Increased cytoplasmic calcium also activates phospholipases and endonucleases [29]. pH-dependent dissociation of the troponin complex may be another important factor in early cTn release from injured myocardium (see Figure 1).
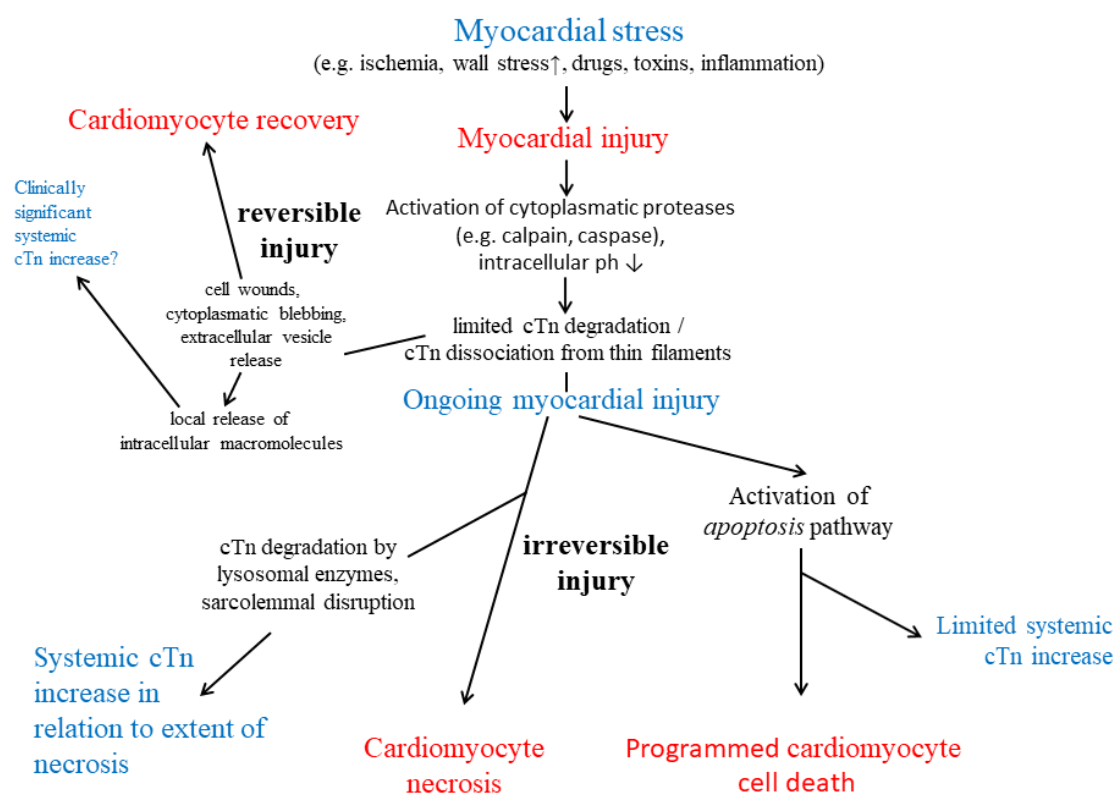

Figure 1. Potential cardiac troponin release mechanisms from injured human myocardium. Three possible mechanisms of cardiac troponin increase in the systemic circulation are summarized, i.e. cardiomyocyte necrosis, which is the only generally accepted cause, cardiomyocyte apoptosis, and reversible myocardial injury by cTn leakage via a temporarilly leaky pllasma membrane (via cell wounds, cytoplasmatic blebbing, extracellular vesicle formation).Abbreviations: cardiac troponin (cTn).

The pathophysiological background of the limited N-terminal intramyocardial proteolysis of cTn by caspase and calpain may be that cTns are involved in the sensitivity of the myocardium to decreases in intracellular pH, and thus, this may be an early specific functional adaptive response to myocyte injury rather than a simple destructive degradation [21–24]. cTnI is also a substrate of caspase-3, which has a key role in apoptosis, it targets the N-terminal region of cTnT as well [24].

Additional proteolysis of troponins may occur in the interstitial space e.g. by matrix metalloproteinase-2 [30].

Once the integrity of the cardiomyocyte plasmalemma is disrupted, cardiomyocytes rapidly release intracellular macromolecules into the interstitial space. In addition, cytoplasmic blebbing may already occur during the reversible phase of cell injury [28]. These blebs contain intracellular macromolecules, such as degraded cTn, and detach with resealing of the plasma membrane without cell death in cell culture experiments and may be responsible for limited cTn release from cardiomyocytes [28]. In contrast, sarcolemmal disruption with subsequent massive release of intracellular molecules into the interstitial space is thought to morphologically indicate the "point of no return" of cell injury, i.e. the occurrence of necrosis.

In addition, intramyocardial posttranslational modifications of cTn occur, such as oxidation or phosphorylization, which may enhance or inhibit the susceptibility to proteolysis [21,23,24,31–33]. Thrombin-mediated cTnT degradation at the N-terminal end has been describd as well [34], which could be involved in the degradation of circulating cTnT in blood and has to be considered when testing serum samples. The currently commercially available routine hs-cTnT assay, however, utilizes antibodies which target to the amino acid 125-147 fragment is not affected by thrombin-mediated cTnT degradation [35].

In summary, cTn degradation in response to myocardial injury starts within cardiomyocytes. cTnI and cTnT show a rapid release after myocardial injury, comparably fast to cytosolic proteins, such as myoglobin or creatine kinase isoenzyme MB [19,20,26,36–38]. The sustained increase in cTn after myocardial infarction is probably a combination of slow washout and prolonged local tissue degradation. Unrestricted blood flow, e.g. from invasive early reperfusion of the infarct-related coronary artery, results in more rapid extraction and clearance of cTn from damaged myocardium [27,39,40]. The only generally accepted mechanism of cTn release into the systemic circulation in humans currently is myocardial necrosis.

Experimental data on cTn release from reversibly injured myocardium

There is evidence from experimental in-vitro models, that macromolecules including cTn are released without histological evidence for cardiomyocyte necrosis [28,41–45]. These observations could be confirmed by more recent thoroughly conducted large animal studies in pigs by Weil et al. [46,47], which confirm previous in-vitro and in-vivo reports. After applying only 10 minutes of left anterior descending artery (LAD) occusion cTnI increases were found in a porcine heart model, which was associated with reversible myocardial dysfunction but without evidence of myocardial necrosis in tissue analysis [46]. Apart from ischemia or hypoxia, mechanical stretch in response to pressure or volume overload may trigger the activation of proteases associated with intracellular troponin degradation and release of troponin fragments from injured cardiomyocytes without evidence of necrosis despite reversible ventricular dysfunction [47]. In this model, intravenous phenylepinephrine infusion lead to preload-induced myocardial injury by acutely increased wall tension with apoptosis but without signs of necrosis [47]. In addition, it was previously reported in cell culture experiments, that tachycardia may stimulate integrins, triggering release of cTnI in the absence of necrosis [42], and additionally the cTn content of cardiomyocytes already decreases before necrosis occurs [48].

Thus, reversible myocardial injury could be also an option of small, limited cTn increases in humans using hs-cTn assay for testing. However, there is often a contiuum of apoptosis and necrosis in response to myocardial stress, and the lack of histological data and the limited sensitivity of imaging compared with hs-cTn testing will make it very difficult or even impossible to prove cTn release from reversibly injured myocardium in humans [49,50]. It has been postulated that for every 1 μg of human myocardium injured, the cTnI and cTnT concentrations increase by approximately 4 ng/L in peripheral blood samples [51].

Clinical data suggesting possible cTn release from reversibly injured myocardium

From the publication of the first research radioimmunoassay for the detection of cTnI [52] the analytical sensitivity of present hs-cTn assays improved dramatically from 500 ng/L to approximately

1-3 ng/L [35]. This advancement in the analytical performance of cTn assays with maintained cardiospecificity lead to a dramatic improvement in the clinical sensitivity for the detection of myocardial injury, which could not be imagined at the beginning of clinical cTn research. Originally, cTn could not be detected in healthy individuals, but with hs-cTn testing cTn can be detected in >50% of healthy individuals [51]. As analytical interferences have been ruled out [53,54], detectable hs-cTn concentrations in humans with normal hearts suggest a constant limited turnover of cTn and/or cardiomyocytes with cTn release into the systemic circulation. In a functioning sarcomere, protein synthesis, processing, and degradation occur continuously as a part of physiological turnover [55]. cTnT undergoes rapid turnover with a half-life of approximately 3.5 days within cardiomyocytes [56]. If not incorporated into myofilaments, cTnT is rapidly degraded to avoid toxic effects [55,56].

As mentioned previously, it is probably impossible to prove cTn release from reversibly injured myocardium in humans. However, there are increasing reports in the literature on cTn release in clinical scenarios in which myocardial necrosis is very unlikely [57–61]. For example, cTnI release measured by a very sensitive assay has been demonstrated in vivo in humans following nuclear perfusion scintigraphy with peak concentrations associated with the extent of myocardial ischemia during stress testing [57]. In addition, it has been reported that even normal individuals have elevated circulating cTn levels in response to dobutamine stress or exercise testing [58,59]. Small, but significant cTn increases in coronary sinus blood samples within 30 minutes were found after brief episodes of incremnental rapid atrial pacing using a protocol with comparable myocardial stress to moderate brief physical exercise [60]. This cTnT release in coronary sinus blood was mirrowed subsequently in a delayed, small but significant hs-cTnT concentration increase in peripheral blood samples after 3 hours (doubling to tripling of baseline values) even in the subgroups without significant coronary artery disease (CAD) or without net myocardial lactate production as a proof of myocardial ischemia. In patients without significant CAD and no net myocardial lactate production hs-cTnT concentrations stayed within the upper reference limit (URL) in all patients.

Challenges in the interpretation of hs-cTn after elective percutaneous coronary interventions in clinical practice

cTn when measured with hs-cTn assays increases significantly in individuals with angiographically normal coronary arteries even after LAD occlusion of only 30 seconds duration in peripheral venous blood samples, a setting in which necrosis is highly unlikely [61]. cTn increases started as early as 15-30 minutes after balloon occlussion. The highest cTn values were found at the end of the blood sampling period at 4 hours after LAD occlussion with the highest relative increases found after 90 seconds lasting LAD occlussion (all participants developed angina, assay dependent increase ranging from about 3-8 fold of baseline values). This observation explains, why cTn increases are found in almost all and even completely uneventful patients after elective percutaneous coronary interventions (PCI) (see figure 2), which makes the clinical interpretation of cTn increases in the individual symptomatic patient without knowing baseline values difficult.
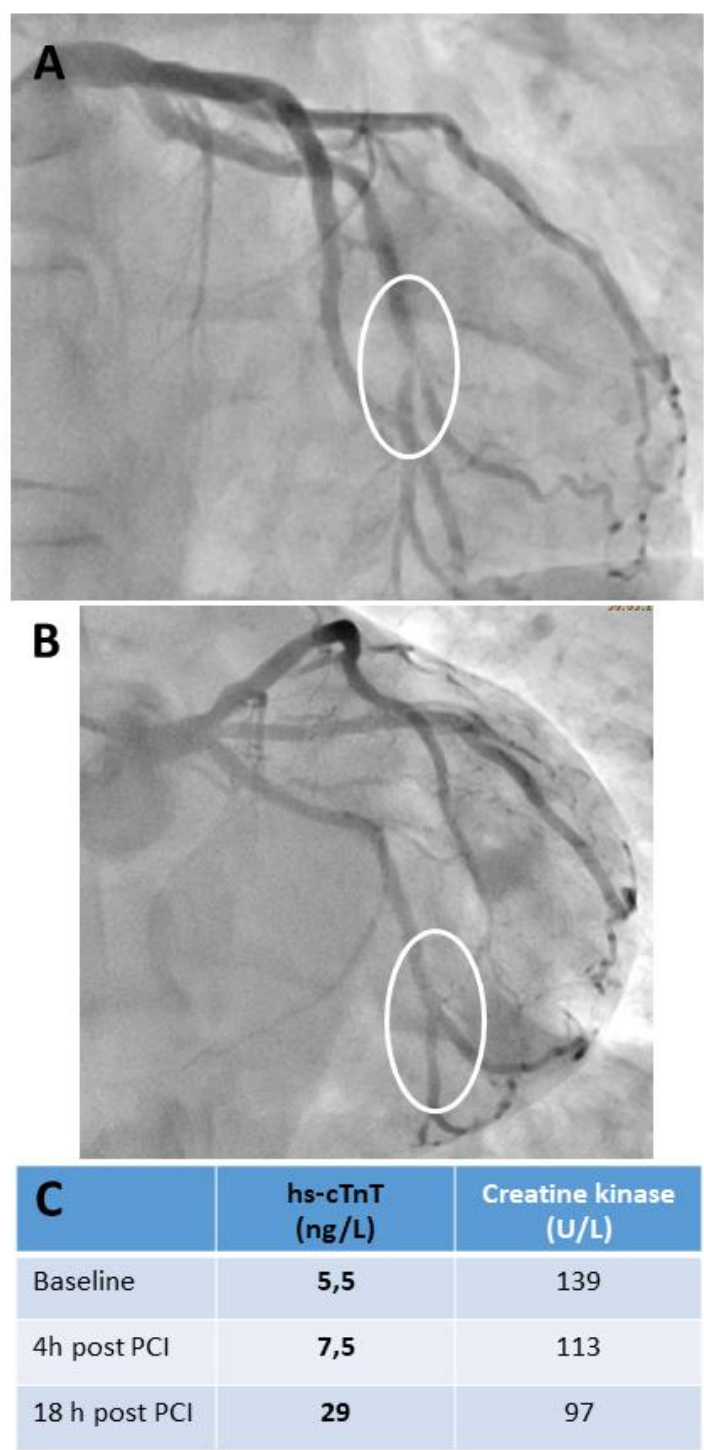
Figure 2. Cardiac biomarker release in a patient with an uncomplicated bifurcation percutaneous tranluminal coronary intevention for unstable angina. This 55-year old male with known CAD was admitted for symptoms of unstable angina. He underwent PCI of the left circimflex coronary artery 11 years ago in another hospital. He showed no signs of acute myocardial ischemia in serial ECGs before and after PCI. Serial hs-cTnT testing in the emergency department before PCI revealed concentrations within the normal range without a significant change. After treatment in the emergency department the patient was asymptomatic, the criteria of an AMI were not fulfilled. Urgent coronary angiography on the next day revealed a subtotal in-stent restenosis at the distal

end of the stent involving the distal circumflex artery bifurcation (Medina 1/1/1, marked with a circle, the angiography of the left coronary artery is shown in A). All other coronary arteries showed no significant stenoses. PCI was performed by dilatation and stent implantation in the marginal branch and dilatation of the smaller distal RCX with subsequent treatment with a drug eluting balloon after succsessful predilatation with an excellent primary result without any complications (B, marked with circle). The biomarker time courses are listed in C. Interestingly, hs-cTnT showed no significant increase within 4 hours after PCI, but it increased above the upper reference limit of 14 ng/L on the morning of the next day. This case nicely demonstrates the importance of a clear specification on the point in time of a proposed cTn decision limit for diagnosing peri-interventional AMI after elective PCI. Notably creatine kinase activities were within the reference limit throughout even showing a constant fall of activities (the patient was a construction worker of tunnel building with heavy physical activities at work). Abbreviations: high-sensitivity troponin T (hs-cTnT), percutaneous coronary intervention (PCI), circumflex artery (RCX), coronary artery disease (CAD), acute myocardial infarction (AMI).

Baseline values correlate with the severity and complexity of CAD and may be already increased [7,60,62]. In general, the cTn increase after elective PCI is related to CAD severity and the complexicity of the performed interventions (see supplemental figure 1).

Apart from the obvious cases of peri-interventionel AMI with complications seen in coronary angiography (see supplemental figure 2) its diagnosis is not always straightforward in clinical practice. The clinical application of the criteria of the 4th Universal Definition of Myocardial in Infarction is sometimes demanding as it requires the careful consideration of cTn increase together with clinical symptoms, angiographic, ECG or imaging evidence for acute myocardial ischemia [3,7]. However, it has been shown that the diagnosis of peri-interventional type 4a AMI complicating elective PCI has prognostic implications [63], it was associated with an about 2-fold higher cardiovascular 1-year event rate and an about 3-fold higher, but still low 1-year mortality (3%) in patients with normal cTn baseline concentrations [63,64]. Other societies (see Table 3) preferred a simpler approach being based on cTn release with markedly higher cTn decision limits in combination with gross ECG evidence for occurrence of AMI (e.g. development of new pathological Q waves) [65,66]. A major limitation of all suggested cTn decision limits is that the time dependence of cTn release after myocardial injury is not considered (see figure 2). No clear criteria on blood sampling regimens and the point in time after PCI at which these decision limits are to be used are given in all current recommendations [3,65,66]. Therefore, all recommendations have to be reworked considering these issues, although still limited data on the optimal blood sampling regimen after elective PCI is available so far. From a practical point of view, it is suggested to test cTn at baseline, which is also useful for risk stratiufication [7,62–64], and, if clinically indicated, 4-6 hours after PCI, and in the morning of the next day, if the patient stays overnight in the hospital.

Table 3. Criteria suggested for the diagnosis of peri-procedural AMI in elective PCI.

	cTn baseline ≤URL	cTn baseline >URL	Clinical criteria (≥1 needed)
UDMI Type 4a AMI [3]	>5x URL plus ECG or imaging criteria	Increase >20% + >5x URL plus ECG or imaging criteria	New signs of myocardial ischemia as evidenced by ECG, imaging, or coronary flow-limiting complications
SCAI clinically	≥70x URL or ≥35x	≥70x URL or ≥35x	New Q waves in ≥ 2 contiguous leads, New persistent LBBB

relevant AMI [65]	URL plus ECG criteria	URL plus ECG criteria	
ARC-2 peri-procedural AMI [66]	≥35x URL plus ECG or imaging criteria	≥35x URL plus ECG or imaging criteria	New Q waves or equivalents, evidence in imaging, coronary flow-limiting complications

Abbreviations: percutaneous coronary intervention (PCI), Universal Definition of Myocardial Infarction (UDMI), Society for Cardiovascular Angiography and Interventions (SCAI), Academic Research Consortium (ARC), acute myocardial infarction (AMI), upper reference limit (URL), left bundle branch block (LBBB), cardiac troponin (cTn), electrocardiogram (ECG).

Table 4. Summary of current knowledge on circulating cardiac troponin complexes and cardiac troponin forms in relation to the cause of myocardial injury .

	Intact and truncated cTnTIC complexes	Free partially truncated (HMM) cTnT	Intact and truncated cTnIC complexes	Free heavily truncated (LMM) cTnT
AMI	x	x#	x*	x
ESRD			x	x
Post heavy endurance exercise (e.g. marathon)			x	x

*Main form of circulating cTnI after AMI (acute phase). # Main form of circulating cTnT after AMI (acute phase)cTnI is mainly found in blood as a partially degraded cTnIC complex in response to all. etiologies of myocardial damage, some is mainly partially degraded cTnTIC complex (e.g. early after AMI), and little is free degraded cTnI. By contrast, according to preliminary clinical results, HMM cTnT is mainly only found in blood in AMI patients during the acute phase. Abbreviations: acute myocardial infarction (AMI), end-stage renal disease (ESRD), low-molecular mass (LMM), high molecular mass (HMM), cardiac troponin I (cTnI), cardiac troponin T (cTnT).

In summary, the distinction between type 4a AMI, PCI-related acute myocardial injury indicated by cTn release not fulfilling these criteria, and chronic myocardial injury indicated already by increased baseline cTn concentrations before PCI without a significant increase thereafter can be challenging in individual patients undergoing elective PCI (see supplemental figures 1 and 2), particularly, if baseline concentrations are not available. This distinction is clinically important, and careful integration of all available clinical data is essential for correct classification. Current clinical practice usually is to test cTn after PCI only when clinically indicated by symptoms or complications during PCI.

Challenges in the clinical interpretation of cTn in symptomatic athletes after competitions or training sessions involving heavy endurance exercise

Of even greater debate on a potential clinical significance are cTn increases in asymptomatic endurance athletes (e.g. marathon runners) after competitions or heavy training sessions with hs-cTn concentrations above the URL and a rising and/or falling pattern [59,67–72]. Peak concentrations usually do not increase above 3-times the URL and occur seceral hours after finishing, and concentrations return into the URL within 48-72 hours after exercise [69]. This frequent cTn release

in asymptomatic athletes makes the interpretation of cTn very challenging in athletes who develop symptoms during or after competitions or training sessions (see figure 3).
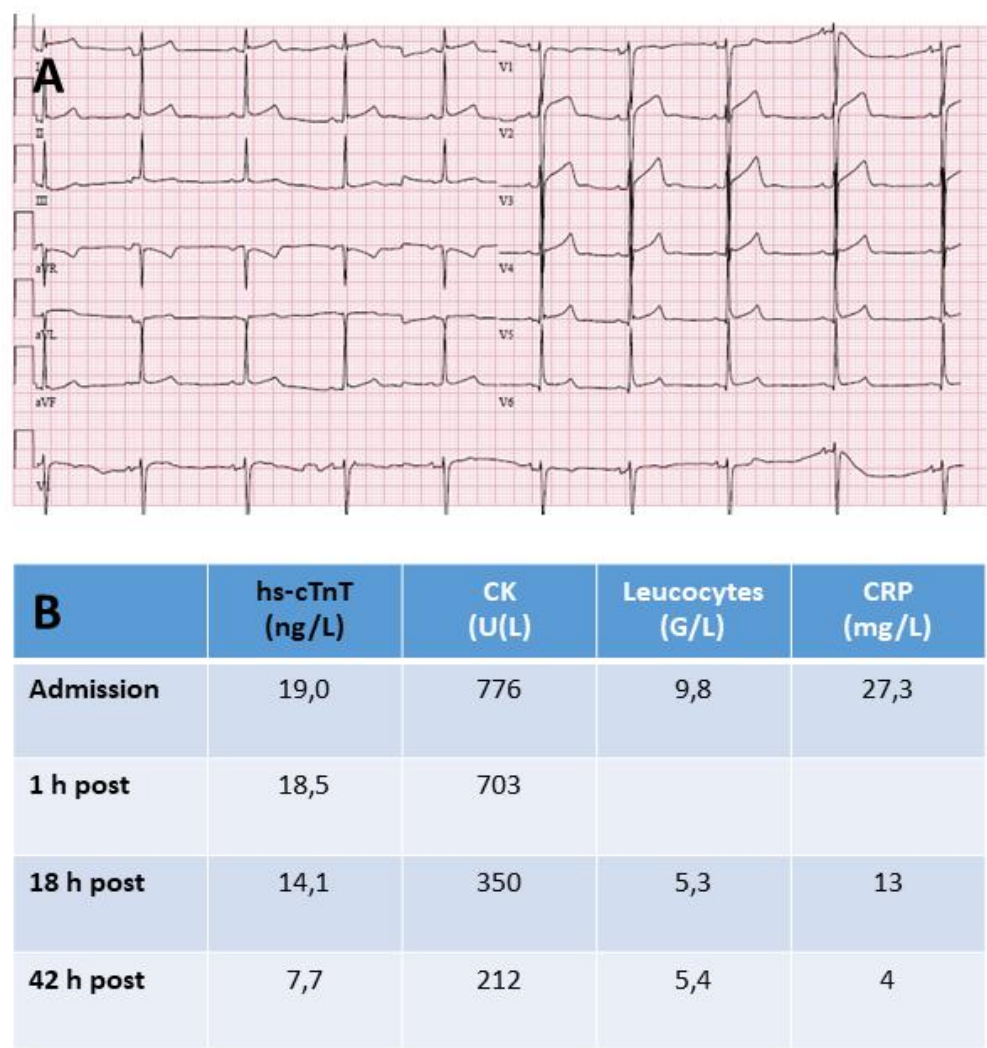

B	hs-cTnT (ng/L)	CK (U(L)	Leucocytes (G/L)	CRP (mg/L)
Admission	19,0	776	9,8	27,3
1 h post	18,5	703		
18 h post	14,1	350	5,3	13
42 h post	7,7	212	5,4	4

Figure 3. Cardiac troponin T interpretation in a 39-year old male amateur cyclist, who had to abort a marathon cycling event after approximately 20 hours. The patient was admitted to the emergency department with the key symptoms dyspnoe worsened on exertion and body position and breathing-related angina. His ECG showed signs of early repolarisation (A) without change in serial recordings. The chest X-ray performed on admission

showed no pathologies, in particular no signs of pneumonia. His key laboratory parameters are listed in B. A coronary computed tomography angiography revealed a calcium score of 0 with normal coronary arteries without arteriosclerotic plaques and coronary anomalies. As typical for long-term endurance sportsman the left ventricular stroke volume was increased and the left ventricle slightly dilated, the left ventricular ejection fraction and right ventricular function were normal. These findings were confirmed by echocardiography. Before start of the competition he was fit without any signs of infection, on admission he was reporting cough, the physical examination including oxygenation and body temperature were normal. hs-cTnT was slightly increased on admission with a constant fall, and concentrations were within the URL before discharge. The maximum concentration was found on admission and was <3-fold URL consistent with an exercise-induced cTn release. The high creatine kinase activities are to be expected with this high skeletal muscle workload. However, because of his advanced age coronary computed tomography angiography was performed to rule-out coronary artery disease. C-reactive protein was slightly increased on admission, which is consistent with a heavy endurance exercise workload, it returned to normal limits until discharge. Leucocytes were within reference limits, but the differential blood count in the morning after admission revealed a relative lymphocytosis of 71%. The patient wanted to be discharged as he was from abroad. His discharge diagnosis was suspicion of a viral respiratory infection. Acute myocarditis was unlikely but cardiac magnetic resonance imaging for ruling it out definitively was not possible before discharge, this imaging modality was recommended to be performed at home in case of persistent symptoms. Abbreviations: high-sensitivity troponin T (hs-cTnT), creatine kinase (CK), C-reactive protein (CRP), upper reference limit (URL), electrocardiogram (ECG)

The symptomatic athlete should be assessed as any other high-risk patient presenting to the emergency department. However, the usual post-exercise increase in cTn makes its interpretation more difficult. Serial cTn testing with considering the magnitudes of cTn increase and the rate of change, and, in the absence of unequivocal ECG findings of acute myocardial ischemia, the combination with imaging, in particular coronary computed tomography angiography (CCTA), are essential to rule out acute myocardial ischemia as the cause of symptoms particularly in older (>35 years) male recreational athletes.

Most healthy individuals have elevated cTn after extreme endurance exercise [59,68–72]. After prolonged endurance events (e.g. marathon, triathlon, ultramarathon) post-race typically mild and transient acute reversible systolic dysfunction of the right ventricle but not the left ventricle has been described [69,73,74]. There was no close correlation between post-race cTnI concentrations and right ventricular ejection fraction decreases [73]. In marathon runners the extent of cTn release did not correlate with myocardial injury [69,73] in imaging as well as the time course of cTn concentrations differed from AMI [69,73]. In athletes there is no association of post-exercise cTn release with worse prognosis [69,73–75]. This appears to be different in elderly physical active individuals from the general population [76]. There is experimental data supporting the possibility of cTn release from reversibly injured myocardium in athletes. In a rat animal model of an endurance exercise challenge cTnT release was only associated with reversible changes in cardiomyocyte structure [67]. This is confirmed by a reported small cTnT release during and after a marathon in healthy completely asymptomatic humans on a motorized treadmill [68]. cTnT increased in all 9 participants during running, at completion or within 1 hour cTnT returned to baseline values. All but one participant showed a further small increase within a 24 hour recovery period starting several hours after finishing, 5 participants still had increased cTnT concentrations 24 hours after exercise. The release of cTnT within the first 60 minutes of running indicates that exercise-induced cTn release is not necessarily restricted to prolonged endurance exercise. It appears very unlikely that these minor cTn increases reflect myocardial necrosis in this healthy, extremely fit individuals. It may be part of an adaptive process [22]. A high training volume and a long duration in training appear to be protective for post-exercise cTn release [72]. Exercise-induced cTn release itself, however, appears to be related to the overall cardiac workload (duration and in particular intensity) of the competition or training session [69]. Exercise-induced cTn release did not differ significantly in patients with rule-out of CAD (calcium score 0, no plaques in CCTA) and patients with signs of coronary arteriosclerosis in CCTA

but without evidence of exercise-induced myocardial ischemia and, therefore, clearance to perform exercise [70]. However, despite being asymptomatic clinically, the exercise-induced cTnI release in individuals with hemodynamically significant CAD was reported to be higher [77].

Although cardiac magnetic resonance imaging (MRI) studies in participants of marathons found no myocardial edema or late gadolinium enhancement despite increased cTn concentrations after exercise [69,73,74], its limited sensitivity does not rule out necrosis as the cause of cTn release from the heart. It has been speculated, that long-term exercise training could produce myocardial damage from repetetive high-intensity exercise sessions, which could explain, why lifelong endurance athletes have more late gadolinium enhancement compared with their physically less active peers [69,78]. There was a relation between the amount of late gadolinium enhancement with the number of race competitions and years of training as well [78].

In summary, brief periods of myocardial ischemia, without obvious myocardial infarction or imaging evidence of necrosis, may cause low-level hs-cTn increases in peripheral blood, and cTn is released after intensive myocardial workload, even among individuals without objective evidence of myocardial ischemia or CAD. Whether these limited cTn increases are due to reversible myocardial injury remains speculative. Although release of cTn could be demonstrated in cell culture experiments from reversibly injured cardiomyocytes by the formation of blebs that bud off from the plasma membrane [28,45], the clinical implication of this observation remains unclear. Experimental studies demonstrated [46,47] that cTnI was released from porcine myocardium in the absence of detectable tissue necrosis, but not in the absence of other forms of cell death, as apoptosis was detected in myocardium.

3. Clearance of cTnI and cTnT from the Circulation in Humans

There are three possible mechanisms of elimination of circulating proteins in blood, i.e. enzymatic degradation within the circulation, metabolism in organs with a high metabolic rate (e.g. liver, pancreas, kidneys, skeletal muscles, endocytosis by the reticuloendothelial system), and elimination via glomerular filtration and excretion in urine. Thus, in cases of impaired clearance from blood (e.g. renal failure, hypothyroidism) prolonged biomarker increases may be found or higher baseline values may be seen then usual.

Most proteins released from injured myocardium, including cTn, appear to be catabolized in tissues with a high metabolic rate, in particular in the liver probably by scavenger receptor mediated endocytosis and subsequent degradation in lysosomes [79]. In this respect, the reports of cTnT degradation by thrombin [34], a rather indiscriminate serine protease, which cleaves cTnT between R68 and S69, add interesting information and may explain the heparin interference of a previous generation of cTnT assays [34,80]. However, the significance of thrombin for cTnT elimination remains to be shown. Small molecules, such as myoglobin, pass through the glomerular filtration membranes of the kidneys and can be found in the urine [81]. These proteins are mainly reabsorbed and subsequently metabolised in the tubular epithelial cells. Thus, in the presence of impaired renal clearance from blood prolonged increases in such biomarkers are found. Intact free cTnT is too large for glomerular filtration, but smaller cTnT fragments would be small enough for renal clearance. By contrast, cTnI appears to be mainly found in blood as part of complexes (see below) which are too large for glomerular filtration. At high cTnT concentrations (e.g. after AMI) extra-renal cTnT clearance dominates [79], but at low concentrations (<100 ng/L, e.g. in patients with chronic cardiac diseases) renal clearance appears to dominate based on experiments where renal perfusion was reduced [82]. As cTn drop to lower concentrations such as those often observed in patients with stable cTn elevations recent data suggest that cTnT clearance becomes slower and more renal dependent [82]. Smaller, circulating, degraded cTn fragments which are predominantly found in blood after chronic myocardial injury (see below) would be small enough for glomerular filtration [82]. However, local degradation and tubular reabsorption could preclude the detection of cTnT and cTnI in urine. At these low steady-state concentrations, cTnT levels are roughly twice as high if kidney function is reduced by 50% [82]. However, other mechanisms such as increased myocardial stress and wall

tension related to a variety of different causes may also contribute to the cTnT elevations seen in patients with chronic renal failure [6,8].

When cTn enters the bloodstream, if follows an exponential 2-phase model with a distribution and elimination phase. Initially, cTn half life times of about 1-2 hours similar to that of myoglobin or CKMB were estimated [14,40,83]. Once cTnT and cTnI reach the circulation, they are initially cleared with a short half-life of about 0.5 hours in dogs and rats [83]. In a recent, carefully designed clinical study [84] with autologous re-transfusion of plasma, which was obtained by plasmapheresis during the subacute phase of AMI, several weeks thereafter the median half-life of cTnT calculated in humans was about 2 hours with a clearance of about 80 ml/min, the half life of cTnI was longer with a median of about 4 hours with a clearance of about 40 ml/min. By this study design the problem of ongoing release of cTn from damaged myocardium is avoided. However, different cTn fragments may have different half-lifes [82,85]. Recently, the half-life of high molecular mass (long-form) cTnT was reported to be about 9 hours, which was calculated from concentration time course analyis of AMI patients [85]. This approach, however, may overestimate the true half-life because of ongoing cTn release from the damaged myocardium.

4. Circulating Forms of Cardiac Troponins in Human Blood

The biochemical and pathophysiological background with post-translational modifications (e.g. phosphorylation, oxidation), intramyocardial N-terminal proteolysis of cTnI and cTnT by caspase and calpain and dissociation of cTn complexes and free cTn forms from the thin filament in response to myocardial injury with the potential ability of rebinding to neighboring filaments, as well as further degradation in the interstitial space and the blood after release from cardiomyocytes is very complex and challenging (see above [10–12,30–34,45–48]). Serum or plasma is a complex matrix with many abundant proteins, such as albumin, and comparably low concentrations of cTn. This is a major analytical challenge for the characterization of circulating cTn forms using standard techniques of proteomics, such as mass spectrometry [86–88], the criterion method of proteomics, or gel filtration, because highly sensitive methods are needed. There are in principal two primary approaches, i.e. antibody based techniques and physical seperation techniques, such as western blotting, gel filtration chromatography, and mass spectrometry, which all have strengths and limitations (e.g. denaturing conditions, analytical sensitivity). For example, specific sample preparation and purification procedures are needed to eliminate this background noise of abundant blood proteins and to enrich cTn in the sample to be able to be detected by mass spectrometry [86,87]. Nonetheless its analytical sensitivity is still limited and very high cTn concentrations in samples are needed [87]. A denaturing technique is not suitable for distinguishing the different forms and sizes of the ternary cTnTIC complex or cTnIC complex as a whole.

An obviously easier approach appears to be the development of specific and sensitive immunoassays for the detection of particular circulating cTn forms [85] after characterization of the relevant circulating cTn forms released in response to different causes of myocardial injury by more sophisticated analytical techniques. Thus, for practical reasons, sensitive and specific sandwich immunoassays are the methods of choice for research and potential future routine use [85,89,90]. They have the potential to be adopted for routine use on automated, high-throughput platforms.

The currently available data on circulating cTn forms is summarized in table 3. It is based on different analytical approaches (mainly Western blotting and gel filtration chromatography, sandwich immunoassays) with their method-specific limitations, and it is also mainly based on stored samples. Testing of stored samples has limitations compared to testing of freshly drawn blood samples as storage conditions and freeze thaw cycles may affect in-vitro stability and consequently circulating degradation forms within samples. In addition, in serum samples thrombin generation may affect in particular cTnT forms, and in EDTA plasma samples the anticoagulant may have effects on cTn complexes [91]. Ideally the reported results should be confirmed by using fresh heparinized plasma samples for testing.

Published results [92–109] can be summarized as follows: Intact cTn forms disappear rapidly from the circulation in the first hours after AMI. After AMI, mainly binary truncated cTnIC with a varying quantity of ternary mostly truncated cTnICT complexes are found [92–94,96,97]. The so called "large size" c TnIC and cTnTIC complexes are only found early after AMI. 75-100% of the total cTnI content in serum of post-AMI patients is part of a complex [92–94,100,102,106]. By contrast, cTnT also circulates in a significant amount as a free unbound form [101,103,104]. Intact cTnT is rapidly degraded at the N-terminal end, and early after AMI it has been described mainly as a high molecular mass (HMM, ≥29kDa), "long" form including complex bound cTnT [97,104,105]. Over time, heavily truncated cTnT forms (14-16 kDa) increase probably by degradation at both the C-terminal and N-terminal ends [101,103,104]. In AMI patients, cTnI primarily exists as truncated cTnIC and truncated cTnTIC complexes. For cTnI degradation the data is less consistent, but degradation at both ends has been reported as well [102,106,107]. By contrast to the N- and C-terminal regions, the central portions of cTnI and cTnT appear to be stable [94,97,102,104,109]. These epitopes appear to the best targets for the development of routine cTn immunoassay with high analytical sensitivity and high in-vitro stability [35].

Interestingly, the cause of myocardial injury may impact circulating cTn forms (see table 2). HMM, long cTnT forms predominate in the acute phase of AMI, whereas in chronic end-stage renal diseases or in individuals after strenous endurance exercise (e.g. marathon running or triathlon) only LMM cTnT forms could be detected [101,104,105]. These observations show potential for future routine use of cTn composition detection in blood samples to define the cause of myocardial injury and to increase the specificity of cTn testing for AMI.

Commercially available routine hs-cTn assays are targeting measurement of total cTnI or cTnT

The practical relevance of complex formation, post-translational modifications of cTn and degradation of cTn is that they may lead to changes in the availability of specific epitopes and thus to different recoveries of cTn variants in different cTn assays. The antibodies of currently commercially available hs-cTn assays target epitopes within stable central regions of the cTnI and cTnT molecules [35]. Therefore, most currently commercially available assays detect total cTnI or cTnT including intact cTn (free or complex-bound) and a comprehensive but varying mixture of cTn degradation products [35].

Research cTn assays detecting specific circulating cTn forms:

Recently immunoassays to quantify intact or only moderately degraded cTnT (so called "long-form") have been developed [85,89,90], that do not detect heavily degraded so called "small-form" cTnT in blood samples. In pilot studies, the ratio of "long-form" to total cTnT demonstrated discrimimation potential of acute from chronic myocardial injury and of different aetiologies of myocardial injury [90,105,110–114]. Similar findings have not been reported for cTnI yet [106]. However, a hs-cTnI/hs-cTnT ratio in blood samples could have similar discriminatory power for seperating type 1 AMI from other causes of myocardial injury [115,116]. These promising results of pilot studies of cTn composition testing, however, have to be confirmed in large-scaled clinical studies testing fresh blood samples.

5. Are There Clinically Relevant Differences Between cTnI and cTnT?

When critically looking on the published literature on this topic, it has to be considered, that cTnI and cTnT assays are neither standardized nor harmonized, and absolute values are, therefore, hard to compare. The only way to make meaningful comparisons is to transform absolute values into multiplies of the URL of each assay. However, even by doing this, there may be still a bias, if one cTn assay has a markedly higher analytical sensitivity then the other assay, and, therefore, URLs should be used, which were calculated from the same reference population, such as the American Association of Clinical Chemistry and Laboratory Medicine reference population biobank, using the same statistical method for URL calculation. In addition, comparisons were mainly made on stored samples, and differences in the in vitro stabilities of cTns must be excluded as the simplest reason for observed discrepancies. In many studies, samples were stored for months or even years before

measurement. Thus, more information is frequently needed, including immediate cTn measurements without sample storage and studies with cardiac MRI imaging as the most sensitive imaging modality currently available to assess myocardial injury to confirm reported differences in clinical sensitivities and specificities of cTnI and cTnT in specific clinical scenarios, e.g. in patients with chronic skeletal muscle myopathies or patients with severe chronic renal failure.

cTnT and cTnI concentration time courses after an AMI

Although cTn show different release kinetics with cTnT having a broader diagnostic window after AMI [27,39,40,117], cTnT and cTnI are considered equally effective in terms of diagnostic performance during the acute phase of AMI [26,118,119]. No clinically relevant differences in their early diagnostic sensitivities for AMI have been published so far. Both cTns generally detect myocardial damage equally well [120].

After AMI with occluded coronary arteries, cTn time courses are influenced by the fact, whether early tissue reperfusion of the infarcted myocardium occurred [27,39,40]. With early reperfusion of the infarct-related coronary artery, cTnT and cTnI peaks are usually found about 12 hours after symptom onset (see supplemental figure 3). Both troponins show a biphasic release pattern with second peak values several days after AMI. However, this feature is much more pronounced in cTnT concentration time courses (see supplemental figure 3), and cTnT increases usually last a couple of days longer than cTnI increases [27]. These second cTnI peaks are much lower than the first peaks (see supplemental figure 4) and may be missed with infrequent (e.g. only daily) blood sampling [27]. In AMI patients with ST-elevations (STEMI) and successfulI primary PCI they were even absent [117]. In case of failed early tissue reperfusion of the infarcted myocardium cTnI peaks occur about 24 hours after symptom onset and the maximum cTnT concentrations are found several days after AMI (see supplemental figure 5) [27,39,40]. The concentration time courses of AMI patients without ST-segment elevations (non-STEMI) resemble those of STEMI with successful primary PCI [121]. cTn peaks and release correlate with infarct size [38,122–124]. The early cTn concentration time courses within the first few hours after symptom onset do not differ significantly on admission between STEMI and non-STEMI patients, and the rate in increase within the first 1-2 hours – although usually higher in STEMI – does not allow a reliable discrimination as well [120,121]. Similarly, they do not allow an accurate discrimination between AMI patients and patients with myocardial injury unrelated to myocardial ischemia as well [120].

Despite the lack of differences of early diagnostic sensitivities for AMI [26,118,119] it has been postulated that cTnI is released faster after myocardial damage [125]. However, as mentioned above, although absolute concentrations of hs-cTnI may be markedly higher, this conclusion is not possible, and differences in absolute values in in-vitro and animal experiments may be just related to differences of cTn assay crossreactivities with cTn of animals in cTn assays which were designed to detect human cTns. The freeze-thawed human myocardial tissue damage model used in one study [125] is very unphysiological. From a pathophysiological point of view it is likely that cTnI and cTnT are released equimolarly after myocardial damage from the thin filaments. However, theoretically an intramyocardial rebindung of cTnT to insoluble structures of cardiomyocytes would be possible.

cTn discrepancies in patients with chronic skeletal muscle diseases:

A potential pathophysiological background of reported discrepancies between cTnI and cTnT concentrations (see figure 4) with much more frequently increased cTnT concentrations in patients with chronically stressed skeletal muscle [126–133] is that during fetal development, cTnT is expressed in cardiac and embryonic and neonatal skeletal muscles [134–137].

	Creatine kinase (U/L)	hs-cTnT (ng/L) URL <14	hs-cTnI (ng/L) URL <34	NT-proBNP (ng/L)
Baseline	6277	41,6	18,9	<50
2-year follow-up	1996	32,8	19,7	<50

Figure 4. Cardiac biomarker testing results of 2 recent visits of a 19-year old male with skeletal muscle dystrophy Becker. His father also suffered from this disease. The diagnosis was additionally confirmed by genetic testing with demonstration of a mutation of the dystrophin gene (deletion of exons 45-47). This patient had no cardiac symptoms throughout his whole life with a normal exercise capacity. The ECGs and echocardiograms performed at both visits were normal. NT-proBNP was normal as well. In addition, cardiac magnetic resonance imaging performed at the last visit was normal as well. Disordant hs-cTnT and hs-cTnI results are frequently seen in patients with chronic skeletal muscle dystrophies, which is consistent with re-expression of the cTnT gene in chronically injured skeletal muscle. It has to be stressed that cardiomyopathy in patients with Becker disease, although frequent, usually does not start to develop before the 3rd decade of life. Abbreviations: high-sensitivity troponin T (hs-cTnT), high-sensitivity cardiac troponin I (hs-cTnI), N-terminal B-type natriuretic peptide (NT-proBNP), upper reference limt (URL), electrocardiogram (ECG), B-type natriuretic peptide (BNP).

cTnT is downregulated in skeletal muscle during development, and after birth, cTnT gradually disappears from skeletal muscle. Healthy adult human skeletal muscle does not contain cTnT. By contrast, cTnI has not been reported to be expressed outside the heart so far [10,15]. When human skeletal muscle is chronically injured, such as in patients with chronic skeletal muscle myopathies, it recapitulates embryonic myogenesis with re-expression of fetal proteins, potentially including cTnT isoforms [134]. Re-expression is likely dependent on disease severity, and re-expressed proteins can be released into the circulation from damaged skeletal muscles, such as in patients with chronic myositis, myopathies or storage diseases.

Messenger ribonucleid acid (mRNA) coding cTnT in human biopsy samples from patients with chronic skeletal muscle diseases was repeatedly reported in the literature [128,130,132,133,138–141]. Data on re-expression of cTnT in humans on the protein level is mainly based on immunohistochemistry and western-blotting [128,133,138,139]. Given the inherent problems of specificity of these technologies, these observations are not a definitive proof of re-expression. More recently, however, cTnT expression in skeletal muscle biopsy specimens of patients with Pompe disease was demonstrated by detection of cTnT fragments using nanoflow LC-MS mass spectrometry and by simultaneous detection of cTnT mRNA [130]. By contrast, cTnT was not detected in skeletal muscle of healthy controls, and Wens et al. [130] detected cTnT isoform 6, which is the cTnT isoform expressed in healthy hearts, in skeletal muscle of some patients with Pompe disease. This identifies skeletal muscle of a potential source of circulating cTnT in these patients in case of missing cardiac involvement. In contrast, Schmid et al [131] reported no evidence of cTnT re-expression in skeletal muscle biopsies from patients with myopathies and myosis by mass spectrometry, although significantly more patients had elevated peripheral blood cTnT concentrations compared with cTnI. These conflicting results, may be explained by disease dependent differences in cTnT concentrations within skeletal muscle and/or on differences in the analytical sesnitivities of mass spectrmetry based analytical methods used in both studies. cTnT mRNA expression in skeletal muscle specimens was not tested in the study of Schmid et al. [131].

In summary, there is accumulating evidence of cTnT expression in several human chronic skeletal muscle diseases, and cTnI is the most cardiac-specific marker in these rare patient populations. It is easier to interpret when AMI is suspected in these patients although serial testing is required anyway to confirm acute myocardial injury by a significant change in cTnI concentrations.

In addition, with increasing age of patients with skeletal muscle myopathies the frequency of cardiac involvement increases as well, which may lead to chronic increases in both cTnT and cTnI.
cTnI and cTnT in chronic renal failure patients

As already discussed in detail above, discrepancies of cTnI and cTnT concentrations are frequent in patients with advanced renal failure [142–144], which may be at least in part due to differences in renal clearances of cTn fragments from blood [79,82]. In hemodialysis patients the changes in cTns during hemodialysis depend on the used membrane and modality of dialysis [145,146], which both influence the permeabilities and clearances of proteins including cTn fragments. In addition, the adherences of cTn complexes and cTn fragments to the dialyzer membrane may be different and membrane dependent, and the recovery of cTn fragments is assay depent as well. In addition, myocardial stress due to a variety of causes (e.g. CAD, hypertension, left ventricular hypertrophy, heart failure) in this patient population may lead to a stable chronic increase in both cTnI and cTnT. cTnI increases, however, are less frequent [142–144]. Ricchiuti et al. [140] reported cTnT mRNA expression and cTnT protein expression by Western blotting analysis without cTnI expression in about 50% of skeletal muscle specimens of hemodialysis patients in whom abdominal wall, back muscles and arm muscles were tested. It was not reported which muscle specimens tested positive. By contrast, Haller et al. [147] reported the absence of cTnT expression in abdominal wall or back skeletal muscle biopsy specimen of 5 patients with end-stage renal failure. However, truncal skeletal muscles are typically not involved in uremic skeletal myopathy. Despite more frequently seen cTnT increases and irrespective of the mechanisms of increase, both hs-cTnT and hs-cTnI maintain their prognostic value in patients with chronic renal failure [142]. Similar to chronic skeletal muscle diseases, the cardiacspecificity of cTnI for the detection of acute myocardial injury appears to be higher in this patient population as well, although additionally serial testing of cTnI is also required for AMI diagnosis to document a significant change.

6. Summary and Conclusions

Currently, cTnI and cTnT remain the most accurate laboratory paramaters for routine laboratory diagnosis of myocardial injury, which are hard to be replaced in the future as well. In general, there are no clinically relevant differences in the diagnostic performances of cTnI and cTnT. However, after AMI cTnT tends to stay increased longer than cTnI, and in specific, but rare patient populations with chronic skeletal muscle diseases or chronic renal failure, the clinical specificity of cTnI for the detection of acute myocardial injury is higher. Even mild forms of myocardial injury can be reliably detected by using hs-cTn assays for measurement. Unexpectedly elevated cTn test result should not be neglected, because analytical interferences with false positive test results are very rare. The extreme analytical sensitivity of hs-cTn assays substantially improved the negative prediciive value of cTn in patients admitted with suspected acute coronary syndromes. However, this comes at the cost of clinical specificity for AMI. A promising approach to improve the clinical specificity for type 1 AMI is studying circulating specific cTn degradation forms by the development of sensitive and specific immunoassays for the detection of particular cTn forms, e.g. the so called "long-form" of cTnT or the calculation of a cTnI/ cTnT ratio in blood samples. However, these results of promising pilot studies have to be confirmed in large clinical studies with testing fresh blood samples.

Supplementary Materials: Figure S1: Peri-interventional cardiac troponin T interpretation in a 60 year-old male with known coronary artery disease with succsessful, uncomplicated chronic total occlusion (CTO) percutaneous coronary intervention (PCI) of the right coronary artery for stable angina, Figure S2: A patient with angiographically documented peri-interventional type 4a acute myocardial infarction, Figure S3: Cardiac troponin T concentration time course in a patient with successful primary percutaneous coronary intervention of a dominant right coronary artery, Figure S4: Cardiac biomarker time courses in a 45-year old male patient with ST-segment elevation acute myocardial infarction, Figure S5: Cardiac troponin T concentration time course in a patient with

inferior wall ST-segment elevation acute myocardial infarction with no reflow after primary percutaneous coronary intervention.

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