High-sensitivity cardiac troponins in heart and kidney diseases: from lab to clinic

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High-sensitivity cardiac troponins
in heart and kidney diseases:
from lab to clinic

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High-sensitivity cardiac troponins in heart and kidney diseases: *from lab to clinic*

PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
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Eline Pieter Maria Cardinaels

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Promotor:

Prof. dr. M.P. van Dieijen-Visser

Co-promotoren:

Prof. dr. O. Bekers

Dr. ir. A.M.A. Mingels

Beoordelingscommissie:

Prof. dr. L.J. van Loon (voorzitter)

Dr. S.C. Bekkers

Prof. dr. J.F. Glatz

Prof. dr. G.C.M. Kusters (Jeroen Bosch Ziekenhuis, 's-Hertogenbosch)

Prof. dr. C. Mueller (University Hospital Basel, Switzerland)

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GENERAL INTRODUCTION

Acute myocardial infarction (AMI), also known as a heart attack, is a prevalent cardiovascular disease and a major cause of hospitalisations and deaths [1-3]. AMI reflects the loss of cardiac tissue as a result of prolonged ischemia that originates from an imbalance between blood supply and demand [4]. Ultimately this will result in the manifestation of symptoms such as chest pain and dyspnea. Blood supply is typically decreased because of a ruptured atherosclerotic plaque in the coronary arteries (coronary artery disease; CAD), subsequently forming a thrombus that can occlude the coronary blood vessel (FIGURE 1). However, also conditions other than CAD can provoke myocardial damage and AMI, such as coronary vasospasms, endothelial dysfunction and revascularization procedures.

FIGURE 1. Representation of normal coronary arteries in a healthy heart (A) and the presence of coronary artery disease (CAD) leading to acute myocardial infarction (AMI) (B).

Cardiac biomarkers are an essential component of the diagnostic criteria for myocardial infarction [4]. Ideally, to make a correct and early diagnosis, a cardiac biomarker should be heart specific and early detectable in the patients' blood [5]. Many previous biomarkers (such as creatine kinase and lactate dehydrogenase) have the disadvantage that they are not solely expressed in the heart. Therefore, in the early 1990s, the search for a better cardiac biomarker has led to the discovery of cardiac specific isoforms of troponin T (cTnT) [6, 7] and troponin I (cTnI) [8].

(Patho-)physiology of cardiac troponins

Troponins are components of the sarcomeres, where they regulate the contraction and relaxation of muscle tissue [9] (FIGURE 2). They are composed of three subunits: troponin T, which anchors the protein-complex to tropomyosin; troponin I, facilitating the binding of myosin heads to actin strains; and troponin C, the calcium-binding subunit that initiates muscle contraction. Troponins T and I both have a unique isoform that is exclusively expressed in cardiac muscle (cTnT and cTnI, respectively), whereas troponin C (TnC) has not. Structural characterisation of cTnT and cTnI revealed two elongated proteins with a relatively low molecular weight of approximately 38 kDa and 24 kDa, respectively [9, 10].

FIGURE 2. Physiology of the healthy myocardium. The myocardium is composed of bundles of myofibrils, which in turn are comprised of repeating units of sarcomeres. Subsequently, each sarcomere contains myosin and actin filaments which are bound at regular sites to the cTnT-I-C complexes.

Within the cardiomyocytes, cardiac troponins (cTn) are complexed with the cardiac myofibrils, although there is some evidence for a smaller soluble fraction (5-10% of total cTn amount) in the cytoplasm [11-13]. Recently, it was shown that this cytosolic fraction is probably not a fixed percentage, but may depend on a seemingly reversible binding between cTn and myofibrillar proteins [14]. Also, in vitro studies have demonstrated that the presence of unbound cTnT induces cytotoxicity to the cardiomyocytes [15], questioning whether such soluble fractions truly exists.

Ischemic damage to cardiomyocytes causes increased cell permeability and necrosis, resulting in a rapid release of cTn molecules into the patients' blood circulation (FIGURE 3).

FIGURE 3. Following myocardial damage, cardiac troponins are released into the patients' blood stream. The cTn proteins can be measured in blood samples collected from the patient.

Once released into the blood, cTnT and cTnI can be measured using antibody-dependent assays (FIGURE 4). Serial cTnT measurements in AMI patients' sera reveal a distinct biphasic release pattern. This cTnT-curve initially displays a rapid increase in just a few hours after AMI, and a second prolonged elevation or plateau phase in which cTn concentrations remain abnormal for up to two weeks [11, 16, 17]. It is hypothesized that the initial release originates from the cytosolic cTnT pool, which are easily released into the blood circulation [11, 13]. The sustained cTnT concentrations are presumed to be the result of slower release of myofibril-bound cTn molecules. In contrary to cTnT, cTnI concentrations more rapidly decreases after reaching peak concentrations, resulting in a monophasic release curve [17].

cTnT-assay

cTnI-assay

FIGURE 4. Measuring principles of cTnT and most cTnI assays. Generally, circulating cTn proteins in the patients' blood are captured and detected using antibodies specifically directed against cTn. Serial cTnT or cTnI measurements in AMI patients' blood result in a biphasic or monophasic release curve, respectively (PROTECT study, Maastricht University Medical Center, unpublished data).

Ischemia is also known to activate proteolytic enzymes that can result into post-translational modifications of cTnT and cTnI proteins, such as phosphorylation and degradation [18]. A heterogeneous mixture of molecular forms of cTnT and cTnI proteins have been characterized in the blood of AMI patients [5]. For cTnI, free and complex forms with TnC (cTnI-C complex) and to a lesser extent with cTnT (cTnT-I-C complex) were observed [19-21]. Also fragments of cTnI as well as cTnT have been described [16, 22, 23]. Although the presence of cTnT fragments were strongly disputed by Bates and colleagues, who found cTnT to mainly circulate in the intact free form [24]. Whether cTnT fragments are present in the patient's circulation therefore remained a matter of debate.

Cardiac troponin as the diagnostic biomarker for acute myocardial infarction

According to consensus documents of multiple international societies in cardiology [4, 25, 26] and biochemistry [27, 28], cTnT and cTnI are since 2000 the preferred biochemical markers to diagnose AMI because of their tissue specific expression in the myocardium. In addition, they should be accompanied with clinical symptoms or imaging signs of ischemia, that can be assessed by a number of different means such as physical examinations, changes on electrocardiography (ECG) and imaging evidence of new loss of viable myocardium [4]. AMI patients can be subdivided according to the presence or absence of ST-elevation on the ECG, resulting in ST-elevation MI (STEMI) and non-ST-elevation MI (non-STEMI). Because of the absence of ECG changes, NSTEMI is more difficult to diagnose and mainly relies on the presence of elevated cTn concentrations [6, 29].

The diagnostic cTn cutoff for myocardial infarction was set at the 99th percentile upper reference limit (URL) of a healthy reference population (FIGURE 5) [25]. It was presumed that the amount of false positives is minimized in this way, as it is more common for other biomarkers to use the 97.5th percentile URL. The diagnostic cutoff should also be measured with sufficient precision, preferably not exceeding a 10% coefficient of variation (CV) for the cTn assays although up to 20% CV remains clinically acceptable [28, 30].

The cTnT assay is produced by a single manufacturer, while multiple companies (>10) market cTnI assays. Each manufacturer uses different

antibody pairs [31], resulting in variable assay results [32]. Due to the heterogeneity in cTnI assays, it is strongly endorsed for each assay to report their own 99th percentile concentration [33].

FIGURE 5. Illustration of the 99th percentile cTn concentration of a reference population of healthy individuals, used as the diagnostic cutoff for AMI.

The era of high-sensitivity cardiac troponin assays

At the time that the 99th percentile cutoff was defined, none of the commercially available cTn assays were able to measure this concentration with sufficient precision (<10% CV) [32, 34]. Ongoing improvements in technology over the past several years have advanced the analytical detection of cTn in the blood, permitting the detection of very low circulating levels of cTn with a higher precision [35-37]. The increased assay sensitivity is achieved by several modifications of the existing immunoassays. For instance, the hs-cTnT assay (Roche Diagnostics) requires a larger sample volume than the previous assay (50 μ L instead of 15 μ L). Moreover, signal amplification is obtained by the use of highly optimized antibody-ruthenium conjugates and interference by heterophilic antibodies has been reduced by using a chimeric mouse-human antibody as the detector antibody [34].

The introduction of hs-cTn concentrations resulted in an earlier detection of AMI. Several large clinical trials have demonstrated that the high-sensitivity assays have superior diagnostic performance in patients with suspected acute coronary syndromes in comparison to the contemporary assays [38-41]. Improved sensitivities have been reported for the hs-cTnT assay of Roche Diagnostics (from 83% to 95%) [40] and hs-cTnI assay of Abbott Diagnostics (from 79% to 82%) [39] (TABLE 1). Overall, this has enabled the earlier detection of the onset of AMI [40-42].

However, increased analytical sensitivity has come at the cost of decreased specificity (TABLE 1) and thus presenting additional diagnostic challenges for clinicians. Though cTn elevations signifies damage to the myocardium, cTn is not specific for one particular cardiovascular disease, such as AMI. Any condition that causes injury to the cardiomyocytes can result into increases in circulating hs-cTn concentrations, for example heart failure and myocarditis [44]. Furthermore, hs-cTn also accumulates in patients with chronic kidney disease [45], including end-stage renal disease (ESRD) [46, 47] but are usually stable and modest in such cases. The exact mechanisms that lead to these elevated cTn concentrations are not yet known, but it seems likely that a decrease in renal clearance contributes to this. In principle, cTnT and cTnI (intact or fragments) are small enough to be filtered by the kidneys [48]. Nevertheless, also in these patients, baseline hs-cTn concentrations remain strongly associated with increased cardiovascular risk and mortality [49-51].

Acute processes typically manifest with a rising and/or falling pattern, whereas chronic diseases generally do not. Subsequently, serial hs-cTn testing has demonstrated promising results in distinguishing acute from chronic disease [39, 52, 53] by assessing absolute or relative changes, though absolute changes appear to be superior especially in the lower measuring range [54, 55]. Many different absolute (5-7 ng/L) and relative (20-50%) deltas have been reported, and increasingly earlier time-frames for monitoring is currently suggested (3-6 versus 1-2 hours) [54, 56].

TABLE 1. Analytical and diagnostic characteristics of the contemporary and high-sensitivity assays of Roche Diagnostics and Abbott Diagnostics, adapted from de Lemos [43].

	Roche		Abbott Architect	
	Contemporary cTnT	Hs-cTnT	Contemporary cTnI	Hs-cTnI
Analytical characteristics^A				
Limit of detection, ng/L	10	5	9	1.1–1.6
10% CV cutoff, ng/L	30	13	32	5.2
99 th percentile cutoff, ng/L	<10	14	28	26.2
Diagnostic characteristics^B				
Area-under-the- ROC-curve (95%CI)	0.90 (0.86-0.94)	0.96 (0.94-0.98)	0.92 (0.90-0.94)	0.96 (0.95-0.97)
Sensitivity (95%CI) ² , %	83 (76-90)	95 (90-98)	79 (75-84)	82 (77-87)
Specificity (95%CI) ³ , %	93 (91-95)	80 (77-83)	95 (93-96)	92 (90-94)
Positive predictive value (95%CI) ⁴ , %	72 (64-79)	50 (43-56)	81 (76-85)	75 (70-80)
Negative predictive value (95%CI) ⁵ , %	97 (95-98)	99 (97-100)	94-92-95)	95 (93-96)

^AAccording to the overview of IFCC [31]
^Baccording to studies performed by Reichlin et al [40] for cTnT and Keller et al [39] for cTnI
¹Sensitivity= true positives/(true positives + false negatives)
²Specificity= true negatives/(true negatives + false positives)
³Positive predictive value= true positives/(true positives + false positives)
⁴Negative predictive value= true negatives/(true negatives + false negatives)

Low levels of hs-cTn concentrations as a risk stratifying tool

Hs-cTn assays are capable of measuring hs-cTn concentrations in most healthy reference subjects [35-37]. Several large clinical trials have demonstrated that low hs-cTnT [57-60] and hs-cTnI [61-64] concentrations, even below the diagnostic cut-off, are associated with the presence and severity of a wide range of cardiac abnormalities, including CAD [65, 66], cardiac morphology and function [67, 68]. Also, the association of hs-cTn concentrations with a higher risk on developing cardiovascular events and all-cause mortality has been well-established in numerous populations [57-64, 69], from general cohorts [68] to stable chest pain patients [62]. Even when adjusted for traditional cardiovascular risk factors such as smoking, diabetes mellitus and hypercholesterolemia, hs-cTn concentrations remain important risk predictors. However, whether all detectable hs-cTn concentrations represents myocardial necrosis, remains to be elucidated. As such, other mechanisms have been proposed for these low levels of circulating cTn [70], for instance physiological renewal or remodelling of the heart [71], and cTn leakage from reversibly damaged cardiomyocytes during transient ischemia [72, 73].

Thesis outline

With the introduction of more sensitive cardiac troponin assays in the clinic, clinicians are often challenged when interpreting troponin values, especially in the lower measuring range. This thesis will shed light on important questions concerning the high-sensitivity assays and offers insight in the underlying reasons for hs-cTn elevations.

An overview of 99th percentile upper reference limits and underlying reference populations is given in **chapter two**. In this chapter the challenges of defining a "cardio-healthy" reference population are discussed.

The interpretation of baseline hs-cTn values in the individual patient is complicated not only by cardiac disease but also renal dysfunction. As of yet it is unknown to what extent renal function contributes to higher cardiac troponin concentrations in patients with chest discomfort, in whom circulating hs-cTnT concentrations are mainly attributed to the presence and severity of atherosclerotic plaques or echocardiographic abnormalities. In-depth understanding of the relative contribution of renal function, next to cardiovascular disease, is examined in **chapter three**. In line with this, **chapter four** describes the effect of heart failure and renal dysfunction on hs-cTn concentrations, measured in an aged population (>65 years) of nursing home residents.

ESRD patients often exhibit elevated cTn levels that are more stable over time. To date, it remains unclear how dialysis affects cTn results, as both intradialytic increases and decreases have been reported. In **chapter five** hs-cTn concentrations before and after different modalities of hemodialysis are therefore investigated.

There is evidence that cTn proteins can be subjected to posttranslational modifications, such as degradation. Which circulating cTnT forms are detected by the clinical assay in the blood of AMI and ESRD patients are investigated in **chapter six** and **chapter seven**, respectively.

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**A COMPREHENSIVE REVIEW OF UPPER REFERENCE LIMITS
REPORTED FOR (HIGH-) SENSITIVITY CARDIAC TROPONIN
ASSAYS: THE CHALLENGES THAT LIE AHEAD**

Cardinaels E, Mingels A, Jacobs L, Meex S, Bekers O and van Dieijen-Visser M.

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Abstract

Cardiac troponins (cTn) are the preferred markers for the diagnosis of acute myocardial infarction (AMI). The guidelines recommend the use of the 99th percentile upper reference concentration of a healthy population as the diagnostic cutoff for AMI. However, a broad range of upper reference limits is still employed, complicating the diagnosis of AMI.

This overview is meant to assist laboratory specialists to define an appropriate cutoff value for the diagnosis of AMI. Therefore we provide an overview of the analytical performance and upper reference limits of seven (high-)sensitivity cTn assays: Roche high-sensitivity cTnT and ADVIA Centaur, Stratus CS, Dimension Vista, Vitros ECI, Access and Architect cTnI assays.

It is shown that none of the reference populations completely met the guidelines, including those in package inserts. 40% of the studies collected less than the advised minimum of 300 subjects. Many studies (50%) did not report their inclusion criteria, while lower 99th percentile limits were observed when more stringent selection criteria were applied. Higher troponin cutoffs were found in men and elderly subjects, suggesting gender- and age-specific cutoffs would be considered. Therefore, there is still need for a large, rigorously screened reference population to more accurately establish cTn upper reference limits.

List of Abbreviations: cTn, cardiac troponin; ACS, acute coronary syndrome; NACB, National Academy for Clinical Biochemistry; ESC, European Society of Cardiology; ACC, American College of Cardiology; AMI, acute myocardial infarction; cTnT, cardiac troponin T; cTnI, cardiac troponin I; CV, coefficient of variation; hs-cTnT, high-sensitivity cTnT; LoB, limit of blank; LoD, limit of detection; RCV, reference change value; CV_a, analytical coefficient of variation; CV_i, within-subject coefficient of variation; II, index of individuality; CV_o, between-subject coefficient of variation; IFCC, International Federation of Clinical Chemistry and Laboratory Medicine;

Introduction

Cardiac troponins (cTn) were generally recommended as the biochemical gold standard for the investigation of patients with acute coronary syndrome (ACS) in 1999 by the National Academy of Clinical Biochemistry (NACB) [1], and in 2000 by the European Society of Cardiology and the American College of Cardiology (ESC/ACC) [2]. Both clinical [3] and analytical aspects [4, 5] of these guidelines were revised in 2007, in order to establish a more universal definition of acute myocardial infarction (AMI). According to these guidelines a rise and/or fall of cardiac troponin T (cTnT) or I (cTnI) with at least one value above the 99th percentile of a healthy reference population is the hallmark of myocardial damage. To complete the diagnosis, cTn changes must be accompanied with at least one of the following clinical factors: the presence of ischemic symptoms, electrocardiographic (ECG) alterations (for example ST-T changes, left bundle branch block or development of pathological Q waves), or imaging evidence of either new loss of viable myocardium or new regional wall motion abnormality [3, 6]. Importantly, the guidelines recommend a total imprecision (CV) of 10% at the 99th percentile reference limit [5]. In a recent overview, Apple classified the available assays [7] as “not acceptable” (CV >20%), “clinically usable” (CV 10-20%) or “guideline acceptable” (CV ≤10%) [8]. In addition, assays were categorized into 4 levels depending on whether <50% (level 1), 50-75% (level 2), 75-95% (level 3) or >95% (level 4) of reference concentrations had detectable cTn concentrations below the 99th percentile. Only nine out of twenty-two assays were labeled “guideline acceptable” and nine were defined as “clinically usable” [8]. However, as recently pointed out, misclassification of patients when using CVs between 10% and 20% at the 99th percentile is considered to be insignificant [9].

Despite the introduction of guideline acceptable assays and the recommendation to use the 99th percentile value of a reference population as cutoff for AMI, a broad range of cutoff concentrations are in use, complicating the diagnosis of AMI for clinicians. A major cause of this heterogeneity is lack of assay harmonization and non-standardized selection of healthy reference populations [10-12]. This issue will become even more prominent with the ongoing development of increasingly sensitive assays that detect measurable

cTn levels in almost everybody. Noteworthy, recent data indicate that even low levels of cTn can predict major adverse cardiac events, pointing out the importance of reliable diagnostic cutoffs [13, 14].

Most laboratories do not have the resources to determine their own 99th percentile cutoff. For appropriate statistical determination of the 99th percentile cutoff a theoretical minimum of 120 reference subjects has been proposed [15]. In order to reliably assess the 99th percentile with an uncertainty <5%, a sample size of 300 subjects is required [5, 6]. Furthermore, the distribution of gender and age in the reference population should resemble those in a typical group of AMI patients, and should ideally have negative exercise stress tests and normal cardiac function as assessed by non-invasive imaging. The CARMAGUE study pointed out that $\pm 50\%$ of the laboratories employ cutoff values from the package insert, 15% adopt values from peer-reviewed studies [16], and sometimes arbitrarily chosen values are used. A structured overview of all studies may be a helpful tool that facilitates the diagnostic process and assists laboratories to define appropriate cutoff values.

In the present review, we provide an extensive summary of seven cTn assays, frequently used in clinical practice, that can measure the 99th percentile cutoff with a high precision, including five “guideline acceptable” assays: 1) high sensitivity cTnT (hs-cTnT, Roche Diagnostics), 2) ADVIA Centaur TnI-Ultra (Siemens Healthcare Diagnostics), 3) Stratus CS Acute Care cTnI (Siemens Healthcare Diagnostics), 4) Dimension Vista cTnI (Siemens Healthcare Diagnostics) and 5) Vitros ECI cTnI ES (Ortho Clinical Diagnostics) assays. We also included two “clinically usable” assays to complete the overview: 6) Access AccuTnI (Beckman Coulter) and 7) Architect cTnI assay (Abbott Diagnostics). Point-of-care tests and research assays were excluded. Between October 2010 and October 2011, we searched the MEDLINE electronic database for publications in English, using the key words “troponin”, “troponin assay”, and “99th percentile”. We paid special attention to analytical assay characteristics and the 99th percentile cutoffs. We also discuss selection criteria of the reference population, gender/age specific differences in cTn concentrations, and biological variation within or between subjects. These are increasingly

prominent subjects of debate since the introduction of the high sensitivity assays.

Hs-cTnT Assay, Roche Diagnostics

The analytical evaluation of the assay established by the manufacturer (Roche Diagnostics, Mannheim, Germany) described a limit of blank (LoB) of 3 ng/L and limit of detection (LoD) of 5 ng/L. As depicted in TABLE 1A, other studies reported LoDs that ranged from 1 to 5 ng/L [17-21]. According to the package insert a 10% CV was measured at 13 ng/L (version 2010). Most authors found the same value or even lower 10% CV cutoffs [18, 22-26]. Only one study reports a noticeable higher 10% CV of 18.5 ng/L [27]. These discrepancies in LoB, LoD, and 10% CV can be the result of using various methods of estimation [11].

The reference populations were selected by either health questionnaires [21], laboratory tests [17, 22], or both approaches in combination with echocardiography [25, 27]. In most studies inclusion criteria were not described [18-20, 23, 24]. The recommended inclusion of at least 120 cardio-healthy reference subjects, required for reliable estimation of the 99th percentile, was met in most studies, except in Koerbin et al and Vasatova et al using 104 and 73 subjects, respectively [19, 25].

The 99th percentile concentration according to the package insert was 14 ng/L and calculated from 533 healthy subjects. This value was reproduced in four independent studies [18, 20, 21, 24]. However, in literature, slightly lower 99th percentile limits (± 12 ng/L) were described in a large reference cohort (n=1061) of healthy subjects using EDTA plasma samples [17] or a small population of 104 subjects, broadly screened by means of questionnaires to exclude possible risk factors, presence of cardiac diseases and cardiovascular medications, followed by stress echocardiography and routine chemistry tests [25]. In our lab, a cutoff of 16 ng/L was found [22] and similar 99th percentile cutoffs were also reported by others, using smaller reference populations [26], comprised of 66% males [19].

Gender related differences in cTnT levels have been documented using the hs-cTnT assay [18, 21, 22, 24, 25, 27]. All studies except one [27] reported higher

cTnT concentrations in males ($P<0.01$). From the reported concentrations in TABLE 1, we calculated a mean 99th percentile cutoff of 15.0 ng/L for males and 9.6 ng/L for females [21, 22, 24, 25]. It has been suggested that this could be due to gender specific differences in the pathophysiology of ACS [21], or variations in mean heart sizes between men and women [22]. Several studies indicated higher cTnT levels in elderly [18, 22, 25]. Significantly higher cTnT levels were found in people over 60 years, compared to younger subjects, both in the male and female cohort ($P<0.01$) [25]. These findings provide an independent replication of a previous report [28].

Taken together, the 99th percentile cutoff varied between 12 to 17 ng/L. Moreover, in all studies but one, the imprecision was $\leq 10\%$.

Architect cTnl assay (Abbott Diagnostics)

The lowest cTn concentration that can be measured with an **analytical imprecision** of 10% was reported to be 32 ng/L by the manufacturer (Abbott Diagnostics, Abbott Park, IL, USA) and others [22, 29-31] (TABLE 1B). In the remaining publications, the 10% CV values were indicated to be higher, ranging from 40 ng/L to 75 ng/L [32-35].

The screening methods for the **reference populations** were primarily not specified [30-32, 36]. For this assay, only three reference populations were screened by means of medical history check, ECG and blood laboratory tests [29, 34, 35]. One study included less than the advised minimum of 300 subjects [34]. The reference population, used by the manufacturer, consisted of 224 apparently healthy subjects.

For the **99th percentiles cutoff** a concentration of 28 ng/L was found by the manufacturer and many others report similar results [30, 31, 33, 36]. Six independent studies, report cutoffs lower than 20 ng/L [22, 32, 34-36]. Only one study reported noticeably higher 99th percentile cutoffs in a reference population of 442 subjects, screened by means of medical history check, ECG and routine blood analysis [29].

Age and gender related differences in 99th percentile cutoffs were not statistically significant among these studies.

In summary, upper reference limits varied between 13 and 30 ng/L, while for the 10% CV cutoff, values between 30 and 76 ng/L were reported.

Access AccuTnl assay (Beckman Coulter)

Evaluation of **the analytical sensitivity** by the manufacturer (Beckman Coulter, Woerden, the Netherlands) indicated a LoD of 4 ng/L according to the package insert (version 2006). 60 ng/L is the lowest cTnl concentration which can be measured with a 10% CV. Various studies reported similar [37-39] or somewhat higher values: 104 ng/L [34]; 101 [35] and 75 [40]. 20% CVs were obtained at 43 ng/L, 34 ng/L and 15 ng/L respectively (TABLE 1C). Recently, the AccuTnl assay was optimized resulting in a lower 10% CV value of 14 ng/L [41, 42].

As described previously, **the reference populations** consisted mostly of apparently healthy subjects, with no details on how the subjects were screened [36, 38, 39, 43-45]. In the various studies, health questionnaires were applied either alone [35, 46], or in combination with ECG and routine blood analysis [29, 34, 40, 42, 47]. Eggers et al clearly showed that a lower cutoff was found when screening for cardiovascular diseases (28 ng/L) in comparison to the unscreened reference population (44 ng/L) [41].

Similar **99th percentile cutoff** as indicated by the package insert (40 ng/L) were mostly described using a wide variety of reference populations [29, 34, 36, 37, 39, 44, 45, 47]. Lower cutoffs were found when less than 300 subjects were included [38, 43, 48] or in a relatively young population [35]. On the other hand, higher 99th percentile concentrations (60-80 ng/L) were also measured [40, 42]. A sample matrix effect was observed between serum and plasma [40], although others opposed this finding [29, 47, 48].

Gender-related differences in the 99th percentile cutoffs were reported to be statistically significant in three studies [41, 44, 46]. Ethnicity was also reported to affect the 99th percentile cutoff [46], although later on this observation was contradicted in another study [44].

Conclusion. Wide variations in 10% CV values and 99th cutoffs are observed ranging from 14-104 ng/L and 13-80 ng/L respectively. In some but not all studies the 99th percentile was measured with a CV < 10%, indicating guideline

acceptance. However, many reported cutoffs were not in agreement with the manufacturer's statements.

ADVIA Centaur Tnl-Ultra Assay, Siemens Healthcare Diagnostics

Analytical sensitivity. Four studies with the new generation ADVIA centaur reported a LoD of 6 ng/L [35, 49-51], and one study found a slightly higher LoD of 8.5 ng/L [52]. The package insert (Siemens Healthcare Diagnostics, Frimley, Camberley, UK, version 2009) stated that the LoD of the ADVIA Centaur Tnl-Ultra assay is 6 ng/L and 30 ng/L is the minimum concentration that could be measured with an analytical imprecision of <10%. This value was reproduced in four studies [35, 42, 51, 53], but higher 10% CV cutoffs ranging from 45 ng/L to 67 ng/L [34, 49, 50, 52, 54] were also reported (TABLE 1D).

A cardio-healthy reference population was selected by laboratory tests [49, 54], sometimes in combination with echocardiography [34, 42, 50, 55]. Three studies excluded cardiac diseases and their possible risk factors by means of medical history examinations and/or health questionnaires [35, 52, 53]. The majority of studies included >300 subjects. Only two studies included less than the recommended 300 subjects [34, 52] from which one study did not meet the minimum inclusion of 120 subjects [34].

The 99th percentile cutoff measured by the manufacturer is 40 ng/L, for both serum and plasma samples. Similar values were found in two studies [50, 51] but 1.5 to 2 fold higher 99th percentile concentrations have also been reported for lithium heparin plasma and serum samples [42, 49, 52-55]. Venge et al attributed their higher 99th percentile cutoff (80 ng/L) to different inclusion criteria of the reference population, or the use of samples that had been frozen for a substantial length of time [42]. It should be noted that an older population was used in this study. A majority of the subjects were male and three outliers were excluded with no reported reason. Tate et al included only 108 subjects in the reference population, rigorously screened for cardiovascular risk factors, cardiac diseases, cardiovascular medications, stress echocardiography, blood and urine routine tests and heterophilic antibodies against cTn, which could explain the relatively low 99th percentile concentration of 21 ng/L found both in plasma and serum samples [34]. Even lower 99th percentile cutoffs (13 ng/L and 18 ng/L for

plasma and serum, respectively) were reported in a relatively young reference population with a mean age of 30 years [35].

Gender dependent differences were also reported, with significantly higher cTnI concentrations in men than in women ($P < 0.05$) [35, 49, 54, 55]. Statistically significant correlations between age and cTnI concentrations were reported ($R = 0.268$; $P < 0.0001$) [49, 54], and in line with this observation older subjects had higher cTnI levels than the younger subjects ($P \leq 0.0002$) [42, 55]. No significant differences in 99th percentile cutoffs were found among multi-ethnic Asian populations [53].

In conclusion, 10% CV and 99th percentile cutoffs ranged between 30 - 67 ng/L and 13 - 87 ng/L, respectively. Most, but not all studies [34, 35, 50] concluded that the ADVIA Centaur TnI-Ultra Assay is guideline acceptable.

Stratus CS Acute Care cTnI Assay, Siemens Healthcare Diagnostics

The analytical evaluation of the assay established by the manufacturer (Siemens Healthcare Diagnostics) yielded a LoB of 30 ng/L and a LoD of 60 ng/L, while other studies reported LoDs ranging from 10 to 20 ng/L [56-59]. According to the package insert 60 ng/L is the minimum concentration that can be measured with an analytical imprecision of $< 10\%$ (version 2001). In literature similar results were found [57-59] (TABLE 1E).

The reference population was not specified in three studies, including the package insert, [57, 58]. One study screened for history of cardiac disease, hematochemical alterations and hyperpyrexia [59]. One study performed a history check, ECG, treadmill tests, stress echocardiography or angiogram [56]. Three studies, including those of the manufacturer, analyzed sample sizes that were smaller than the minimum number of 120 subjects [57, 59]. Only Christenson et al included more than the recommended 300 subjects [58].

The 99th percentile concentration provided by the manufacturer is 70 ng/L. This value was reproduced by Christenson [58], but lower cutoff concentrations of 30 ng/L [59] and 50 ng/L [57] were also reported with an accompanying 10% CV/99th percentile ratio of 2.33 and 1.20 respectively. These lower estimations may be due to the inclusion of younger subjects, and the small sample size.

In conclusion, only one reference population included a sufficiently large sample size and reported the assay to be guideline acceptable [58]. More studies are however needed for the Stratus CS assay to make valid conclusions.

Dimension Vista cTnI Assay, Siemens Healthcare Diagnostics

Analytical validation of the Dimension Vista cTnI assay by the manufacturer (Siemens Healthcare Diagnostics) yielded a LoB of 15 ng/L and a total imprecision (CV) below 10% at 40 ng/L (version 2008). Only one independent study validated the sensitivity of the Dimension Vista cTnI Assay, and reported a LoB, LoD and 10% CV cutoff of 11, 15, and 36 ng/L, respectively [60].

The reference population collected by the manufacturer included 199 people with no clear description of the applied screening methods. Arrabola et al included 350 individuals (73% male) after examination of the patient's medical history [60] (TABLE 1F).

The 99th percentile cutoff calculated by the manufacturer is 45 ng/L. Arrabola et al reported a lower cutoff (22 ng/L), and a 10% CV/99th percentile ratio of 1.64.

No significant **gender and age related differences** in cTnI levels are currently described for this assay. However, caution is required in interpretation of the limited available evidence [60].

In conclusion. Although more studies are required to confirm these results, the Dimension Vista cTnI Assay is not guideline acceptable according to the current evidence in literature [60].

Vitros ECI Troponin I ES Assay, Ortho Clinical Diagnostics

Analytical validation of the Vitros ECI cTnI ES assay by the manufacturer (Ortho Clinical Diagnostics, Buckinghamshire, UK) yielded a LoB of 7 ng/L and a LoD of 12 ng/L. The package insert (version 3.0) states that the lowest concentration measurable with an imprecision (CV) less than 10% is 34 ng/L. Tate and colleagues replicated this result [34], but other studies reported 10% CV cutoffs of 40 ng/L [61] and 93 ng/L [35] (TABLE 1G).

The reference populations used by Tate et al and La'ulu et al have already been described above (section on the TnI-Ultra assay) [34, 35]. Apple and colleagues recruited serum samples of 2992 healthy subjects, without additional

specification, as well as lithium heparin plasma samples from 2000 healthy subjects, aged between 18-66 years and of which 75% were male [36].

The 99th percentile cutoff calculated by the manufacturer is 34 ng/L, which corresponds exactly to the 10% CV cutoff value. Apple et al calculated the 99th percentile in two independent reference populations [36]. In one cohort with serum samples they replicated the cutoff value from the manufacturer, in the second reference population with heparin plasma samples a 99th percentile concentration of 31 ng/L was reported [36]. Other studies by Tate et al, and La'ulu et al calculated much lower 99th percentile concentrations in populations that were extensively screened [34], or comprised relatively young subjects [35]. However, the concentrations reported at an imprecision of 10% CV were markedly higher than the 99th percentile concentration in these studies.

In conclusion, various 10% CV values and 99th percentile cutoffs were observed. All studies but one [36] reported a 10% CV/99th percentile ratio >1. Therefore, it remains controversial whether this assay can measure the 99th percentile with a precision of 10%, as quoted by the package insert.

How were reference populations established? A summary

Despite all efforts, heterogeneity in the functional sensitivity and 99th percentile cutoffs is clearly demonstrated in TABLE 1. The reported upper reference limits deviated from the cutoffs provided by the manufacturer, due to variations in sample size, health condition, age, gender and ethnicity. In addition, (pre-) analytical factors may have contributed to cutoff discrepancies e.g. serum versus plasma samples [11, 62] or the use of different platforms. Variations in the determination (e.g. using variable number of days, replicates or different platforms) of the functional sensitivity, could explain the range of 10% or 20% CV cutoffs that have been reported in literature.

The broad variation is apparent from the reviewed studies (66 studies; 53 publications) with sample sizes from <100 to >10,000 subjects. Most studies (40%) included less than the advised minimum of 300 subjects and 15% of the studies collected even less than 120 subjects. Notice that with a sample size of 120, 300 or 1000 subjects the 99th percentile cutoff can be strongly affected by respectively only 1, 3 or 10 subjects.

Another concern is the variation in inclusion criteria among studies. The majority of studies (50%) reported no inclusion criteria, suggesting that no extensive screening was performed. In 26% of the studies, the reference populations were screened by means of medical history questionnaires and/or laboratory tests. In 24% of the cases it was combined with ECG or cardiac imaging techniques such as stress echocardiography or angiography. Herein, no reference population met the selection criteria described in the guidelines [6]. However, in four studies the guidelines were nearly accomplished, reporting a reference population comprised of more than 300 individuals and screened by means of non-invasive imaging [29, 42, 50, 55]. It must be noted that in two of these studies the same reference population was used (SWISCH study) [29, 42]. Importantly, studies that most extensively screened their reference population, reported lower 99th percentile cutoff concentrations [25, 29, 34, 41, 47, 50]. Evidently, in smaller reference populations more stringent screening methods can be performed, improving the composition of the reference population [25, 27, 34, 59]. This is counterbalanced by the fact that a small sample size makes the 99th percentile cutoff more prone to outliers.

Ideally, the distribution of gender and age in the reference population resembles those of a typical group of AMI patients [6, 15]. However, age distribution in the reference populations varied widely among studies. A majority chose a broad age distribution (25-75 years), but some reference populations were composed of merely younger [35, 60] or older subjects [42, 50]. Only two publications use a group of reference individuals in the same age category as AMI patients (>45 years) [42, 50]. The 99th percentile cutoff concentration had a tendency to increase with age [22, 25, 42, 50, 54]. This supports recent ROC analysis from Reiter et al who reported that optimal diagnosis for AMI was achieved at higher cutoff concentrations in elderly (54 ng/L) as compared to younger subjects (17 ng/L) [63]. Moreover, gender specific differences in the 99th percentile cutoff concentration are frequently reported. The cTnT and cTnI assays measured respectively 1.7- and 1.2-fold higher cTn levels in men than women [21, 22, 24, 25, 34, 49, 54, 55]. Also ethnicity was reported to affect the 99th percentile cutoff [46], although others contradict this finding [19, 44]. Taken together, these findings add to the existing body of evidence that gender and age influence cTn

levels [64]. Future research should reveal whether gender- and age-specific cutoff values can improve diagnostic or prognostic performance.

Current status on biological variation

The NACB recommends using the rising and/or falling pattern of cardiac troponin obtained by serial measurements. They propose a $\geq 20\%$ change in cTn concentration to be suggestive of AMI [4]. A twenty percent difference exceeds 3 analytical standard deviations, but it neglects the contribution of the biological variation to cardiac troponin levels when measuring in the lower range [65]. A better approach may be the reference change value (RCV). The RCV is established in healthy individuals and calculated as follows:

$RCV = z \times \sqrt{2} \times \sqrt{CV_A^2 + CV_I^2}$; where z is the standard deviation for a certain probability [65], and CV_A and CV_I represent the analytical and biological variation respectively. When troponin concentrations are measured at two time points, and the subtraction of both values exceeds a critical value, defined by the RCV, the change is considered significant. Using this approach, Wu et al established the log-normal RCV for a hs-cTnI assay (Erenna Immunoassays, Singulex). They concluded that an increase of 48% or a decrease of 32% over a four hour period (short term), and +81% or -32% change over an eight week period (long term) is required [66]. Log-normal RCVs have also been calculated for cTnT using the hs-cTnT assay [20]. For short term changes, the cTnT concentration must rise 85% to exceed the critical difference, whereas for long term changes a >3 fold increase is required.

Another way to express the biological variation is the index of individuality (II). The II can be calculated by the simplified formula: CV_I/CV_G (within subject variation/between subject variation) [67]. By definition, reference intervals are more useful for biomarkers with a high II (>1.4) than for markers with a low II (<0.6) [68]. For cTnI, the CV_I is smaller than the CV_G , both for short-term and long-term measurements (9.7% versus 57%, and 14% versus 63% respectively) [66]. This results in a short-term II of 0.21 and a long term II of 0.39. When the II is low as for cTnI, it has been proposed to subdivide the reference population into more homogenous reference cohorts, which will improve the II and the usefulness of the marker in clinical practice [67]. The short- and long-term II for

cTnT were higher, 0.84 and 1.4, respectively (CV_I , 48% and 94%; CV_G , 86% and 94% for short- and long-term, respectively) [20]. Overall, cTnT seems to be more useful than cTnI, but with only one cTnI and one cTnT study published, more studies are required for a more decisive conclusion.

Conclusion and future perspective

In general, large heterogeneity in 99th percentile cutoff values of cTn complicates the diagnosis of myocardial infarction. Major causes are lack of cardiac troponin assay harmonization, and non-standardized selection of individuals in healthy reference populations. We reviewed the literature on reference populations for seven (high-)sensitivity assays used in clinical practice and found that the upper reference limits deviated from the package inserts for all assays, although this was less apparent for the hs-cTnT assay.

As for now, the best way to establish a reference population is still debatable. From all reviewed literature, no reference populations were collected according to the guidelines [5, 6] although three nearly met the required standards [29, 42, 50, 55]. A total of 40% of the studies collected less than the advised minimum of 300 subjects for reliable estimation of the 99th percentile cutoff and 50% of the studies did not report their inclusion criteria. Moreover, there is important suggestive evidence that substantially lower cutoff values were calculated in studies that applied the most stringent selection criteria to their reference group. The available literature supports the concept that age and gender are important determinants of cardiac cTn levels. Men have on average 1.7- and 1.2-fold higher cTn levels, measured with the cTnT and cTnI assays respectively, and the 99th percentile cutoff concentration increases with age [22, 25, 42, 50, 54].

There is thus need for a large and highly screened population of cardio-healthy subjects that are preferably gender- and age-matched for AMI patients. As proposed by the IFCC, multicentre studies can be the answer to heterogeneity in reference populations: a large number of reference subjects, collected at national scale that can be adopted by clinical laboratories to determine the 99th percentile cutoff values [69]. Alternatively, the optimal cutoff value can be defined by ROC analyses [63], reference change values, or the 97.5th percentile can be chosen as diagnostic cutoff [1], which would be less sensitive to outliers.

Further research should reveal whether more stringent selection criteria and gender/age-specific cutoffs can optimize the use of cTn for diagnostic and prognostic purposes, or whether alternative approaches such as the use of reference change values have added value.

TABLE 1. Analytical characteristics, 99th percentile cutoffs and baseline characteristics of the reference study populations of seven commercially available cTn assays, classified as either guideline acceptable or clinically usable [8]* (*next page*)

* BMI, indicates body mass index; CVD, cardiovascular disease; Li, Lithium; NT-proBNP, N-terminus pro- Brain Natriuretic Peptide; n.s., not significant; -, not reported; ^a, Intra-assay imprecision of 5% at 10 ng/L and 1% at 100 ng/L; ^b, CV at 13.5 ng/L <10%; ^c, Mean $\pm$ SD; ^d, Significant differences in cTnT concentrations between the same decades of age in males and females; ^e, 10% CV cutoffs were all <99th percentile, except for one; ^f, Mean; ^g, Mean (95% Confidence Interval); ^h, as quoted by the manufacturer; ⁱ, median age; ^j, Intra-assay imprecision of 9.6% at 11 ng/L; ^k, 42% Malays, 30% Chinese, 27% Indians; ^l, Mean of ADVIA Centaur CP and ADVIA Centaur platform; ^m, 20% CV cutoff concentration; ⁿ, Mean $\pm$ 2 SD

Publication	Analytical characteristics and 99 th percentile concentrations						Characteristics of Reference population			Gender-specific cutoffs			
	Sample matrix	Analyzer	LoB (ng/L)	LoD (ng/L)	10%CV (ng/L)	99 th perc. (ng/L)	Ratio 10% CV/ 99 th perc.	Subjects (%male)	Age range (years)	Screening methods/Inclusion criteria	99 th perc. Male (ng/L)	99 th perc. Female (ng/L)	P-value
A. hs-cTnT assay (Roche Diagnostics)													
≥ 300 subjects in reference populations													
Latini 2007 [17]	EDTA plasma	Elecsys 2010	-	1	^a	12	-	1061 (-)	-	1. Laboratory tests: NT-proBNP <125 pg/mL	-	-	-
Vasile 2010 [20]	Serum	Elecsys 170	2	4.5	^b	13.5	<1.00	2992 (-)	-		-	-	-
Giannitis 2010 [24]	-	Elecsys 2010 and 170, Cobas e411 and e611	3	5	13	13.5	0.96	616 (50%)	20-71; 44 ± 13.8 ^c	Apparently healthy subjects and blood donors	14.5	10	P<0.01
Roche Diagnostics 2010	-	-	3	5	13	14	0.92	533 (+)	-	Apparently healthy subjects	-	-	-
Beyrau 2009 [18]	-	-	-	2	12	14	0.86	546 (-)	20-71	-	-	-	P<0.05 ^d
Saenger 2011 [21]	-	Elecsys 2010 and 170, Cobas e411 and e611	3	5	^e	14.2	<1.00	533 (50%)	20-71; 37 ^f	1. Medical history questionnaire: <20 years, acute or chronic diseases, pregnancy, medications indicating chronic disease, hospitalizations within the prior 3 months or abnormal BMI	15.5	8.9	P<0.001
Aw 2010 [23]	Serum	Cobas 6000	-	-	11.5	15	0.77	380 (47%)	31-60	Apparently healthy subjects	-	-	-
Mingels 2009 [22]	Serum	Elecsys 2010	1	-	9	16	0.56	479 (55%)	51(26-71) ^g	1. Laboratory tests: Cardiac biomarkers < mean + 3 SD	18	8	P<0.01
<300 subjects in reference populations													
Koertlin 2010 [25]	Serum	Cobas e411	3 ^h	5 ^h	11.9	12.5	0.95	104 (55%)	25-74	1. Medical history questionnaires; 2.Stress echocardiography and angiography; 3. Routine laboratory test in blood and urine	12.9	11	P<0.01
Collinson 2010 [27]	Serum	Elecsys 2010	-	-	18.5	15.5	1.19	248 (42%)	53 ⁱ	1. Medical history examinations to exclude history of vascular disease, diabetes mellitus, hypertension, heavy alcohol intake and cardiac medication; 2. Laboratory tests (blood glucose and creatinine) 3. Echocardiography	-	-	n.s.
Chenevier-Gobeaux 2011 [26]	Li heparin plasma	Elecsys 2010	-	-	9.2	16.9	0.54	213 (36%)	21-90; 52±15 ^c	1. Medical history check; 2. clinical examination 3. routine blood analysis	-	-	P<0.0001
Vasitova 2010 [19]	Serum	Elecsys 2010	-	3	^j	17	-	73 (66%)	56 ± 13 ^c	Cardio-healthy blood donors	-	-	-

Table 1. (continued)

Publication	Sample matrix	Analyzer	LoB (ng/L)	LoD (ng/L)	10%CV (ng/L)	99 th perc. (ng/L)	Ratio 10% CV/ 99 th perc.	Subjects (%male)	Age Range (years)	Screening methods/Inclusion criteria	99 th perc. Male (ng/L)	99 th perc. Female (ng/L)	P-value
B. Architect cTnI assay (Abbott Diagnostics)													
≥ 300 subjects in reference populations													
Mingels 2009 [22]	serum	i2000SR	-	-	32	13	2.46	479 (55%)	51 (26-71) ^a	1. Laboratory tests: Cardiac biomarkers < mean + 3 SD	13	12	n.s.
LaJolla 2010 [35]	Li Heparin Plasma	i1000SR	-	<10	49	18	2.72	400 (40%)	18-65 30 ^b	1. Medical history examinations to exclude CVD, cardio-active medications, cancer, diabetes mellitus, hypertension, hypercholesterolemia and hyperlipidaemia	35	17	n.s.
	Serum		-			13	3.79				-	-	-
	Li Heparin Plasma	i2000SR	-	<10	76	20	3.80				40	17	n.s.
	Serum		-			16	4.75				-	-	-
Apple 2007 [36]	Serum	i2000 system	-	-	-	25	-	2992 (-)	-	Apparently healthy subjects	-	-	-
Dupuy 2009 [31]	-	-	-	-	32	27	1.19	-	-	-	-	-	-
Abbott Diagnostics (2004)	-	-	-	-	32	28	1.14	449 (50%)	18-63	Apparently healthy subjects	33	13	-
Lam 2006 [30]	Serum plasma	Ci8200	-	4	30	30	1.00	475 (66%)	16-82	Healthy blood donors	-	-	-
James 2006 [29]	EDTA plasma	-	4	30	30	1.00	480 (-)	-			-	-	
			-	-	32	38	0.84	442 (67%)			1. Health questionnaire; 2. Clinical examination; 3. ECG; 4. routine blood analysis	-	-
<300 subjects in reference populations													
Apple 2007 [36]	Heparin plasma	i2000 system	-	-	-	12	-	224 (75%)	18-66	Apparently healthy subjects	-	-	-
Hubl [32]	serum	Ci8200	-	4	40	20	2.00	130 (-)	-	1. Exclusion of pregnancy, cardiac, pulmonary, gastrointestinal, endocrinological, renal, hepatic or metabolic disease	-	-	-
Tate 2008 [34]	Li heparin plasma		-	-	55	20	2.75	108 (57%)	25-74	1. Medical history examinations to exclude diabetes mellitus, hypertension, CVD, hyperlipidaemia and cardio-active treatment. 2. Stress echocardiography 3. Routine Laboratory tests on blood and urine	15	21	-
	Serum	Ci8200	-	-		21	2.62				14	18	-
Tovey 2010 [33]	-	-	-	-	50	30	1.67	153 (52%)	30-94	1. ECG; 2. Routine laboratory tests	-	-	-

Table 1, (continued)

Publication	Sample matrix	Analyzer	LoB (ng/L)	LoD (ng/L)	10%CV (ng/L)	99 th perc. (ng/L)	Ratio 10% CV/ 99 th perc.	Subjects (%male)	Age range (years)	Screening methods/Inclusion criteria	99 th perc. Male (ng/L)	99 th perc. Female (ng/L)	P-value
C. AccuTn assay (Beckman Coulter)													
≥ 300 subjects in reference populations													
Zaninotto 2009 [39]	serum	Access 2 UniCel Dxl 800	-	-	48.6	34	1.42	679 (77%)	18-71 (6%≥60y)	Healthy blood donors	34.8	26.6	n.s.
Beckman Coulter 2006	-	Access	4	60	40	40	1.5	-	-	-	-	-	-
James 2006 [29]	EDTA plasma	-	-	-	-	40	-	442 (67%)	-	1. Health questionnaire; 2. Clinical examination; 3. ECG; 4. routine blood analysis	-	-	-
Verge 2003 [47]	EDTA plasma	-	-	-	-	40	-	436 (67%)	-	1. Health questionnaire; 2. Clinical examination; 3. ECG; 4. routine blood analysis	-	-	-
Eggers 2009 [41]	EDTA plasma	Access	-	6	14	28	0.31	1005 (50%)	≥ 70	General population older than 70 years	72	28	P=0.03
Apple 2004 [44]	Serum	Access	-	-	-	50	-	374 (51%)	30-84 (49)	General population older than 70 years screened for cardiovascular disease, left ventricular hypertrophy, ECG abnormalities, or elevated NT-proBNP	33	25	n.s.
Apple 2003 [46]	Heparin plasma	Access	-	-	-	80	-	696 (45%)	18-84	Apparently healthy subjects	60	30	P<0.001
Apple 2007 [36]	Serum	Access	-	-	-	40	-	2992 (-)	-	1. Health questionnaires	100	34	P=0.034
Verge 2009 [42]	EDTA plasma	Access	-	-	14	80	0.18	442 (67%)	-	Apparently healthy subjects	-	-	-
			-	-	-	-	-	-	-	1. Health questionnaire; 2. Clinical examination; 3. ECG; 4. routine blood analysis	-	-	-
<300 subjects in reference populations													
Eriksson 2005 [38]	Serum	Access	-	-	60	15	4.00	144 (-)	-	Patients without cardiac-related symptoms	-	-	-
Bevne 2004 [48]	Li heparin plasma / serum	Access	-	-	-	16	-	52 (73%)	60 ± 16	Non-ACS patients	-	-	-
La'ulu 2010 [35]	Li heparin plasma	Access 2	-	10 ^a	101	18	5.61	400 (40%)	18-65 (30) ^b	1. Medical history examinations to exclude CVD, cardio-active medications, cancer, diabetes mellitus, hypertension, hypercholesterolemia and hyperlipidaemia	33	52	n.s.
Verge 2001 [43]	Serum	Access	-	-	30	20	1.50	122 (43%)	26-73 (41) ^c	Apparently healthy subjects	-	-	-
Tate 2008 [34]	Li heparin plasma	Access2	-	-	104	30	3.47	105 (57%)	25-74	1. Medical history examinations to exclude diabetes mellitus, hypertension, CVD, hyperlipidaemia and cardio-active treatment 2. Stress echocardiography 3. Routine Laboratory tests on blood and urine	40	30	n.s.
Le Moal 2007 [45]	EDTA plasma	-	-	-	-	40	2.60	130 (57%)	18-60 (42±10) ^a	Healthy donors with no history of heart disease	30	30	n.s.
Uetwiller-Geiger 2002 [37]	Serum	Access	-	4	60	40	1.50	254 (35%)	19-88	14% non-cardiac related surgery patients; 20% healthy volunteers; 66% outpatients.	-	-	-
Apple 2007 [36]	Heparin plasma	Access	-	-	-	40	-	254 (75%)	18-66	Apparently healthy subjects	-	-	-
Song 2008 [40]	Heparin plasma	Access 2	-	0	75	61	1.23	259 (51%)	30-68	1. Routine laboratory tests on blood; 2. Medical history examinations to exclude diabetes mellitus, hypertension, thyroid disease, gastric ulcer, cancer and use of other medications.	-	-	n.s.
	Serum		-	-	-	83	0.90				-	-	n.s.
	Heparin plasma	Dxl 800	-	8	58	50	1.16				-	-	n.s.
	serum					63	0.92				-	-	n.s.

Table 1. (continued)

Analytical characteristics and 99 th percentile concentrations							Characteristics of Reference population			Gender-specific cutoffs			
Publication	Sample matrix	Analyzer	LoB (ng/L)	LoD (ng/L)	10%CV (ng/L)	99 th perc. (ng/L)	Ratio 10%CV/99 th perc.	Subjects (%male)	Age range (years)	Screening methods/Inclusion criteria	99 th perc. Male (ng/L)	99 th perc. Female (ng/L)	P-value
D. ADVIA Centaur TnI-Ultra (Siemens Healthcare Diagnostics)													
≥ 300 subjects in reference populations													
Lalulu 2010 [35]	Li heparin plasma	ADVIA Centaur	-	6 ^b	39	18	2.17	400 (40%)	18-65 (30) ^c	1. Medical history examinations to exclude CVD, cardio-active medications, cancer, diabetes mellitus, hypertension, hypercholesterolemia and hyperlipidaemia.	-	-	P=0.028
Collinson 2009 [50]	Serum	ADVIA Centaur	-	6	45	39	1.15	309 (41%)	45-80	1. Heart rate and blood pressure measurements 2. Spirometry 4. ECG 5. Echocardiography	-	-	n.s.
Siemens Healthcare Diagnostics 2009	Li heparin; EDTA plasma; serum	ADVIA Centaur CP	-	6	30	40	0.75	648 (-)	17-91	Apparently healthy subjects	-	-	-
Keller 2009 [51]	-	-	-	6	30	40	0.75	5000 (51%)	35-74 (55.5±10.9) ^e	General population	-	-	n.s.
Shaneshwar 2010 [53]	Serum	ADVIA Centaur	-	-	36	61	0.59	442 ^a (53%)	18-73	1. Health questionnaires	-	-	n.s.
Clerico 2008 [55]	Li heparin plasma	ADVIA Centaur CP	-	-	-	72	-	692 (45%)	11-89 45.3±17.3 ^c	1. Medical history questionnaires 2. Clinical examinations 3. ECG 4. laboratory tests	81	65	P<0.0001
Prontera 2008 [49]	Li heparin Plasma	ADVIA Centaur (CP)	-	6	57	72	0.79	645 (43%)	15-89; 47.4±16.2 ^e	1. Clinical Examinations 2. Laboratory tests	12 ^f	8 ^f	P<0.0001
Venge 2009 [42]	Serum	ADVIA Centaur	-	-	30	80	0.38	442 (67%)	40-76	1. Medical history questionnaires to exclude chronic disease and medication 2. Clinical examinations 3. ECG 4. Routine laboratory tests	-	-	-
Prontera 2007 [54]	Li heparin plasma	ADVIA Centaur (CP)	6	-	67 ⁱ	87	0.77	418 (49%)	16-89 50.7±16.6 ^e	1. Clinical Examinations 2. Laboratory tests	15 ±18 ^d	9 ±14 ^d	P<0.0001
<300 subjects in reference populations													
Tale 2008 [34]	Li heparin plasma	ADVIA Centaur	-	-	45	21	2.14	108 (57%)	25-74	1. Medical history examinations to exclude diabetes mellitus, hypertension, CVD, hyperlipidaemia and cardio-active treatment 2. Stress echocardiography 3. Routine Laboratory tests on blood and urine.	22	19	n.s.
Van de Kerkhof 2008 [52]	Li heparin plasma	ADVIA Centaur	-	8.5	50	60	0.83	221 (50%)	14-86 59 ^g	1. Medical history questionnaires to exclude myocardial disease	18	27	n.s.
											-	-	-

Table 1. (continued)

Analytical characteristics and 99 th percentile concentrations							Characteristics of Reference population			Gender-specific cutoffs			
Publication	Sample matrix	Analyzer	LoB (ng/L)	LoD (ng/L)	10%CV (ng/L)	99 th perc. (ng/L)	Ratio 10% CV/ 99 th perc.	Subjects (%male)	Age range (years)	Screening methods/Inclusion criteria	99 th perc. Male (ng/L)	99 th perc. Female (ng/L)	P-value
E. Stratus CS Acute Care cTnI assay (Siemens Healthcare Diagnostics)													
≥ 300 subjects in reference populations													
Christenson 2004 [58]	Li heparin whole blood	Stratus CS	-	10	60	70	0.86	345 (-)	-	Apparently healthy subjects	-	-	-
<300 subjects in reference populations													
Di Seno 2005 [59]	Li heparin whole blood	Stratus CS	-	15	70	30	2.33	105 (59%)	18-77 50 ± 16*	1. Medical history investigations to exclude ACS 2.Hematochemical alterations 3. Exclusion of hyperpyrexia.	-	-	-
Alliner 2000 [57]	Li heparin whole blood	Stratus CS	-	20	60	50	1.20	85 (67%)	19-75	Apparently healthy subjects	-	-	-
Siemens Healthcare Diagnostics 2001	Li heparin whole blood	Stratus CS	-	30	60	70	0.86	101(-)	-	Apparently healthy subjects	-	-	-
Heeschen 1999 [56]	Li heparin whole blood	Stratus CS	-	10	30**	80**	<1.00	161(-)	-	1. Medical history examinations to exclude CVD; 2. ECG; 3. Treadmill tests 4. Stress echocardiography or angiogram.	-	-	-
F. Dimension Vista cTnI assay (Siemens Healthcare Diagnostics)													
≥ 300 subjects in reference populations													
Arebola 2010 [60]	Serum	Dimension Vista	11	15	36	22	1.64	350 (73%)	18-65	1. Medical history examinations 2. Routine laboratory tests on blood	-	-	n.s.
<300 subjects in reference populations													
Siemens Healthcare Diagnostics 2008	-	Dimension Vista	15	-	<40	45	<0.89	199(-)	-	Apparently healthy subjects	-	-	-

Table 1. (continued)

Publication	Analytical characteristics and 99 th percentile concentrations					Characteristics of Reference population			Gender-specific cutoffs				
	Sample matrix	Analyzer	LoB (ng/L)	LoD (ng/L)	10%CV (ng/L)	99 th (ng/L)	Ratio 10% CV/ 99 th perc.	Subjects (%male)	Age Range (years)	Screening methods/Inclusion criteria	99 th perc. Male (ng/L)	99 th perc. Female (ng/L)	P-value
G. Vitros ECI cTnI ES assay (Ortho Clinical Diagnostics)													
≥ 300 subjects in reference populations													
La Jolla 2010 [35]	Li heparin plasma	Vitros ECI	-	12 ^a	93	18	5.17	400 (40%)	18-65 30 ^a	1. Medical history examinations to exclude CVD, cardio-active medications, cancer, diabetes mellitus, hypertension, hypercholesterolemia and hyperlipidaemia	17	26	n.s.
	Serum					13	7.15						
Ortho Clinical Diagnostics 2007-2009	Li heparin; EDTA plasma; serum	Vitros ECI	7	12	34	34	1.00	>10 000 (-)	-	-	-	-	-
	Li heparin plasma					31	1.10						
Apple 2007 [36]	Li heparin plasma	Vitros ECI	-	12 ^a	34 ^a	34	1.00	2992 (-)	-	-	-	-	-
	Serum					34	1.00						
<300 subjects in reference populations													
Tate 2008 [34]	Li heparin plasma	Vitros ECI	-	12 ^a	34	16	2.13	108 (57%)	25-74	1. Medical history examinations to exclude diabetes mellitus, hypertension, CVD, hyperlipidaemia and cardio-active treatment 2. Stress echocardiography 3. Routine Laboratory tests on blood and urine	15	14	n.s.
	Serum					15	2.27						
Saw 2009 [61]	-	Vitros ECI	-	-	40	39	1.03	311 (-)	20-72	Blood donors	-	-	-

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THREE

HIGH-SENSITIVITY CARDIAC TROPONIN CONCENTRATIONS IN PATIENTS WITH CHEST DISCOMFORT: *IS IT THE HEART OR THE KIDNEYS AS WELL?*

Cardinaels E, Altintas S, Versteylen M, Joosen I, Jellema L, Das M, Wildberger J, Crijns H, Bekers O, van Dieijen-Visser M, Kietselaer B* and Mingels A*

**, both authors contributed equally*

Submitted

Abstract

Background

The extent of renal influence on hs-cTn concentrations is still unknown in patients suspected for coronary artery disease (CAD) and is necessary for appropriate interpretation of low-level hs-cTn concentrations. We therefore simultaneously investigated the renal and cardiac contribution to hs-cTn concentrations in chest discomfort patients from the cardiology outpatient department.

Methods

A cross-sectional analysis was performed in 1864 patients with symptoms of chest discomfort who underwent cardiac computed tomographic angiography (CCTA). Serum samples were analyzed using hs-cTnT and hs-cTnI assays. Renal function was measured by the estimated glomerular filtration rate (eGFR), established from serum creatinine and cystatin C. On follow-up, the incidence of cardiovascular events was assessed.

Results

Median hs-cTnT and hs-cTnI concentrations were 7.2(5.8-9.2)ng/L and (2.6(1.8-4.1) ng/L, respectively. Both hs-cTn measurements were more strongly associated with eGFR (hs-cTnT:st β :-0.289; hs-cTnI:st β :-0.222) than with cardiac imaging parameters, such as coronary calcium score (hs-cTnT:st β :0.078; hs-cTnI:st β :0.092), CCTA plaque severity score (hs-cTnT:st β :0.074; hs-cTnI:st β :0.072) and left ventricular mass (hs-cTnT:st β :0.139 ;hs-cTnI:st β :0.234) (all $p \leq 0.01$ and after adjustment for traditional risk factors). Moreover, the association between eGFR and hs-cTn remained equally strong among patients with no (hs-cTnT:st β eGFR:-0.295; hs-cTnI:st β eGFR:-0.228), mild (hs-cTnT:st β eGFR:-0.290; hs-cTnI:st β eGFR:-0.176) and moderate-to-severe CAD (hs-cTnT:st β eGFR:-0.293; hs-cTnI:st β eGFR:-0.249) (all $p < 0.001$). Both hs-cTnT and hs-cTnI remain independent prognostic markers for cardiovascular events, irrespective of renal function and CAD.

Conclusion

In patients with chest discomfort, we identified decreases in renal function and the extent of CAD as two separate contributors of troponin accumulation. Awareness of these renal influences is therefore warranted when interpreting hs-cTn concentrations.

Introduction

Identifying chest pain patients at risk for cardiovascular events remains an ongoing challenge [1]. A promising and cost-effective way to identify those “vulnerable” patients is the use of cardiac troponins [2, 3]. Because of their unique cardiospecificity, cardiac troponins are considered the preferred biochemical markers to detect myocardial injury and to diagnose acute myocardial infarction (AMI) in particular [4]. Since the introduction of high-sensitivity cardiac troponin (hs-cTn) assays, more accurate detection of low levels of circulating cardiac troponins became feasible [5]. The use of hs-cTn concentrations significantly improved the diagnostic performance in patients with acute cardiac risk[6]. Moreover, hs-cTn concentrations turned out to have an important prognostic value for acute cardiovascular events, even below the diagnostic cut-off and beyond traditional cardiovascular risk factors [7-9].

Unfortunately, the shift to more sensitive assays is accompanied by a reduction in specificity, as circulating hs-cTn levels are elevated in many other conditions besides AMI[7, 10]. Renal dysfunction is one of those conditions in which elevated cardiac troponin concentrations are commonly detected [11, 12]. Recently it was shown in chronic kidney disease (CKD) patients that elevated hs-cTn concentrations are indeed associated with reduced renal function [13]. Therefore, the interpretation of baseline hs-cTn values in the individual patient is complicated not only by cardiac disease [14-16] but also renal dysfunction. As of yet it is unknown to what extent renal function contributes to higher cardiac troponin concentrations in patients with chest discomfort, in whom circulating hs-cTnT concentrations are mainly attributed to the presence and severity of atherosclerotic plaques [2, 3] or echocardiographic abnormalities [17-19]. In-depth understanding of the relative contribution of renal function, in addition to cardiac parameters, is of utmost importance for the use of hs-cTn values in clinical practice.

In this study, we therefore evaluate the association of serum hs-cTnT and hs-cTnI concentrations with renal function (as assessed by the estimated glomerular filtration rate (eGFR)), cardiac imaging measures (cardiac computed tomographic angiography (CCTA) and echocardiography), and the incidence of cardiovascular events in patients who visited the cardiology outpatient

department with symptoms of chest discomfort. Particularly, the renal and cardiac contribution to hs-cTnT and hs-cTnI concentrations will be examined simultaneously.

Materials and methods

Study cohort

We analyzed a cohort of 1864 consecutive patients who were enrolled in the Maastricht Biomarker CT Study (ClinicalTrials.gov, NCT01671930). This cohort is comprised of patients presenting with (a)typical chest pain with a low-to-intermediate pretest probability who were referred for CCTA from the cardiology outpatient department for the evaluation of coronary artery disease (CAD), in accordance with the current guidelines[1]. Included were patients of whom serum was collected. Excluded were patients with previous history ACS and patients on dialysis (due to application of contrast fluids). Additional specifications of this population have previously been published [2, 20]. This study was conducted according to the principles of the Declaration of Helsinki and approved by the local ethics committee. Written informed consent was obtained from all patients.

Biochemical analysis

Serum samples were collected immediately before CCTA, processed within 2 hours and directly stored at -80 °C until analysis. Total cholesterol, triglycerides, high-density and low-density lipoprotein concentrations were measured as previously described [2]. Serum creatinine, cystatin C and cTnT concentrations were measured on the Cobas 6000 analyzer (Roche Diagnostics) in a fresh aliquot. Creatinine concentrations were assessed using the enzymatic method (Roche). Cystatin C was measured using a new particle-enhanced turbidimetric assay (Gentian AS), that was standardized against the certified ERM-DA471/IFCC cystatin C reference material [21]. The glomerular filtration rate was estimated using the Chronic Kidney Disease Epidemiology Collaboration equations[22] using serum creatinine and cystatin C concentrations. cTnT concentrations were determined using the high-sensitivity cTnT assay (Roche; lotnumber 167650), with a 99th percentile upper reference limit of 14 ng/L and a

10% coefficient of variation cut-off at 13 ng/L. Gender-specific cutoffs were reported at 14.5 ng/L and 10 ng/L for males and females, respectively [5]. cTnI measurements were performed on the ARCHITECT i2000SR platform using the precommercial ARCHITECT STAT high-sensitivity troponin I (hs-cTnI) assay (Abbott Laboratories). According to the manufacturer, a 10% CV was reached at 4.7 ng/L and the 99th percentile cut-off concentration at 26.2 ng/L for the overall population. Gender-specific cut-offs at 34.2 ng/L and 15.6 ng/L were also defined for males and females, respectively.

Cardiac computed tomographic angiography

CCTAs were performed from December 2007 through December 2012 and analyzed as previously described [20]. In brief, CCTA were analyzed by two experts who were blinded from hs-cTn results. The coronary calcium score (CCS) was quantified by the Agatston method[23] and luminal plaque severity as assessed by CCTA was scored as no, mild (<50% stenosis), moderate (50-70% stenosis) and severe (>70% stenosis) CAD.

Echocardiography

Echocardiography was performed in a subset of 549 patients (31%) within a period of 3 months from the CCTA scan by an expert echocardiographer, who was blinded for hs-cTn concentrations. Transthoracic images of the left ventricle (LV) were acquired to assess morphology, function and mass (Philips IE 33, Philips Medical Systems). LV function and -mass were calculated by off-line image analysis using Xcelera software package (Philips Medical Systems), according to current ESC/AHA guidelines [24]. Left ventricular hypertrophy was defined as an LVmass >95 g/m² in females or LVmass >115 g/m² in males [25].

Cardiovascular events

Electronic patient records were monitored for the occurrence of cardiovascular events by two reviewers. Survival time was defined as the period from date of CCTA to date of the first cardiovascular event or June 2013. The composite study endpoints were defined in advance as major adverse cardiovascular events, encompassing cardiovascular mortality, acute coronary syndromes including AMI and unstable angina requiring hospitalization; and late coronary

revascularization (>90 days after CCTA), such as percutaneous coronary interventions and coronary artery bypass grafting [26]. No records could be retrieved of 175 subjects, therefore 1689 patients (91%) of the total cohort were included for follow-up analysis.

Statistical analysis

Comparisons of baseline characteristics were performed using the T-test for continuous variables with a normal distribution, Mann-Whitney U-test for non-normal distributed continuous variables and Chi square test for categorical variables. Data are presented as proportions, means $\pm$ standard deviations, and data with a non-normal distribution are given as the median (interquartile range, IQR). Pearson R correlation factors were calculated with the natural logarithm (Ln) of hs-cTnT and hs-cTnI to normalize their skewed distribution.

To assess the independent association of renal and cardiovascular parameters with hs-cTn concentrations, linear regression analyses were performed with either Ln(hs-cTnT) or Ln(hs-cTnI) as the dependent variable. Only the cardiovascular risk factors that were significantly associated to higher hs-cTnT quartiles (TABLE 1) were entered into the multivariable model. R^2 was calculated to measure the performance of the model, and the R^2 change to address the additive effect of eGFR to the model. Univariable and multivariable cox-proportional hazards models were performed to investigate the association with cardiovascular events. The discriminative ability of each predictor was assessed using the receiver-operating characteristic (ROC) curves.

Statistical analysis was performed with SPSS 20.0 (SPSS). Two sided p-values of <0.05 were considered statistically significant.

Results

Distribution and determinants of hs-cTnT and hs-cTnI

Median (IQR) hs-cTn concentrations in this cohort were 7.2 (5.8-9.2) ng/L for hs-cTnT and 2.6 (1.8-4.1) ng/L for hs-cTnI. Of all patients, 6.6% (n=123) were above the cutoff of hs-cTnT (14 ng/L) and 2.1% (n=30) above the cutoff of hs-cTnI (26.2 ng/L). The majority of all patients (72%) had a normal renal function (eGFR>90 mL/min/1.73m²) and 98% had an eGFR above 60 mL/min/1.73m².

The baseline characteristics are presented in TABLE 1 and illustrate that increasing quartiles of hs-cTnT and hs-cTnI were highly associated with traditional cardiovascular risk factors such as advancing age, male sex and hypertension. The association between Ln(hs-cTnT) and Ln(hs-cTnI) was only moderate (Pearson R: 0.635; $p < 0.001$ and cohen κ of 0.27; $p < 0.001$; SUPPLEMENTAL TABLE 1).

Independent association of cardiovascular disease and renal function with hs-cTn concentrations

Univariable regression analysis demonstrated that hs-cTn concentrations are significantly associated with eGFR (hs-cTnT: R:-0.396; hs-cTnI: R:-0.251; SUPPLEMENTAL FIGURE 1), regardless of the algorithm that is used to estimate GFR (SUPPLEMENTAL FIGURE 2). Also, both hs-cTnT and hs-cTnI concentrations are significantly correlated with CCS (hs-cTnT: R:0.279; hs-cTnI: R:0.213) and CCTA plaque severity (hs-cTnT: R:0.307; hs-cTnI, R:0.230) (TABLE 2 and SUPPLEMENTAL FIGURE 3, ALL $P < 0.001$).

As displayed by FIGURE 1, when adding renal and CT parameters as explanatory variables for hs-cTn concentrations in a multivariable regression model, both eGFR, CCS as CCTA plaque severity were identified as independent predictors (TABLE 2, unadjusted multivariable model).

TABLE 1. Baseline characteristics in the overall population and by median hs-cTnT and hs-cTnI concentrations.

Determinant	All patients	hs-cTnT		hs-cTnI	
		≤7.2 ng/L	>7.2 ng/L	≤2.6 ng/L	>2.6 ng/L
Traditional cardiovascular risk factors					
Age, years	55.8±11.0	51.8±9.9	60.4±10.2	53.0±10.7	58.1±10.6
Males, %	56.0	41.3	69.2	42.7	62.6
Body mass index, kg/m ²	27.0±4.4	26.6±4.3	27.3±4.3	26.3±4.4	27.3±4.3
Smokers, %	22.6	24.5	21.0	23.3	22.3
Diabetes, %	7.3	5.6	8.9	6.9	7.6
Family history, %	38.8	45.1	33.0	43.6	36.3
Systolic blood pressure, mmHg	142.2±19.3	139.1±19.0	145.3±19.2	138.1±18.2	144.5±19.6
Diastolic blood pressure, mmHg	80.2±11.3	79.2±10.6	80.8±12.0	78.3±10.8	80.9±11.6
Total cholesterol, mmol/L	5.4±1.2	5.5±1.1	5.3±1.2	5.4±1.1	5.5±1.2
High density lipoprotein, mmol/L	1.3±0.5	1.3±0.4	1.3±0.5	1.4±0.4	1.3±0.5
Low density lipoprotein*, mmol/L	3.4±1.0	3.4±1.0	3.3±1.1	3.3±1.0	3.4±1.1
Triglycerides*, mmol/L	1.8±1.2	1.7±1.1	1.8±1.3	1.6±1.0	1.9±1.2
Estimated GFR					
CKD EPI _{Creat-CysC} , mL/min/1.73m ²	99.4±18.4	105.7±16.2	92.1±18.7	105.1±17.7	95.2±18.5
CCTA					
Coronary calcium score, AS	4.0(105.4)	0.0(0.0-25.3)	37.5(0.0-219.1)	0.0(0.0-38.2)	16.3(0.0-167.7)
Moderate-to-severe plaque, %	23.7	14.3	32.1	17.4	26.7
					<0.001

* indicates 250 missing values; AS, indicates Agatston score

FIGURE 1. Median hs-cTnT (A) and hs-cTnI (B) concentrations according to estimated GFR categories and coronary calcium score or CCTA plaque severity. AS, agatston score

Similar standardized β_{eGFR} ($\text{st}\beta_{\text{eGFR}}$), $\text{st}\beta_{\text{CCS}}$ and $\text{st}\beta_{\text{CCTA}}$ coefficients were observed in the univariable versus unadjusted multivariable models, indicating that CT parameters were hardly influenced by the addition of eGFR when predicting hs-cTnT or hs-cTnI concentrations (TABLE 2). After adjustment for traditional cardiovascular risk factors, eGFR (hs-cTnT: $\text{st}\beta$:-0.289; hs-cTnI: $\text{st}\beta$:-0.222; $p<0.001$), CCS (hs-cTnT: $\text{st}\beta$:0.078; hs-cTnI: $\text{st}\beta$:0.092; $p<0.001$) and CCTA plaque severity score (hs-cTnT: $\text{st}\beta$:0.074; hs-cTnI: $\text{st}\beta$:0.072; $p<0.001$) remained significantly associated with hs-cTn concentrations (TABLE 2, adjusted multivariable model 1 and 2, respectively). Also here, $\text{st}\beta_{\text{eGFR}}$ values remain unchanged (TABLE 2, adjusted multivariable models 1 and 3).

TABLE 2. Univariable and multivariable linear regression analysis, demonstrating the independent influence of eGFR on hs-cTn concentrations beyond CT parameters.

	Univariable models			Unadjusted multivariable model			Adjusted multivariable models							
	St β	P-value	R ²	St β	P-value	R ²	Model 1		Model 2		Model 3			
Dependent variable: $\text{Ln}(\text{hs-cTnI})$														
eGFR	-0.396	<0.001	0.156	-0.347	<0.001	0.224	-0.289	<0.001	0.323			-0.290	<0.001	0.338
Coronary calcium score	0.279	<0.001	0.077	0.140	<0.001					0.078	0.002	0.089	<0.001	
CCTA plaque severity score	0.307	<0.001	0.094	0.158	<0.001					0.074	0.004	0.062	0.012	
Age							0.254	<0.001		0.357	<0.001	0.201	<0.001	
Female sex							-0.336	<0.001		-0.278	<0.001	-0.307	<0.001	
BMI							0.007	0.726		0.047	0.022	0.009	0.659	
Diabetes							0.066	0.001		0.056	0.006	0.061	0.002	
Family history							-0.025	0.197		-0.046	0.023	-0.033	0.085	
Systolic blood pressure							0.057	0.014		0.052	0.031	0.055	0.017	
Diastolic blood pressure							-0.028	0.224		-0.030	0.206	-0.033	0.151	
Total cholesterol							-0.042	0.032		-0.043	0.035	-0.036	0.069	
Dependent variable: $\text{Ln}(\text{hs-cTnI})$														
eGFR	-0.251	<0.001	0.063	-0.212	<0.001	0.104	-0.222	<0.001	0.145			-0.222	<0.001	0.159
Coronary calcium score	0.213	<0.001	0.045	0.113	<0.001					0.092	<0.001	0.101	<0.001	
CCTA plaque score	0.230	<0.001	0.052	0.122	<0.001					0.072	0.011	0.062	0.026	
Age							0.095	0.001		0.158	<0.001	0.038	0.186	
Female sex							-0.261	<0.001		-0.207	<0.001	-0.231	<0.001	
BMI							0.031	0.174		0.061	0.006	0.032	0.145	
Diabetes							-0.022	0.319		-0.032	0.152	-0.028	0.202	
Family history							-0.013	0.544		-0.032	0.158	-0.022	0.317	
Systolic blood pressure							0.098	<0.001		0.094	<0.001	0.096	<0.001	
Diastolic blood pressure							-0.017	0.522		-0.019	0.467	-0.022	0.400	
Total cholesterol							0.018	0.435		0.018	0.431	0.025	0.266	

The independent contribution of eGFR to the prediction of hs-cTnT and hs-cTnI concentrations was also demonstrated by significant and identical R^2 changes (hs-cTnT: 0.056, hs-cTnI: 0.030; all $p < 0.001$) when adding eGFR either to the baseline model or to adjusted multivariable model 2.

Furthermore, the association of eGFR with hs-cTn concentrations remained equally strong when subdividing this cohort into patients with no (hs-cTnT: $\text{st}\beta_{\text{eGFR}}$: -0.295; hs-cTnI: $\text{st}\beta_{\text{eGFR}}$: -0.228), mild (hs-cTnT: $\text{st}\beta_{\text{eGFR}}$: -0.290; hs-cTnI: $\text{st}\beta_{\text{eGFR}}$: -0.176) and moderate-to-severe CAD (hs-cTnT: $\text{st}\beta_{\text{eGFR}}$: -0.293; hs-cTnI: $\text{st}\beta_{\text{eGFR}}$: -0.249) (all $p < 0.001$), confirming the independent influence of eGFR on hs-cTn concentrations beyond CAD severity (TABLE 3). This finding was visible but less apparent for hs-cTnI than for hs-cTnT.

Considering the hs-cTnT concentrations above the 99th percentile concentrations, we found that 47% were suffering from significant CAD (presence of obstructive plaques or CCS > 400) and 53% had decreased renal function ($\text{eGFR} < 90 \text{ mL/min/1.73m}^2$). Moreover, 72% of the patients with elevated hs-cTnT concentrations were diagnosed with CAD or had a decreased eGFR (SUPPLEMENTAL TABLE 2). For hs-cTnI, this was 43%, 30% and 58%, respectively. Similar findings were observed when applying gender-specific cutoffs (data not shown).

Comparable regression analyses were performed including echocardiographic parameters as explanatory variables for hs-cTn concentrations. Univariably, hs-cTnT and hs-cTnI were significantly associated with the echocardiographic measures LVEF (hs-cTnT: R : -0.151, $p = 0.001$; hs-cTnI, R : -0.142, $p = 0.002$) and LVmass (hs-cTnT: R : 0.253; hs-cTnI: R : 0.309; $p < 0.001$) (SUPPLEMENTAL TABLE 3 and SUPPLEMENTAL FIGURE 3). In line with previous results, $\text{st}\beta_{\text{eGFR}}$ coefficients were only influenced by the confounding effects of traditional cardiovascular risk factors and not by any of the measured CT parameters or echocardiographic parameters (SUPPLEMENTAL TABLE 4).

In total, 30% and 19 % of hs-cTnT and hs-cTnI variation, respectively, could be explained by the most important independent predictors: eGFR, CCS, LVmass, age and male sex (SUPPLEMENTAL TABLE 4).

TABLE 3. Association between eGFR and hs-cTnT or hs-cTnI concentrations in patients without CAD (n= 756), mild (n= 667) and moderate-to-severe CAD (n= 441), indicating almost identical unstandardized and standardized β values for eGFR ($st\beta_{eGFR}$) across these different categories.

<u>CCTA plaque severity</u>	Dependent variable: $Ln(hs-cTnT)$		Dependent variable: $Ln(hs-cTnI)$	
		$St\beta_{eGFR}(95\%CI)^*$	R^2	
No CAD	eGFR	-0.295(-0.373;-0.218)†	0.237	$St\beta_{eGFR}(95\%CI)^*$
				-0.228(-0.313;-0.144)†
			0.107	
Mild CAD	eGFR	-0.290(-0.368;-0.213)†	0.297	$St\beta_{eGFR}(95\%CI)^*$
				-0.176(-0.264;-0.088)†
			0.094	
Moderate-to-severe CAD	eGFR	-0.293(-0.386;-0.200)†	0.275	$St\beta_{eGFR}(95\%CI)^*$
				-0.249(-0.350;-0.148)†
			0.151	

*, indicates $St\beta_{eGFR}$ obtained from the regression model containing the variables eGFR, age, gender, BMI, Family history, Systolic BP, diastolic BP and total cholesterol;

†, $p<0.001$

Algorithm between eGFR and hs-cTn concentrations

In patients with an eGFR between 120-130 mL/min/1.73m², median hs-cTnT and hs-cTnI concentrations were 6.0 ng/L and 2.0 ng/L, respectively (TABLE 4). Moreover, when eGFR was halved to 60 mL/min/1.73m², a doubling of both hs-cTn concentrations was observed (hs-cTnT: 9.9 ng/L; hs-cTnI: 4.1 ng/L). A decrease in eGFR of 10 mL/min/1.73m² corresponded in this population to a mean relative increase of 9% and 11% or mean absolute increase of 0.8 ng/L and 0.4 ng/L in hs-cTnT and hs-cTnI concentrations, respectively.

Hs-cTn remain significant and comparable prognostic markers for cardiovascular events

Over a mean follow-up period of 2.9±1.5 years, 54 cardiovascular events (3.2%) were observed, encompassing 33 patients who underwent late revascularization, 18 patients that suffered from a non-fatal acute coronary syndrome and 3 patients who died due to cardiac disease (decompensatio cordis and myocardial infarction) or vascular causes (cerebrovascular attack). From all traditional risk factors, only age, gender and smoking were significantly different in the event versus non-event group (SUPPLEMENTAL TABLE 5).

As depicted in FIGURE 2, univariable Cox regression analysis showed that the incidence of cardiovascular events was significantly associated with hs-cTnT and hs-cTnI. Moreover, both hs-cTnT and hs-cTnI remained highly significant predictors for cardiovascular events when adjusted for eGFR and CCS or CCTA plaque severity .

ROC analysis demonstrated no significant difference between hs-cTnT (area-under-the-curve, AUC (95% confidence intervals): 0.671(0.599-0.742)) and hs-cTnI (0.649(0.571-0.727)) in the prediction of cardiovascular events (p>0.05, data not shown).

TABLE 4. Observed and calculated change in hs-cTnT and hs-cTnI concentrations across eGFR categories.

Observed hs-cTn delta					Calculated hs-cTn delta				
eGFR (mL/min/1.73m ²)	Number of patients	Median hs-cTnI- concentration (ng/L)	Absolute hs-cTnI delta (ng/L)†	Relative hs-cTnI delta (%)†	eGFR (mL/min/1.73m ²)	hs-cTnI- concentration (ng/L)*	Absolute hs-cTnI delta (ng/L)†	Relative hs-cTnI delta (%)†	
120-130	134	6.0	+0.5	+8.0	120	6.2	+0.6	+8.8 %	
110-120	319	6.5	+0.3	+4.5	110	6.8	+0.6	+9.3 %	
100-110	468	6.8	+0.5	+7.1	100	7.5	+0.7	+8.5 %	
90-100	349	7.3	+0.5	+6.6	90	8.2	+0.8	+7.9 %	
80-90	249	7.8	+1.0	+12.0	80	8.9	+0.9	+9.2 %	
70-80	169	8.8	+1.1	+11.8	70	9.8	+0.9	+8.4 %	
60-70	65	9.9			60	10.7			
		Mean delta	+0.7 ng/L	+8.3%		Mean delta	+0.8 ng/L	+8.7 %	

eGFR (mL/min/1.73m ²)	Number of patients	Median hs-cTnI- concentration (ng/L)	Absolute hs-cTnI delta (ng/L)†	Relative hs-cTnI delta (%)†	eGFR (mL/min/1.73m ²)	hs-cTnI- concentration (ng/L)†	Absolute hs-cTnI delta (ng/L)†	Relative hs-cTnI delta (%)†	
120-130	134	2.0	+0.3	14.0	120	2.5	+0.3	+10.7 %	
110-120	319	2.3	+0.2	8.3	110	2.8	+0.3	+9.7 %	
100-110	468	2.5	+0.3	11.3	100	3.1	+0.4	+11.4 %	
90-100	349	2.8	+0.3	10.2	90	3.5	+0.4	+10.3 %	
80-90	249	3.1	+0.4	12.1	80	3.9	+0.5	+11.4 %	
70-80	169	3.5	+0.6	15.8	70	4.4	+0.5	+10.2 %	
60-70	65	4.1			60	4.9			
		Mean delta	+0.4 ng/L	+12.0%		Mean delta	+0.4 ng/L	+10.6 %	

*calculated from regression lines, as displayed in supplemental figure 1, $\ln(\text{hs-cTnT}) = -0.009 * (\text{eGFR}) + 2.910$ and $\ln(\text{hs-cTnI}) = -0.011 * (\text{eGFR}) + 2.243$ †, per 10 mL/min/1.73m² decrease

FIGURE 2. Hazard ratios and 95% confidence intervals (CI) for cardiovascular events as predicted by hs-cTnT or hs-cTnI either unadjusted or adjusted to eGFR and the cardiac imaging parameters: coronary calcium score (CCS) and CCTA plaque severity score. *, indicates a p-value <0.001

Discussion

The present study provides new insights into the interpretation of hs-cTn concentrations in patients with chest discomfort, identifying renal function and CAD as independent and strong contributors to circulating hs-cTn concentrations. In fact, in this population eGFR exhibited limited confounding effects on the association between hs-cTn and cardiac parameters.

Significant influence of renal function on hs-cTnT and hs-cTnI concentrations

We found that hs-cTnT and hs-cTnI were strongly correlated with eGFR in patients with chest discomfort, in which 98% had a renal function >60 mL/min/1.73m². In this cohort, a decrease in eGFR of 10 mL/min/1.73m² indicated a $\pm 10\%$ increase in hs-cTn concentrations, equivalent to an absolute increase of 0.8 ng/L and 0.4 ng/L for hs-cTnT and hs-cTnI, respectively. Although these findings need to be validated in other populations, it clearly

illustrates that a decreased renal clearance, even within normal boundaries, can affect hs-cTn concentrations and questions whether baseline hs-cTn concentrations should be reported without the access to an eGFR.

As observed in moderate-to-severe CKD patients [13], hs-cTnT was more strongly correlated with eGFR than hs-cTnI. In addition, the association between hs-cTnT concentrations and eGFR were stronger than any known association between hs-cTnT and cardiac parameters, such as coronary plaque severity [2, 3, 27] or left ventricular structure [16, 18, 28]. Interestingly, this is in contrast to hs-cTnI, in which the association with LVmass was greater than with eGFR. These findings suggest that hs-cTnT could be more susceptible to changes in renal clearance than hs-cTnI.

Renal and cardiac parameters as two separate contributors to hs-cTn

Importantly, in a cohort of patients with normal eGFR values, we demonstrate for the first time that the extent of CAD and echocardiographic characteristics of the left ventricle did not interfere with the magnitude of the association between eGFR and hs-cTn concentrations. As a consequence, in patients with a normal to a mildly impaired kidney function, decreases in renal function and the extent of CAD can be considered as two separate contributors of hs-cTn accumulation. This independent association with renal function might not only be valuable for the evaluation of stable chest pain patients [1] but also chronic heart failure and acute chest pain patients [4].

Furthermore, we show that a significant amount of the patients with elevated hs-cTn concentrations suffered from decreased renal function, besides the presence of CAD. These findings can explain the reduced diagnostic performance of hs-cTn seen in AMI patients with lower renal function in comparison to those with normal renal function [29, 30]. Further research should therefore focus on the influence of mild CKD when acquiring the diagnostic hs-cTn cutoffs as established from reference populations (99th upper reference limit).

The significant association between eGFR and hs-cTn concentrations, independent from cardiac pathologies, is in line with the observation that cTnT and cTnI are cleared by the kidneys [31, 32]. However, we cannot exclude that a

decreased renal function may exert additional myocardial stress that is not identified by cardiac imaging, leading to subsequent cardiac troponin release.

Prognostic importance of hs-cTnT and hs-cTnI besides CCTA and renal function

In this study, serum hs-cTnT as well as hs-cTnI were significant prognostic markers for the development of cardiovascular events, as previously demonstrated [8, 9, 28, 33]. Although hs-cTnT concentrations were twice as high as hs-cTnI concentrations, we found the overall prognostic performance of hs-cTnT and hs-cTnI to be comparable (AUC_{ROC} : 0.670 versus 0.650 for hs-cTnT and hs-cTnI, respectively).

Hs-cTnT was shown to be a strong prognostic marker for cardiovascular events, despite the influence of a decreased renal function [34, 35]. We found that even when adjusted to eGFR, both hs-cTn concentrations still hold an important prognostic value besides important cardiovascular imaging parameters such as CCS and CCTA plaque severity score. In fact, we were able to explain a quarter to one third of the variability in hs-cTn concentrations, suggesting that other mechanisms could contribute to the overall hs-cTn level as well.

Study limitations

A limitation to our survival analysis is that this is a low-risk population and consequently a low number of adverse cardiovascular events were observed. Also, we cannot rule out that CCTA outcomes were used in the decision to perform a CABG and PCI in these patients. However, we corrected for this bias by excluding the procedures that were performed within 90 days after CCTA. Furthermore, urine samples were not available for the assessment of albuminuria.

Conclusion

In conclusion, we identified in patients with symptoms of chest discomfort that renal function has a significant influence on circulating hs-cTnT and hs-cTnI concentrations. Moreover, we found that cardiac pathology and decreases in renal function are two independent contributors for hs-cTn accumulation. Prospective studies must establish whether baseline hs-cTn concentrations

should be accompanied by GFR-assessment. Notwithstanding, hs-cTnT and hs-cTnI remained significant predictors of cardiovascular events, even on top of cardiovascular disease and renal function.

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SUPPLEMENTAL FIGURE 1. Scatterplots of the association of eGFR with Ln (hs-cTnT) (A) and Ln (hs-cTnI) (B).

SUPPLEMENTAL FIGURE 2. Association of hs-cTnT (blue) and hs-cTnI (grey) with GFR estimated by the MDRD equation (A) and the CKD EPI equations using serum creatinine (B), serum cystatin C (C) concentrations or both (D). *p-value < 0.05 in comparison with eGFR 31-60 mL/min/1.73 m² group. Pearson R correlation factors with the natural logarithm of hs-cTnT and hs-cTnI are given.

SUPPLEMENTAL FIGURE 3. Association of hs-cTnT (blue) and hs-cTnI (grey) with the coronary calcium score (CCS) (A), CCTA plaque severity (B), and echocardiographic parameters, LVEF (C) and LVH (D). *p-value < 0.05 in comparison with the non-diseased group. Pearson R correlation factors with the natural logarithm of hs-cTnT and hs-cTnI are given. AS, indicates Agatston score

SUPPLEMENTAL TABLE 1. Contingency table of subjects with elevated hs-cTnT concentrations by elevated hs-cTnI concentrations

	Hs-cTnT < 4th quartile	Hs-cTnT in 4th quartile
Hs-cTnI < 4 th quartile	1202	193
Hs-cTnI in 4 th quartile	169	300
Cohen's kappa= 0.49 (p<0.001)		
	Hs-cTnT ≤14 ng/L	Hs-cTnT >14 ng/L
Hs-cTnI (≤ 26.2 ng/L)	1725	99
Hs-cTnI (>26.2 ng/L)	16	24
Cohen's kappa=0.27 (P<0.001)		
	Hs-cTnT ≤ 14.5 ng/L (males) and ≤ 10 ng/L (females) ^a	Hs-cTnT > 14.5 ng/L (males) and > 10 ng/L (females) ^a
Hs-cTnI ≤ 34.2 ng/L (males) and ≤ 15.6 ng/L (females)	1675	144
Hs-cTnI > 34.2 ng/L (males) and > 15.6 ng/L (females)	16	29
Cohen's kappa=0.24 (P<0.001)		

^a Gender-specific cutoffs according to literature (5)

SUPPLEMENTAL TABLE 2. The presence (%) of CAD and renal dysfunction in patients with elevated hs-cTnT and hs-cTnI concentrations.

	Patients with hs-cTn > 99th percentile cutoff		Patients with hs-cTn > gender specific 99th percentile cutoff	
	Hs-cTnT (n=123)	Hs-cTnI (n=40)	Hs-cTnT (n=173)	Hs-cTnI (n=45)
	Percentage (n)	Percentage (n)	Percentage (n)	Percentage (n)
Obstructive plaque	42% (52/123)	38% (15/40)	39% (68/173)	36% (16/45)
Obstructive plaque or CCS > 400	47% (58/123)	43% (17/40)	43% (75/173)	42% (19/45)
eGFR < 90 mL/min/1.73m ²	53% (65/123)	30% (12/40)	61% (105/173)	27% (12/45)
Obstructive plaque or CCS > 400 or eGFR < 90 mL/min/1.73m ²	72% (88/123)	58% (23/40)	75% (129/173)	56% (25/45)

SUPPLEMENTAL TABLE 3. Baseline characteristics of subpopulation (N=549/1876 patients) in which echocardiography was performed, by median hs-cTnT and hs-cTnI concentrations.

Determinant	All patients	hs-cTnT		P-value	hs-cTnI		P-value
		≤7.2ng/L (N=231)	>7.2ng/L (N=318)		≤2.6ng/L (N=163)	>2.6ng/L (N=386)	
Left ventricular ejection fraction, %	60.0±7.3	60.7±5.9	59.4±8.2	0.037	60.8±5.5	59.6±8.0	0.056
Left ventricular mass, g	169.3±45.0	158±37	178±49	<0.001	155±34	176±48	<0.001
Left ventricular mass index, g/m ²	87.4±20.0	83.2±16.6	90.7±21.8	<0.001	82.5±16.1	89.7±21.2	<0.001

SUPPLEMENTAL TABLE 4. Adjusted multivariable linear regression analysis for the influence of CT and echocardiographic parameters and eGFR on hs-cTn concentrations (N=549/1864). Almost identical standardized β values (st β) for eGFR were observed when comparing adjusted multivariable models 1, 3 and 5, indicating the independent influence of eGFR on hs-cTn concentrations beyond echocardiographic parameters.

	Adjusted multivariable models									
	Model 1		Model 2		Model 3		Model 4		Model 5	
Dependent variable: $LN(hs-cTnI)$	St β	P-value	R ²	St β	P-value	R ²	St β	P-value	R ²	P-value
eGFR	-0.244	<0.001	0.261	0.132	0.004	0.241	0.139	0.002	0.256	<0.001
LVmass				-0.074	0.077		-0.065	0.115		0.144
LVEF							0.132	0.002		0.101
Coronary calcium score							0.142	0.001		0.142
Age	0.255	<0.001		0.378	<0.001		0.335	<0.001		0.195
Gender	-0.288	<0.001		-0.212	<0.001		-0.193	<0.001		<0.001
BMI	-0.016	0.700		-0.018	0.676		-0.017	0.687		<0.001
Diabetes	0.076	0.064		0.062	0.135		0.064	0.118		0.242
Family history	-0.040	0.312		-0.037	0.357		-0.037	0.359		0.083
Systolic BP	0.041	0.396		0.026	0.593		0.029	0.542		0.543
Diastolic BP	-0.060	0.200		-0.046	0.337		-0.055	0.240		0.582
Total cholesterol	-0.079	0.053		-0.072	0.081		-0.066	0.109		0.179
										0.118
Dependent variable: $LN(hs-cTnI)$										
eGFR	-0.207	<0.001	0.120	0.225	<0.001	0.141	0.234	<0.001	0.160	<0.001
LVmass				-0.080	0.071		-0.070	0.109		0.238
LVEF							0.150	0.001		<0.001
Coronary calcium score							0.136	0.005		0.100
Age	0.085	0.008		0.185	<0.001		-0.120	0.012		0.157
Gender	-0.253	<0.001		-0.141	0.003		-0.136	0.005		0.804
BMI	0.010	0.821		-0.020	0.654		-0.019	0.669		0.014
Diabetes	-0.019	0.674		-0.032	0.465		-0.030	0.496		-0.134
Family history	-0.019	0.656		-0.009	0.834		-0.009	0.838		-0.025
Systolic BP	0.110	0.037		0.081	0.123		0.084	0.105		0.555
Diastolic BP	-0.071	0.171		-0.047	0.356		-0.058	0.252		0.965
Total cholesterol	-0.031	0.489		-0.020	0.649		-0.013	0.765		0.002
										0.083
										0.102
										0.183
										0.820

SUPPLEMENTAL TABLE 5. Differences in baseline characteristics between cardiovascular event and event-free group.

Determinant	Event (N=54)	No event (N=1635)	P-value
Traditional risk factors			
Males, n (%)	39 (72.2%)	904 (55.3%)	0.014
Age, years	59.7 ± 10.7	56.4 ± 10.8	0.010
BMI, kg/m ²	26.6 ± 3.5	27.0 ± 4.4	0.586
Smokers, n (%)	39.7%	21.7%	<0.001
Diabetes, n (%)	7 (13.0%)	121 (7.4%)	0.129
Fam. history, n (%)	17 (31.5%)	627 (38.3%)	0.307
Systolic BP, mmHg	146.0 ± 17.5	142.7 ± 19.6	0.147
Diastolic BP, mmHg	80.5 ± 11.7	80.0 ± 11.4	0.930
Total cholesterol, mmol/L	5.2 ± 1.4	5.5 ± 1.2	0.318
Cardiac biochemical markers			
hs-cTnT, ng/L	8.9 (6.5)	7.2 (3.3)	0.004
hs-cTnI, ng/L	3.6 (7.4)	2.7 (2.4)	0.024
Renal clearance			
Cystatin C, mg/L	0.86 ± 0.27	0.77 ± 0.18	0.006
Creatinine, µmol/L	82.8 ± 21.8	74.8 ± 15.6	0.009
eGFR _{creat+cysC} , mL/min/1.73m ²	91.2 ± 21.0	98.6 ± 18.3	0.001
CT/CCTA parameters			
Coronary calcium score, AS	206.3 (601.0)	4.8 (111.0)	<0.001
Moderate-to-severe plaque, n (%)	40 (74.1%)	375 (22.9%)	<0.001

FOUR

HIGH-SENSITIVITY CARDIAC TROPONIN T DIFFERS FROM TROPONIN I IN THE ASSOCIATION WITH RENAL DYSFUNCTION AND MORTALITY IN NURSING HOME RESIDENTS

Cardinaels E, Daamen M, Bekers O, ten Kate J, van Suijlen J, Niens M, van Dieijen-Visser M, Brunner-La Rocca HP, Schols J, Mingels A

Submitted

Abstract

Background

High-sensitivity cardiac troponins (hs-cTn) have proven to be valuable risk stratification tools in more general populations of older adults. In the present study we explore the value of measuring hs-cTn in geriatric care, by investigating the major determinants and prognostic importance of hs-cTnT and (hs-)cTnI.

Materials and methods

495 residents from nursing homes (>65 years) of five healthcare organizations were examined. The residents underwent clinical and echocardiographic assessment for the diagnosis of heart failure. cTn was measured using high-sensitivity (hs-)cTnT (Roche), hs-cTnI (Abbott) and less sensitive cTnI (Beckman) assays. The glomerular filtration rate was estimated (eGFR) using serum creatinine and cystatin C concentrations. Data on all-cause mortality were collected upon 1 year follow-up.

Results

Median (IQR) concentrations were 20.6(17.8-30.6) ng/L, 6.8(4.1-12.5) ng/L and 4.0(2.0-8.0) ng/L for hs-cTnT, hs-cTnI and cTnI respectively. In total, 79% had elevated hs-cTnT concentrations, while only 9% and 5% of hs-cTnI and cTnI concentrations were elevated. Most important determinants for higher hs-cTnT and hs-cTnI concentrations were of cardiac and renal origin. Whereas both heart failure (OR:3.4) and eGFR<60mL/min/1.73m² (OR:3.6) were equal contributors to higher hs-cTnT concentrations (all p<0.001), hs-cTnI and cTnI were less associated to renal dysfunction (OR of respectively 1.9 and 2.1; p<0.01) in comparison to heart failure (OR:4.3 and 4.7, respectively, p<0.001). Still, higher hs-cTnT concentrations were a better predictor for all-cause mortality compared to both (hs-)cTnI assays.

Conclusion

In frail nursing home residents, all hs-cTn assays were highly associated with heart failure, while hs-cTnT was more associated with renal dysfunction than both (hs-)cTnI. Despite this, hs-cTnT had a more prominent role in assessing the risk on all-cause mortality.

Introduction

Older adults receiving long-term care in nursing homes are thought to have a high risk on chronic diseases such as heart failure, but still remain a poorly investigated population [1, 2]. Predicting those at risk for chronic diseases and mortality can therefore lead to preventive strategies and enhancements in their quality of life.

Cardiac biomarkers, such as high-sensitivity cardiac troponins (hs-cTn) have proven to be valuable risk stratification tools in more general populations of older adults [3, 4] and are associated with the prevalence of cardiovascular diseases [5, 6]. Measuring hs-cTn is currently an essential component for the diagnosis of acute myocardial infarction (AMI) [7], in which the diagnostic cutoff is set at the 99th percentile upper reference limit (URL) established in a healthy reference population [8, 9]. Elevated hs-cTn concentrations are however commonly found in the elderly [3, 10, 11] even when contributing comorbidities were excluded [12-15] and thereby challenging clinicians with the interpretation of these results [10]. These issues become even more important in frail older adults, such as nursing home residents. Therefore, age-specific cutoffs have been proposed [5, 10, 11, 15, 16], although they are currently not implemented in the guidelines [7, 17].

In the present multicenter study on nursing home residents, we explore the value of measuring hs-cTnT and (hs-)cTnI in geriatric care. Major determinants and prognostic information of hs-cTn concentrations are evaluated in a well-characterized and representative cohort of nursing home residents over 65 years. Moreover, we examined whether higher cut-offs should be applied in these elderly and focus on the comparison between the results of hs-cTnT, hs-cTnI and a sensitive cTnI assay.

Materials and methods

Study population

In this study, 501 nursing home residents from five healthcare organizations in the Netherlands who receive long term care were examined. Residents were eligible when over 65 years of age, not receiving palliative care and not admitted

for short-term rehabilitation (<2 months). Blood samples could not be drawn in 6 residents, therefore data is presented for 495 subjects (SUPPLEMENTAL FIGURE 1). Information regarding comorbidities and current prescribed medication were retrieved from medical records, physical examinations and questionnaires. The presence of heart failure was assessed by a panel of two cardiologists and a geriatrician, who were blinded for (hs-)cTn results. The diagnosis of heart failure was based on clinical examinations (including history, physical examinations, electrocardiography, echocardiography and if in doubt NTproBNP concentrations), patient records and questionnaires. A detailed study protocol has been published previously [18]. One year after inclusion, medical records at the nursing home were searched again for the occurrence of all-cause mortality. Follow-up data was retrieved from 466 residents (94%) due to transfer to non-participating healthcare centres.

This study was conducted according to the principles of the Declaration of Helsinki and approved by the local ethics committee. Written informed consent was obtained from all subjects.

SUPPLEMENTAL FIGURE 1. Flow chart of included residents

Biochemical analysis

Serum samples were collected, processed within 5 hours in aliquots and directly stored at -80 °C until analysis. Serum creatinine, cystatin C, NTproBNP and hs-cTnT concentrations were measured on the Cobas 6000 analyzer (Roche Diagnostics, Mannheim, Germany). Creatinine concentrations were assessed using the enzymatic method (Roche Diagnostics). Cystatin C was measured using a particle-enhanced turbidimetric assay (Gentian AS, Moss, Norway), that was standardized against the certified ERM-DA471/IFCC cystatin C reference material [19]. The glomerular filtration rate was estimated by the Chronic Kidney Disease Epidemiology Collaboration [20] and the Berlin Initiative Study (BIS)-2 equation [21], both using serum creatinine and cystatin C concentrations. Hs-cTnT concentrations were determined using the high-sensitivity cTnT assay (Roche Diagnostics; lotnumber 167650), with a 99th percentile upper reference limit of 14 ng/L and a 10% coefficient of variation (CV) cutoff at 13 ng/L and limit of detection (LoD) at 5 ng/L. Gender-specific cutoffs were reported at 14.5 ng/L and 10 ng/L for males and females, respectively [22]. Hs-cTnI measurements were performed on the ARCHITECT i2000SR platform using the precommercial ARCHITECT STAT high-sensitivity troponin I (hs-cTnI) assay (Abbott Laboratories, Hoofddorp, the Netherlands). According to the manufacturer, LoD and 10% CV cutoff were reached at 1.1-1.9 and 4.7 ng/L, respectively and the 99th percentile cut-off concentration for the overall population was at 26.2 ng/L. Gender-specific cut-offs at 34.2 ng/L and 15.6 ng/L were also defined for males and females, respectively. Also, cTnI was measured using the guideline acceptable Access-3 TnI assay (Beckman Coulter) on the DXL platform. The 99th percentile and 10% CV cut-off concentration were both established at 40 ng/L by the manufacturer. All biochemical analysis were performed in freshly thawed aliquots, except for Beckman cTnI results that were measured after one additional freeze-thaw cycle.

Echocardiography analysis

Echocardiography was performed by an expert echocardiographer, who was blinded for hs-cTn concentrations. Transthoracic images of the left ventricle (LV) were acquired to assess morphology, function and mass (Philips CX-50, Philips

Medical Systems, Best, the Netherlands). LV function and -mass were calculated by off-line image analysis using Xcelera software package (Philips Medical Systems, Best, the Netherlands), according to current ESC/AHA guidelines [23]. Left ventricular hypertrophy (LVH) was defined as an LVmass $>95 \text{ g/m}^2$ in females or LVmass $>115 \text{ g/m}^2$ in males. Echocardiographic analysis could not be performed in 51 residents due to impaired image quality.

Selection criteria of healthier subpopulations

From the complete nursing home cohort (n=495), increasingly healthier subjects were selected using gradually more rigorous selection criteria. In Subgroup 1 (n=322; 65%) all residents with heart failure, diagnosed during this study were excluded from the original cohort. In subgroup 2 (n=196; 40%) all residents with heart failure, LVH, LVEF $<55\%$ and NTproBNP concentrations $>400 \text{ pg/mL}$ (47 pmol/L) were excluded. Subgroup 3 (n=154; 31%) encompassed the residents of subgroup 2, except for those with a history of cardiovascular events, defined as acute myocardial infarction, coronary artery disease, cardiac surgery or revascularisation procedures performed and prior stroke. Finally, subgroup 4 (n=97; 20%) consisted of the residents of subgroup 3, excluding those with known impaired kidney functions or an eGFR $<60 \text{ mL/min/1.73m}^2$.

Statistical analysis

Comparisons of baseline characteristics were performed using the T-test for continuous variables with a normal distribution, Mann-Whitney U-test for non-normal distributed continuous variables and Chi square test for categorical variables. Data are presented as proportions, means $\pm$ standard deviations (SD), and data with a non-normal distribution are given as the median (interquartile range, IQR). Due to the low amount of (<300) residents in the increasingly healthier subpopulations, 97.5th and 95th percentile (hs-)cTn cutoffs were computed instead of the 99th percentile URL. The 95% confidence intervals (CI) of the 97.5th and 95th percentile cut-offs were calculated using 10.000 bootstrap samples. The relative contribution of renal function and heart failure to (hs-)cTn concentrations was assessed by logistic regression analyses with hs-cTnT, hs-cTnI or cTnI in the 4th quartile as the dependent categorical variable. Furthermore, linear regression analysis and Pearson R correlations

were performed with the natural logarithm (Ln) of hs-cTnT and hs-cTnI as dependent categorical variable, to normalize their skewed distribution. The prognostic value of (hs-)cTn concentrations was investigated by univariable and multivariable Cox-proportional hazards models. Kaplan Meier plots were constructed to illustrate survival rates.

Statistical analysis was performed with SPSS 20.0 (SPSS Inc, Chicago, IL, USA). Two sided p-values of <0.05 were considered statistically significant.

Results

Baseline characteristics and the association to (hs-)cTn concentrations

In this study, participants were on average 82 ± 7 years old and 64% was female. Of all residents with heart failure (34%), 48% of the residents suffered from heart failure with a reduced ejection fraction (LVEF<50%). An overview of other baseline characteristics is presented in TABLE 1.

The distribution of baseline hs-cTnT and hs-cTnI concentrations is illustrated in FIGURE 1. Median (IQR) concentrations were 20.6 (17.8-30.6) ng/L, 6.8 (4.1-12.5) ng/L and 4.0 (2.0-8.0) ng/L for hs-cTnT, hs-cTnI and cTnI respectively (TABLE 1). The results of the two cTnI assays were highly correlated (Pearson R(95%CI):0.884 (0.860-0.904), $p < 0.001$), whereas associations between hs-cTnT assay and either hs-cTnI (Pearson R (95%CI):0.580 (0.515-0.638); $p < 0.001$) or the cTnI-assay (Pearson R (95%CI):0.548 (0.483-0.608); $p < 0.001$) were significantly weaker. In total, 79% had elevated hs-cTnT concentrations (>99th percentile), while only 9% and 5% of hs-cTnI and cTnI concentrations were elevated. When applying gender-specific cut-offs 89% and 16%, respectively, had elevated hs-cTnT and hs-cTnI concentrations.

TABLE 1. Baseline characteristics of a cohort of aged nursing home residents. *, indicates more than 10% missing data.

	<i>All residents (n = 495)</i>
Traditional cardiovascular risk factors	
Age (years), mean ($\pm$ SD)	82 ($\pm$ 7)
Male gender, %	36%
Body mass index, mean ($\pm$ SD)	25.1 ($\pm$ 5.2)
Hypertension, %	53%
Diabetes mellitus, %	22%
Hypercholesterolemia, % ¹	29% ¹
smokers, %	14%
History, n (%)	
Prior cardiac event (total)	33%
Prior CAD (angina)	23%
Prior myocardial infarction	16%
Prior cardiac surgery (pacemaker, heart valve)	5%
Prior revascularization (CABG, PCI)	10%
Prior stroke (CVA)	41%
Comorbidities, %	
Heart failure ²	34%
Impaired kidney function	12%
COPD	17%
Dementia	57%
Medication use	
Statins	26%
Beta-blokkers	30%
Digoxin	8%
Diuretics	39%
ACE inhibitors	18%
Nitrates	17%
Ca-antagonists	12%
Echocardiography²	
LVmass (g), mean ($\pm$ SD)	137.2 ($\pm$ 51.8)
LVMI (g/m ²), mean ($\pm$ SD)	78.5 ($\pm$ 26.7)
LVEF (%), mean ($\pm$ SD)	55.3 ($\pm$ 11.8)
LVH, %	14% ²
Biochemistry	
NTproBNP (pmol/L), median (IQR)	48 (20–117)
Creatinine (μ mol/L), mean ($\pm$ SD)	89.5 ($\pm$ 52.1)
Cystatin C (mg/L), mean ($\pm$ SD)	1.3 ($\pm$ 0.53)
eGFR _{CKD EPI} (creat-cys C) (mL/min/1.73m ²), mean ($\pm$ SD)	58.8 ($\pm$ 20.1)
eGFR _{BIS-2} (mL/min/1.73m ²), mean ($\pm$ SD)	53.2 ($\pm$ 16.3)
Roche hs-cTnT (ng/L), median (IQR)	20.6 (17.8–30.6)
Abbott hs-cTnI (ng/L), median (IQR)	6.8 (4.1–12.5)
Beckman cTnI (ng/L), median (IQR)	4.0 (2.0–8.0)

¹data on hypercholesterolemia was present for 440 residents; ² echocardiography results based on 444 residents

FIGURE 1. Distribution of hs-cTnT_{Roche}, hs-cTnI_{Abbott} and cTnI_{Beckman} concentrations in aged nursing home residents.

TABLE 2. Median (IQR) hs-cTnT, hs-cTnI and cTnI concentrations in fragile elderly according to the presence or absence of traditional risk factors as well as heart and kidney dysfunction.

Determinant	Median (IQR) hs-cTnT (Roche)			Median (IQR) hs-cTnI (Abbott)			Median (IQR) cTnI (Beckman)		
	concentrations (ng/L), when determinant is:		P- value	concentrations (ng/L), when determinant is:		P- value	concentrations (ng/L), when determinant is:		P- value
	not present	present		not present	present		not present	present	
Male sex	19.7 (14.2–28.4)	21.8 (16.0–35.4)	0.014	6.5 (4.1–12.6)	7.2 (4.4–12.3)	0.337	4.0 (2.0–9.0)	4.0 (2.0–7.0)	0.756
Hypertension	19.0 (13.3–27.8)	21.8 (16.0–35.1)	<0.001	5.9 (3.8–10.4)	7.2 (4.4–13.5)	0.015	4.0 (2.0–7.0)	4.0 (2.0–9.0)	0.212
Diabetes	20.0 (14.5–30.0)	22.2 (15.7–36.2)	0.044	6.8 (4.1–12.4)	6.6 (4.2–13.1)	0.930	4.0 (2.0–8.0)	4.5 (2.0–9.0)	0.529
Hypercholesterolemia	20.2 (14.5–31.3)	21.1 (15.5–28.8)	0.899	7.1 (4.1–12.7)	6.3 (4.4–11.1)	0.264	4.0 (2.0–8.0)	4.0 (2.0–7.5)	0.374
Smoking	21.0 (15.0–30.7)	18.4 (12.6–30.5)	0.227	6.8 (4.2–12.4)	6.6 (3.7–12.0)	0.997	4.0 (2.0–8.0)	3.0 (2.0–7.0)	0.811
Prior cardiac event (total)	19.3 (14.1–28.3)	23.4 (16.5–35.9)	<0.001	5.9 (3.8–10.5)	8.3 (5.2–18.4)	<0.001	3.0 (2.0–7.0)	6.0 (3.0–12.0)	<0.001
Prior MI	19.8 (14.5–29.4)	23.6 (18.2–45.1)	0.001	6.3 (4.0–11.4)	8.2 (5.8–19.2)	<0.001	4.0 (2.0–7.0)	6.0 (3.0–12.0)	0.001
Prior stroke	20.0 (14.6–31.0)	21.6 (15.5–29.0)	0.475	6.4 (4.1–11.3)	7.3 (4.1–13.7)	0.501	4.0 (2.0–8.0)	5.0 (2.0–8.0)	0.174
COPD	20.0 (14.8–28.8)	23.5 (14.9–35.38)	0.033	6.4 (4.1–11.3)	10.0 (5.0–17.5)	0.017	4.0 (2.0–8.0)	7.0 (2.0–10.0)	0.004
Dementia	21.8 (16.4–33.0)	19.5 (14.3–28.3)	0.008	7.7 (4.3–13.4)	6.4 (4.0–11.3)	0.335	5.0 (2.0–8.0)	4.0 (2.0–7.0)	0.020
Heart failure	18.6 (13.2–25.4)	26.4 (18.7–43.8)	<0.001	5.1 (3.6–8.5)	11.4 (7.2–20.3)	<0.001	3.0 (1.0–6.0)	7.0 (4.0–16.0)	<0.001
HF reduced LVEF	24.0 (17.4–37.2)	25.6 (18.6–35.9)	0.751	11.1 (7.0–18.9)	11.6 (6.6–19.1)	0.947	7 (4.0–16.5)	7 (4.0–16.8)	0.800
LVH	20.1 (14.5–28.6)	21.5 (16.2–31.0)	0.609	6.4 (3.8–11.4)	5.9 (4.1–10.7)	0.010	4.0 (2.0–7.0)	7.0 (2.0–12.0)	0.033
LVEF<40%	19.6 (14.3–28.2)	25.6 (18.3–35.4)	0.022	6.4 (3.8–11.0)	11.4 (5.7–22.3)	0.008	4.0 (2.0–7.0)	7.0 (4.0–19.0)	0.004
LVEF<50%	19.3 (14.1–27.2)	22.5 (17.6–33.2)	0.017	6.3 (3.7–10.4)	9.6 (5.1–18.4)	0.005	4.0 (2.0–6.0)	7.0 (3.0–11.0)	0.002
Impaired kidney function	19.9 (14.5–28.5)	28.9 (19.4–54.9)	<0.001	6.5 (4.1–11.4)	7.8 (4.8–20.6)	0.013	4.0 (2.0–8.0)	6.0 (3.0–26.0)	0.397
eGFR _{ysC+K_{cre}} < 60 mL/min	18.0 (13.2–23.2)	25.2 (16.8–36.4)	<0.001	5.1 (3.7–8.7)	8.1 (4.8–16.6)	<0.001	3.0 (1.0–6.0)	6.0 (3.0–11.0)	<0.001
eGFR _{ais-2} < 60 mL/min	18.1 (13.3–23.3)	25.2 (16.8–36.5)	<0.001	5.6 (4.2–9.9)	12.4 (7.5–25.5)	<0.001	3.0 (1.0–5.0)	5.0 (2.0–10.0)	<0.001
Statins	21.2 (14.6–31.2)	19.8 (15.2–28.6)	0.858	6.8 (4.0–12.7)	6.8 (4.5–12.2)	0.890	4.0 (2.0–9.0)	4.0 (2.0–7.0)	0.559
Beta-blokkers	20.1 (14.5–30.1)	22.3 (16.4–32.6)	0.036	6.4 (3.9–11.3)	7.9 (4.7–16.4)	0.009	4.0 (2.0–8.0)	5.0 (3.0–10.8)	0.017
Digoxin	20.3 (14.7–30.0)	22.6 (16.0–41.5)	0.096	6.4 (4.1v11.4)	14.9 (7.0–22.6)	<0.001	4.0 (2.0–8.0)	10.0 (5.8–33.8)	<0.001
Diuretics	18.8 (13.3–25.4)	25.3 (17.7–39.6)	<0.001	5.7 (3.8–9.6)	9.1 (5.3–18.1)	<0.001	3.0 (2.0–7.0)	6.0 (3.0–12.0)	<0.001
ACE inhibitors	20.0 (14.3–31.0)	22.8 (17.6–30.2)	0.033	6.3 (3.9–11.9)	8.4 (5.7–14.0)	<0.001	4.0 (2.0–8.0)	6.0 (3.0–9.5)	0.005
Nitrates	20.1 (14.6–29.7)	22.5 (15.5–36.0)	0.079	6.3 (4.0–11.4)	8.5 (5.5–17.1)	0.001	4.0 (2.0–8.0)	6.0 (3.0–12.5)	0.002
Ca-antagonists	21.1 (14.8–31.0)	18.1 (14.6–25.5)	0.240	6.8 (4.1–12.6)	6.9 (4.9–10.9)	0.969	4.0 (2.0–9.0)	4.0 (2.0–7.0)	0.713

As depicted in TABLE 2, significantly higher concentrations for all cTn results ($p < 0.001$) were found in residents with a cardiac event in their medical history, a reduced kidney function and on diuretics. Also in residents with heart failure, higher (hs-)cTn concentrations were found although median (hs-)cTn concentrations were not statistically different in subjects with a reduced versus preserved ejection fraction (all $p > 0.05$). Strikingly, higher hs-cTnT but not (hs-)cTnI concentrations were found in males compared to females, although only minor differences were observed. In contrast, (hs-)cTnI but not hs-cTnT were significantly higher in participants with LVH.

(Hs-)cTn concentrations in subpopulations without cardiac, echocardiographic and renal abnormalities

In the total cohort, 97.5th (95%CI) percentile cutoffs were established at 83.9 (71.2-108.1), 58.4 (40.8-123.8) and 52.0 (41.1-88.9) ng/L for hs-cTnT, hs-cTnI and cTnI respectively (FIGURE 2). These cutoffs decreased when subjects with heart failure and other cardiovascular diseases (subgroup 1-3) were excluded. As such, in residents without structural heart disease and prior cardiovascular events (subgroup 3) the 97.5th percentile reduced to 62.8 (38.3-75.2), 25.7 (16.6-45.6) and 19.7 (9.4-218.7) ng/L for hs-cTnT, hs-cTnI and cTnI, respectively.

Additionally, excluding participants with renal dysfunction (subgroup 4) resulted in a further reduction in the 97.5th percentile for hs-cTnT to 56.5 (33.1-78.4) ng/L, but did not affect hs-cTnI (25.9 ng/L) and cTnI (17.8 ng/L).

Interestingly, 97.5 percentile cutoffs in all subgroups were substantially above the reported 99th percentile URL for hs-cTnT (14ng/L), but not for both hs-cTnI (26.2 ng/L) and cTnI (40 ng/L), as depicted in FIGURE 2. Similar trends were seen for the 95th percentile (hs-)cTn concentrations among the different subgroups (FIGURE 2).

FIGURE 2. 97.5% and 95% upper reference limits (95% confidence intervals) of hs-cTnT, hs-cTnI and cTnI in increasingly cardio-and renal healthier subpopulations. *, indicates that 95% CI were constructed using 10.000 bootstrapping sample; Reported 99th percentile URL for each cTn assay are indicated with horizontal lines.

Association of (hs-)cTn concentrations with heart failure and renal dysfunction

Univariable logistic regression analysis demonstrated that the presence of heart failure increased the odds of having hs-cTnT, hs-cTnI and cTnI concentrations in the 4th quartile by 4.3, 4.9 and 5.4 times, respectively (TABLE 3; all $p < 0.001$). Furthermore, the presence of renal dysfunction ($\text{eGFR} < 60 \text{ mL/min/1.73m}^2$) also accompanied with significantly higher (hs-)cTn concentrations, even beyond the presence of heart failure (TABLE 3; unadjusted multivariable model). Whereas both heart failure and decreases in eGFR were equal contributors to higher hs-cTnT concentrations (OR of respectively 3.4 and 3.6, $p < 0.001$), associations of hs-cTnI and cTnI with renal dysfunction were less pronounced (OR of respectively, 1.9 and 2.1; $p < 0.01$) in comparison to heart failure (OR of respectively 4.3 and 4.7, $p < 0.001$). The same results were found when linear regression analyses were performed (SUPPLEMENTAL TABLE 1-2).

TABLE 3. Logistic regression analysis to assess the association of heart failure and eGFR to higher hs-cTn concentrations

	univariable			Unadjusted multivariable		
	OR	95%(CI)	p-value	OR	95%(CI)	p-value
Outcome variable: Roche hs-cTnT in 4th quartile ($>30.4 \text{ ng/L}$)						
Heart failure	4.3	(2.8-6.5)	<0.001	3.4	(2.2-5.4)	<0.001
$\text{eGFR}_{\text{CKDEPI}} < 60 \text{ mL/min/1.73m}^2$	4.5	(2.8-7.3)	<0.001	3.6	(2.2-5.9)	<0.001
Outcome variable: Abbott hs-cTnI in 4th quartile ($>12.4 \text{ ng/L}$)						
Heart failure	4.9	(3.2-7.7)	<0.001	4.3	(2.7-6.8)	<0.001
$\text{eGFR}_{\text{CKDEPI}} < 60 \text{ mL/min/1.73m}^2$	2.6	(1.6-4.0)	<0.001	1.9	(1.2-3.1)	0.007
Outcome variable: Beckman cTnI in 4th quartile ($>8.0 \text{ ng/L}$)						
Heart failure	5.4	(3.4-8.4)	<0.001	4.7	(2.9-7.4)	<0.001
$\text{eGFR}_{\text{CKDEPI}} < 60 \text{ mL/min/1.73m}^2$	2.9	(1.8-4.6)	<0.001	2.1	(1.3-3.4)	0.003

Prognostic value of hs-cTn concentrations for all-cause-mortality.

During the follow-up period of one year, 109 subjects (23.4%) died. These subjects were older, more frequently male and had a higher prevalence of heart failure and impaired kidney disease (SUPPLEMENTAL TABLE 3). Also, significantly higher hs-cTnT, hs-cTnI and cTnI concentrations were found in the residents that died. According to univariable Cox regression analysis, baseline hs-cTnT concentrations were a better predictor for all-cause mortality in comparison to both cTnI assays (FIGURE 3). Subjects with the highest hs-cTnT levels (4th quartile) had a higher hazard ratio for all-cause mortality (HR = 1.8; $p=0.004$), compared to hs-cTnI (HR:1.3 $p=0.300$) and cTnI (HR:1.6; $p=0.041$) concentrations. Even when adjusting for other significant risk factors baseline hs-cTnT remained superior in the prediction for all-cause mortality (SUPPLEMENTAL TABLE 4).

FIGURE 3. Kaplan-meier curves for the estimation of risk on all-cause mortality using hs-cTnT, hs-cTnI and cTnI concentrations.

Discussion

In this multicentre study we found that hs-cTnT concentrations are above the stated normal range, even in those without any cardiovascular and renal disease. This however is not the case of hs-cTnI and cTnI results. Moreover, employing hs-cTnT assays in geriatric care appears to be associated not only by cardiovascular diseases but also renal dysfunction. Despite this, hs-cTnT remained the most important prognostic factor for all-cause mortality.

Hs-cTnT is more affected by age than (hs-)cTnI

In this nursing home cohort, median hs-cTnI and cTnI concentrations were much lower than hs-cTnT. Intriguingly, the reported 99th percentile cutoffs for both (hs-)cTnI assays are set higher than for hs-cTnT, even when established in the same reference population [9, 15, 24]. Consequently, more positive results were observed for hs-cTnT than (hs-)cTnI elevations. Since, 99th percentile URL of hs-cTn are reported to be highly affected by age, gender and selection criteria of the reference population [24, 25], more cardiac and renal healthy subgroups were selected to gain more insight into the distribution of hs-cTn in these elderly subjects. Even in these healthier subpopulations, 97.5th percentile hs-cTnT values were still at least 4 times higher as compared to the healthy reference population (URL at 14 ng/L), while around or below the reported URL for the hs-cTnI (26.2 ng/L) and cTnI (40 ng/L) assays. It has been described that higher hs-cTn cutoffs give a better diagnostic performance in the elderly [5, 10, 11, 15]. This was especially true for hs-cTnT, with optimal cutoffs 4 to 5 fold higher as the established 99th percentile URL in healthy reference populations (14 ng/L) [12, 26]. Together with our results, these findings imply that in elderly (>65 years) a higher cutoff seems necessary for hs-cTnT, but might not be required for hs-cTnI.

Hs-cTnT is more strongly associated with renal function than (hs-)cTnI

The association with heart failure was equally as strong for all cTn assays. Remarkably, (hs-)cTnI appeared to be more associated with left ventricular function and morphology abnormalities than hs-cTnT in this cohort. However, both (hs-)cTnI and hs-cTnT results have been described to highly correlate with

left ventricular measures in the elderly [3, 4]. Therefore, more detailed head-to-head comparisons between hs-cTnT and hs-cTnI assays should be performed to truly unravel these differences.

Furthermore, we found that hs-cTnT was more influenced by renal dysfunction than hs-cTnI. By excluding residents with renal dysfunction, a visible effect on the reduction in hs-cTnT URL was noticed, which is in line with previous observations in other study populations [24, 27], but was not found for (hs-)cTnI. In chronic kidney disease patients, a higher association of eGFR with hs-cTnT in comparison to hs-cTnI has also been reported [28]. By examining the contribution of age, heart failure and renal dysfunction to hs-cTn concentrations, we are the first to identify the magnitude of these determinants. As such, we found in frail nursing home residents that hs-cTnT concentrations were equally associated by the presence of heart failure and renal dysfunction, whereas the correlation of both (hs-)cTnI concentrations with renal dysfunction was only half as strong as with heart failure. We are the first to describe this using two different cTnI assays, suggesting biological differences between the cTnT and cTnI molecules rather than assay-related analytical influences. A plausible explanation for these findings could be a slower clearance of cTnT from the blood circulation by the kidneys in comparison to cTnI.

Hs-cTnT is a better predictor for all-cause mortality than (hs-)cTnI

A stronger association with all-cause mortality was found for hs-cTnT in comparison to both hs-cTnI and cTnI, suggesting a more prominent role for hs-cTnT in risk stratification. Notwithstanding, previous studies have clearly established the prognostic importance of hs-cTnI in the prediction for cardiovascular events and all-cause mortality [4, 12]. Our findings however do extend the results of previous work in acute populations where hs-cTnT outperformed (hs-)cTnI in its prognostic accuracy for the risk on all-cause mortality [27, 29, 30]. More recently, hs-cTnT appeared to have an additional prognostic importance independently from hs-cTnI [31], suggesting that hs-cTnT and hs-cTnI could contain different prognostic information. The rationale behind these findings could be the higher association of hs-cTnT with renal dysfunction.

Limitations

Due to the small sample size, we were not able to compute the diagnostic cut-off at the 99th percentile concentration of the more healthier subgroups.

Also, as the diagnosis of heart failure was assessed by an expert team, other clinical diagnoses were not. Echocardiographic analysis could however not be performed in 51 frail nursing home residents and subsequently the presence of heart failure could not be evaluated in these residents.

Conclusion

In summary, we challenge the application of the 99th percentile cutoff in frail elderly (>65 years), especially for hs-cTnT. We found that hs-cTnT concentrations were elevated in almost all nursing home residents, in contrast to (hs-) cTnI concentrations, regardless from their cardiac and renal health. All hs-cTn assays were highly associated with heart failure and structural heart disease, while hs-cTnT was more associated with renal dysfunction than both (hs-)cTnI and had a more prominent role in assessing the risk on all-cause mortality. In daily practice, hs-cTnT and hs-cTnI results are used interchangeably to diagnose AMI. It however merits further research whether patients equally benefit from hs-cTnT and hs-cTnI measurements and as to whether such clinical benefit is dependent on age.

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SUPPLEMENTAL TABLE 1. Linear regression analysis for the associations of renal and cardiac parameters with hs-cTn concentrations

Univariable			Unadjusted multivariable model 1			Adjusted * multivariable model 1			Unadjusted multivariable model 2			Adjusted * multivariable model 2			
	St(b)	p-value	R ²	St(b)	p-value	R ²	St(b)	p-value	R ²	St(b)	p-value	R ²	St(b)	p-value	R ²
Outcome variable: Ln(hs-cTnT)															
Heart failure	0.346	<0.001	0.118	0.249	<0.001	0.222	0.198	<0.001	0.256	0.287	<0.001	0.167	0.223	<0.001	0.212
eGFR _{CKDEPI}	-0.410	<0.001	0.166	-0.340	<0.001		-0.322	<0.001							
eGFR _{CKDEPI} < 60 mL/min/1.73m ²	0.306	<0.001	0.093							0.236	<0.001		0.219	<0.001	
Outcome variable: Ln(hs-cTnI)															
Heart failure	0.356	<0.001	0.125	0.307	<0.001	0.150	0.280	<0.001	0.172	0.334	<0.001	0.130	0.296	<0.001	0.163
eGFR _{CKDEPI}	-0.259	<0.001	0.065	-0.175	<0.001		-0.105	0.032							
eGFR _{CKDEPI} < 60 mL/min/1.73m ²	0.169	<0.001	0.026							0.089	0.050		0.024	0.617	
Outcome variable: Ln(cTnI)															
Heart failure	0.356	<0.001	0.125	0.310	<0.001	0.153	0.276	<0.001	0.186	0.334	<0.001	0.132	0.290	<0.001	0.176
eGFR _{CKDEPI}	-0.259	<0.001	0.065	-0.179	<0.001		-0.116	0.019							
eGFR _{CKDEPI} < 60 mL/min/1.73m ²	0.175	<0.001	0.028							0.100	0.034		0.042	0.386	

*adjusted to age, gender, hypertension, diabetes, prior cardiac event, dementia, COPD

SUPPLEMENTAL TABLE 2. Linear regression analysis of the contribution of renal and cardiac parameters on higher hs-cTn concentrations, when eGFR was based on BIS-2 algorithm.

Univariable			Unadjusted multivariable model 1			Adjusted * multivariable model 1			Unadjusted multivariable model 2			Adjusted * multivariable model 2			
	St(b)	p-value	R ²	St(b)	p-value	R ²	St(b)	p-value	R ²	St(b)	p-value	R ²	St(b)	p-value	R ²
Outcome variable: Ln(hs-cTnT)															
Heart failure	0.346	<0.001	0.118	0.237	<0.001	0.232	0.189	<0.001	0.268	0.221	<0.001	0.161	0.229	<0.001	0.208
eGFR _{BIS-2}	-0.429	<0.001	0.182	-0.358	<0.001		-0.359	<0.001							
eGFR _{BIS-2} < 60 mL/min/1.73m ²	0.373	<0.001	0.083							0.291	<0.001		0.215	<0.001	
Outcome variable: Ln(hs-cTnI)															
Heart failure	0.356	<0.001	0.125	0.296	<0.001	0.159	0.274	<0.001	0.176	0.314	<0.001	0.150	0.283	<0.001	0.173
eGFR _{BIS-2}	-0.287	<0.001	0.080	-0.200	<0.001		-0.130	0.011							
eGFR _{BIS-2} < 60 mL/min/1.73m ²	0.248	<0.001	0.059							0.173	0.050		0.110	0.025	
Outcome variable: Ln(cTnT)															
Heart failure	0.356	<0.001	0.125	0.298	<0.001	0.162	0.269	<0.001		0.323	<0.001	0.148	0.283	<0.001	0.183
eGFR _{BIS-2}	-0.290	<0.001	0.082	-0.206	<0.001		-0.144	0.005							
eGFR _{BIS-2} < 60 mL/min/1.73m ²	0.229	<0.001	0.050							0.162	0.001		0.104	0.038	

SUPPLEMENTAL TABLE 3. Associations of traditional risk factors, comorbidities, renal function and hs-cTn concentrations with the presence of all-cause mortality

Determinant	All-cause-mortality		
	Alive (n=357)	Dead (n=109)	p-value*
Traditional cardiovascular risk factors			
Age (years), mean ($\pm$ SD)	81.9 $\pm$ 6.8	83.5 $\pm$ 6.4	0.035
Male gender, %	34%	48%	0.009
Body mass index, mean ($\pm$ SD)	25.6 $\pm$ 5.3	24.2 $\pm$ 5.3	0.011
Smoking, %	14%	14%	0.926
Systolic blood pressure, mean($\pm$ SD)	141.2 $\pm$ 24.4	135.0 $\pm$ 28.7	0.021
Diastolic blood pressure, mean($\pm$ SD)	75.6 $\pm$ 13.0	74.5 $\pm$ 17.1	0.402
Diabetes mellitus, %	21%	23%	0.632
Hypercholesterolemia, %	27%	30%	0.926
Prior cardiac event (total) , %	32%	38%	0.246
Prior stroke (CVA) , %	39%	44%	0.315
Comorbidities			
Heart failure, %	31%	43%	0.017
Impaired kidney disease, %	10%	17%	0.066
COPD, %	16%	20%	0.304
Dementia, %	57%	57%	0.956
(hs-)cTn concentrations			
Hs-cTnT concentrations (ng/L), median (IQR)	19.6 (13.9–29.1)	23.2 (16.8–41.0)	0.001
Hs-cTnI concentrations (ng/L), median (IQR)	6.2 (3.9–12.1)	8.5 (5.6–16.7)	0.001
cTnI concentrations (ng/L), median (IQR)	4.0 (2.0–8.0)	6.0 (3.0–13.0)	0.001

SUPPLEMENTAL TABLE 4. Cox-regression analysis to examine the prognostic value of hs-cTnT (Roche), hs-cTnI (Abbott) and cTnI (Beckman) for all-cause mortality when adjusted for significant risk factors.

	HR (95%CI)	p-value
<i>Ln(hs-cTnT) (Roche)</i>		
Model 1	1.58 (1.17–2.15)	0.003
Model 2	1.50 (1.06–2.14)	0.024
<i>Ln(hs-cTnI) (Abbott)</i>		
Model 1	1.10 (0.97–1.24)	0.139
Model 2	1.06 (0.91–1.22)	0.467
<i>Ln(cTnI) (Beckman)</i>		
Model 1	1.15 (1.02–1.29)	0.018
Model 2	1.12 (0.98–1.23)	0.103

Model 1, adjusted for age, gender, BMI, systolic BP; Model 2, adjusted for age, gender, BMI, systolic BP, presence of heart failure, presence of impaired kidney disease (medical history), eGFR.

ACUTE EFFECTS OF CONVENTIONAL AND EXTENDED HEMODIALYSIS AND HEMODIAFILTRATION ON HIGH-SENSITIVITY CARDIAC TROPONINS

Cardinaels E*, Cornelis T*, van der Sande F, Leunissen K, van Dieijen-Visser M,
Mingels A, Kooman J and Bekers O.

**, both authors contributed equally*

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Introduction

Elevated concentrations of cardiac troponin T (cTnT; 37kDa) and I (cTnI; 24kDa), and the N-terminal fragment of the prohormone brain-type natriuretic peptide (NTproBNP; 8.5kDa) have been reported in end-stage renal disease patients and are associated with cardiovascular morbidity and mortality [1, 2]. Also, cTnT, cTnI and NTproBNP correlate with glomerular filtration rate, suggesting a significant role of renal removal and consequences for the clinical interpretation of these cardiac markers [3]. The influence of hemodialysis (HD) on cardiac biomarkers itself remains contradictory. Previous studies have demonstrated intradialytic decreases [4] and increases in NTproBNP [5]. Also, significant reductions as well as increases in cTnT and cTnI concentrations have been reported following dialysis [5-8]. These differences can be attributed to the use of different dialysis modalities and membrane filters [5, 8].

Currently, most patients are treated with 4-hour high-flux HD. However, it has been shown that uremic toxins were better removed when patients were treated with hemodiafiltration (HDF) and extended 8-hour dialysis treatments resulted in more hemodynamic stability [9]. The aim of the present study was to assess the acute effects of conventional and extended high-flux HD and HDF on cardiac biomarkers in the same population as previously described [9], especially focusing on high-sensitivity (hs-) cTnT and hs-cTnI results.

Materials and methods

Study population

Prevalent conventional HD patients were recruited receiving chronic conventional HD treatments, without residual urine production and acute illness such as infection or cardiovascular events. All patients underwent one mid-week session in random order: 4-hour HD (HD4), 8-hour HD (HD8), 4-hour online HDF (HDF4) and 8-hour online HDF (HDF8) sessions; with a 2-week interval between the study sessions. Between the study sessions, these patients received conventional HD (HD4) treatments. All treatments were performed with the Fresenius 5008 Therapy System (Fresenius Medical Care). High-flux FX80 and FX800 dialyzers (both Fresenius) were used for HD and HDF, respectively.

Blood flow and dialysate flow were 300 and 600mL/min, respectively. Substitution flow was 83.3mL/min to achieve a total substitution volume of 15L for HDF4 and 30L for HDF8. Dialysate composition was 1.5 mmol/L calcium, 2 mmol/L potassium, 136-138 mmol/L sodium and 35-38 mmol/L bicarbonate and remain unchanged during the study period. This study (NL34908.068.10/MEC10-2-098) was approved by the local ethics committee, and written informed consent was obtained from each patient.

Biochemical measurements

Blood was drawn from the inlet bloodlines in serum tubes, immediately before and after dialysis. Serum samples were aliquoted, stored at -80°C and thawed prior to analysis. For HDF8, hs-cTnI and NTproBNP could not be measured in 3 patients due to a lack of sufficient serum. cTnT was measured using the hs-cTnT assay (Roche Diagnostics), with a limit of detection at 5ng/L and 10% coefficient of variation (CV) cutoff at 13ng/L and cTnI was measured with the ARCHITECT hs-cTnI assay (Abbott Diagnostics) with a limit of detection of 2.3ng/L and 10%CV cutoff at 4ng/L. NTproBNP concentrations were assessed using the ProBNP-II assay (Roche) with a limit of detection of 0.6pmol/L and CV of 6.8% at 8.78pmol/L. Pre- and post-dialysis measurements were performed using the same assay on the same day. Post-dialysis concentrations were corrected for hemoconcentration by ultrafiltration using serum total protein and albumin measurements (Cobas 8000 analyzer, Roche Diagnostics).

Statistical analysis

Reduction ratios were calculated as follows: $RR = [1 - (\text{pre-dialysis concentration} / \text{post-dialysis concentration})]$. Differences in pre- versus post-dialysis concentrations and reduction ratios (RR) were analyzed using the Wilcoxon signed rank test and pair-wise ANOVA, respectively. Two sided p-values of <0.05 were considered statistically significant. Statistical analysis was performed with IBM SPSS 20.0 statistics program.

Results

Baseline characteristics

Thirteen patients completed the study, who were on dialysis for a mean period of 49(±29) months. Mean age of the patients was 53.6(±20.4) years and 10 patients were males. During the study period, these patients were clinically stable. Additional specifications regarding this study are summarized in SUPPLEMENTAL TABLE 1 and have been previously published [9].

SUPPLEMENTAL TABLE 1. Baseline characteristics as previously published by Cornelis et al [9]

Characteristic	HD4	HD8	HDF4	HDF8
Effective dialysis time (min)	246±4	486±2*§	245±3	487±6*§
Blood volume (L)	67.9±1.6	134.9±2.0*§	68.5±1.1	135.3±1.4*§
Blood flow (mL/min)	283±7	287±4	286±5	288±3
Dialysate flow (mL/min)	569±24	576±8	578±7	572±11
Dialysate temperature (°C)	35.9±0.4	35.9±0.4	35.8±0.4	36.0±0.4
Total ultrafiltration (L)	1.98±0.76	2.31±0.82	1.77±0.63	2.21±0.81
Ultrafiltration rate (mL/h)	500±193	295±111*§	449±160	265±83*§
Substitution volume (L)	-	-	14.6±1.1	29.7±0.4§
Single pool Kt/V	1.49±0.21	3.30±0.54*§	1.60±0.30	3.34±1.05*§
Equilibrated Kt/V	1.36±0.26	3.09±0.51*§	1.41±0.26	3.14±0.99*§

*P<0.05 versus HD4; § p<0.05 versus HDF4

Influence of hemodialysis and hemodiafiltration on hs-cTnT, hs-cTnI and NTproBNP

Median hs-cTnT concentrations were considerably higher than hs-cTnI concentrations before and after hemodialysis (TABLE 1). Moreover, all pre-dialysis hs-cTnT concentrations were above the diagnostic cut-off of 14ng/L. In contrast, only three patients had elevated hs-cTnI concentrations (>26.2ng/L). Also, elevated NTproBNP concentrations (>35pmol/L) were measured in all patients.

No significant differences were found between pre-dialysis hs-cTnT, hs-cTnI and NTproBNP concentrations for different dialysis-modalities ($p>0.05$).

TABLE 1. *hs-cTnT, hs-cTnI and NTproBNP concentrations pre and post dialysis, corrected for hemoconcentration, per modality.*

Hs-cTnT	modality	hs-cTnT _{pre} (ng/L); Median (IQR)	hs-cTnT _{post} (ng/L); Median (IQR)	p-value
	HD4	43.1 (36.1-62.8)	48.0 (31.4-77.1)	0.158
	HD8	41.6 (26.8-57.8)	35.9 (19.5-61.4)	0.534
	HDF4	46.0 (37.2-65.0)	41.8 (24.2-60.3)	0.013
	HDF8	44.1 (33.0-57.3)	33.9 (20.7-61.4)	0.004
Hs-cTnI	modality	hs-cTnI _{pre} (ng/L); Median (IQR)	hs-cTnI _{post} (ng/L); Median (IQR)	p-value
	HD4	11.4 (8.2-17.7)	12.6 (8.6-22.2)	0.347
	HD8	17.2 (7.4-26.5)	13.7 (6.7-24.0)	0.203
	HDF4	18.6 (7.0-24.6)	19.0 (6.1-29.1)	0.917
	HDF8*	22.4 (6.7-34.0)	12.8 (5.7-29.1)	0.028
NTproBNP	modality	NTproBNP _{pre} (pmol/L); Median (IQR)	NTproBNP _{post} (pmol/L); Median (IQR)	p-value
	HD4	427.3 (283.6-754.6)	407.2 (220.8-1112)	0.034
	HD8	393.0 (234.8-574.8)	218.2 (125.4-349.4)	0.008
	HDF4	458.9 (256.1-1170)	249.3 (117.3-591.4)	0.002
	HDF8*	308.6 (245.2-512.9)	102.2 (59.8-179.6)	0.008

*based on serum samples of 10/13 patients

Generally, decreases in hs-cTnT, hs-cTnI as well as NTproBNP were found immediately after dialysis (TABLE 1). However, no significant differences were observed between pre- and post-dialysis hs-cTnT and hs-cTnI concentrations after HD4 and HD8 (TABLE 1; $p>0.05$). In contrast, HDF had a highly significant effect on hs-cTnT concentrations ($p<0.01$), especially following HDF8. Also hs-cTnI demonstrated a significant decrease after HDF8 but not HDF4 (TABLE 1). For NTproBNP, significant intradialytic decreases following all modalities were observed, with the highest reduction after HDF8 (TABLE 1 and FIGURE 1).

When comparing HD4 to HDF8, more patients demonstrated a significant ($>10\%$) decrease in hs-cTnT, hs-cTnI and NTproBNP concentrations following HDF8 (SUPPLEMENTAL FIGURE 1).

SUPPLEMENTAL FIGURE 1. Individual changes in hs-cTnT, hs-cTnI and NTproBNP concentrations following either HD4 and HDF8-treatments. Full lines indicate a $>10\%$ decrease pre versus post-dialysis, while dotted lines indicate less than 10% reduction.

HD4

HDF8

For hs-cTnT, significantly higher RRs were established following HDF compared to HD (FIGURE 1). Interestingly, RR were significantly higher for hs-cTnI after HD8 and HDF8 in comparison to HD4 and HDF4. Overall the highest RR were obtained following HDF8, although mean RR were still lower for hs-cTnT ($20.4 \pm 8.8\%$) and hs-cTnI ($23.0 \pm 16.2\%$) in comparison to NTproBNP ($64.2 \pm 9.5\%$) and other uremic toxins (50-90%; [9]).

FIGURE 1. Mean ($\pm$ SD) reduction ratios of hs-cTnT, hs-cTnI and NTproBNP, corrected for hemoconcentration, following 4 and 8 hour hemodialysis and hemodiafiltration.

Identical results were found when hs-cTnT, hs-cTnI and NTproBNP concentrations were corrected for hemoconcentration by ultrafiltration using albumin concentrations (data not shown).

Discussion

This is the first study to reveal the effects of extended hemodialysis and hemodiafiltration on cardiac markers. Here, we clearly demonstrate a significant reduction in hs-cTnT, hs-cTnI and NTproBNP concentrations following HDF, although RR remained relatively low for both hs-cTn measurements (<25%).

Recently it was shown that hs-cTnT concentrations display a diurnal variation, with peak hs-cTnT concentrations in the morning that gradually decrease during the day [10]. Herein, we found higher RR for hs-cTnT following 4 hour HDF in comparison to 8 hour HD. Consequently, we can conclude that dialysis treatments have an additional effect on hs-cTnT concentrations. However, whether these cTn reductions are due to clearance, still has to be elucidated by measuring in dialysate samples.

Interestingly, the reduction of cTnT was more influenced by the use of convection in comparison to cTnI [9]. In addition, 8-hour dialysis treatments had the largest effects on the RR of cTnI. Whether this is the consequence of differences in cTn release or in clearance from the blood stream merits further research.

In conclusion, this study demonstrated a clear effect of different dialysis modalities on cardiac biomarkers with highest RR's during HDF8. Clinicians should be aware of this intradialytic reduction in cardiac biomarkers when interpreting serial results, especially in the diagnostic context of an acute myocardial infarction. The long-term effects of these dialysis modalities on the levels of cardiac biomarkers should be addressed in future studies.

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TIME-DEPENDENT DEGRADATION PATTERN OF CARDIAC TROPONIN T FOLLOWING ACUTE MYOCARDIAL INFARCTION

Cardinaels E*, Mingels A*, van Rooij T, Collinson P, Prinzen F and van Dieijen-Visser M.

**both authors contributed equally*

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Abstract

Background

Cardiac troponin T (cTnT) is widely used for the diagnosis of acute myocardial infarction (AMI). However, it is still unclear whether degraded cTnT forms circulate in the patient's blood. We therefore aimed to elucidate which cTnT forms are detected by the clinical assay.

Methods

Separation of cTnT forms by gel filtration chromatography (GFC) was performed in sera from 13 AMI patients to examine cTnT degradation. The GFC eluates were subjected to Western blot analysis, using the original antibodies from the Roche immunoassay to mimic the clinical cTnT assay. To investigate the degradation pattern with time, standardized serum samples of 18 AMI patients collected 0-72 hours post-admission were analyzed by Western blot analysis.

Results

GFC analysis of AMI patients' sera revealed two cTnT peaks with retention volumes of 5 and 11 mL. Western blot analysis identified these peaks as cTnT fragments of 29 and 14-18 kDa, respectively. Furthermore, the performance of direct Western blots on standardized serum samples, demonstrated a time-dependent degradation pattern of cTnT, with fragments ranging between 14 and 40 kDa. Intact cTnT (40 kDa) was only present in 3 patients within the first 8 hours after hospital admission.

Conclusion

These results demonstrate that the Roche cTnT immunoassay detects intact as well as degraded cTnT forms in AMI patients' sera during the period of diagnostic testing. Moreover, following AMI, cTnT is degraded in a time-dependent pattern.

Introduction

Cardiac troponin T (cTnT) and cardiac troponin I (cTnI) are superior to other cardiac markers because of their unique cardiospecificity. Consequently, they are considered to be the biomarkers of first choice to diagnose and monitor an acute myocardial infarction (AMI) [1]. Owing to patent restrictions only one cTnT assay (Roche Diagnostics) is currently commercially available, as compared with over twenty different assays for cTnI [2]. In addition, according to a recent survey, the Roche cTnT assay is used in half of European laboratories [3]. Therefore, characterization of cTnT, as detected by this assay, is important to better define its role in clinical decision making.

The cTn T-I-C complex is structurally bound predominantly to the myofibrils with only a minor fraction of cTnT and cTnI (6-8%) present in the cytoplasm as soluble intact protein [4, 5], although this finding has been disputed [6]. Typically, AMI patients undergoing rapid reperfusion demonstrate a first cTnT peak concentration within 24 hours after onset of symptoms, which has been attributed to the fast release of cytosolic cTnT [4, 7]. This peak is followed by a second and persistent cTnT elevation which remains present for 7-14 days and is generally thought to represent the relatively slow dissociation of cTnT from the sarcomeres and release from the cells during necrosis. There is limited knowledge on the posttranslational modifications of cTnT after myocardial damage, in particular degradation of cTnT. On the other hand, degradation products of cTnI have been extensively reported [8-11] and even associated with post-ischemic contractile dysfunction [12, 13].

Previously, we have reported the presence of cTnT fragments on Western blots of human serum from renal failure and post AMI patients using different antibodies as the clinical assay [14, 15]. Other investigations have not confirmed these findings and found only intact cTnT on gel filtration chromatography (GFC) of serum from such patients [16, 17]. To date it is still not clear which cTnT forms circulate in the serum of AMI patients and if they are detected by the Roche cTnT assay.

In this study, we aimed to elucidate whether degraded cTnT forms are present in the circulation following ischemic myocardial damage. We examined AMI patients' sera using a novel approach. Firstly, the patients' sera were

fractionated by GFC, as in previous studies [16, 17]. Secondly, these GFC fractions were subjected to a unique Western blotting technique, in which the capture and detector antibodies of the commercial cTnT assay were incorporated to mimic the working principle of this assay. In a second AMI population, we aimed to characterize the changes of the molecular forms of cTnT with time. Serum samples collected for each patient at standardized time points after symptom onset were subjected directly to this Western blotting technique.

Materials and Methods

Study populations

GFC followed by Western blotting was performed on residual serum samples from 13 patients with acute myocardial infarction followed by rapid revascularization. Blood samples were obtained from the routine laboratory at two time points 3 (± 1) as well as 15 (± 2) hours after admission to the emergency department of Maastricht University Medical Center) and frozen at -80°C . One serum sample was missing from each of two AMI patients, one at 3 hours and one at 15 hours post-admission. As a result, 12 AMI serum samples were analyzed for both time points.

The molecular changes of cTnT with time and their subsequent effect on infarct size estimation were examined in a second well-characterized AMI population. Serum samples of 18 ST-segment elevation myocardial infarction (STEMI) patients were collected at hospital admission and at standardized time points: 4, 8, 12, 24, 36, 48, 60, and 72 hours after hospital admission and immediately stored at -80°C . These patients were enrolled in the control group of a clinical trial investigating the effects of ventricular pacing during revascularization, and thus receiving standard treatment (<http://clinicaltrials.gov/ct2/show/NCT00409604>). Inclusion criteria for the study were as follows: age >18 years, presentation with their first myocardial infarction, admitted to hospital 3 (± 1) hours from symptom onset, treated with percutaneous coronary intervention (PCI) and exhibiting a typical release pattern of cTnT, creatine kinase (CK) and lactate dehydrogenase (LD).

The study was conducted according to the principles of the Declaration of Helsinki and approved by the local ethics committees. Informed consent was obtained from the patients.

Biochemical testing

cTnT was measured on the Elecsys 2010 (Roche) using the 4th generation cTnT assay with a limit of detection $<0.01 \mu\text{g/L}$ and 10% CV cutoff at $0.03 \mu\text{g/L}$. NT-proBNP was also measured on the Elecsys 2010 (Roche) with limit of detection 5.08 ng/L (0.6 pmol/L) and inter-assay CV 6.8% at 74.4 ng/L (8.78 pmol/L). Albumin was measured on the Synchron LX20 (Beckman Coulter) using the microalbumin assay with measuring range of $2.0\text{--}970 \text{ mg/L}$. CK and LD were measured on the Synchron LX20 (Beckman) as well. cTnI was measured using the AxSYM troponin I-Adv assay (Abbott Laboratories) with the 10% CV cutoff at $0.16 \mu\text{g/L}$. All assay characteristics were given by the manufacturer.

Fractionation by GFC

GFC was performed on a HP 1100 system (Agilent Technologies) equipped with a $1.6 \times 60 \text{ cm}$ Sephacryl-S100 column (GE Healthcare) and a diode array detector. The column was equilibrated with 0.26 mol/L NaCl , $2.5 \text{ mmol/L CaCl}_2 \cdot 2 \text{ H}_2\text{O}$, 0.02 mol/L Tris , 6 mmol/L NaN_3 and 1 g/L bovine serum albumin buffer, pH 7.4 [16] and operated at 0.5 mL/min . The void volume (V_0) determination and calibration was conducted using the Gel Filtration Calibration kit (GE Healthcare) containing Dextran Blue (molecular weight (MW), 2000 kDa), conalbumin (MW, 75.0 kDa), ovalbumin (MW, 43.0 kDa), carbonic anhydrase (MW, 29.0 kDa) and ribonuclease (MW, 13.7 kDa), supplemented with myoglobin (MW, 16.9 kDa , Sigma). Dextran Blue (1 mg/ml) and globular protein standards ($3\text{--}4 \text{ mg/ml}$) were dissolved in running buffer, 0.5 mL was loaded on the column, and absorbance was studied at 280 nm , all in duplo. V_0 was determined to be 37 mL .

In addition, purified human cTn T-I-C complex (MW, 77 kDa ; Hytest) and purified free cTnT, cTnI, and troponin C (TnC) (MW, 37 , 24 and 18 kDa , respectively; all Advanced ImmunoChemical) were dissolved as prescribed by

the manufacturer and were further diluted in running buffer or spiked in serum pool of healthy volunteers (cTnT < 0.01 µg/L and cTnI < 0.03 µg/L).

To obtain the standard calibration curve, the gel-phase distribution coefficient ($K_{av} = V_R / (V_c - V_0)$) was plotted against the logarithm of MW of globular proteins and purified cTn standards. V_R signifies the retention volume adjusted to void volume, while V_c indicated column volume and corresponded to 120 mL.

For serum samples, 1.0 mL was loaded on the column. Serum albumin (67 kDa) and serum NT-proBNP were used as internal standards in the patients' sera.

For each sample loaded, fractions of 1.25 mL were collected, kept on ice until immunoassay measurements (≤ 1 hour) and subsequently stored at -80°C .

Purification and Western blot detection of cTnT

Purification and characterization of cTnT was performed using the antibodies from the Roche cTnT-assay (as kindly provided by Roche Diagnostics, www.roche.com). The capture cTnT antibody (M11.7, epitope a.a.r. 136-147) was used for immunoprecipitation of cTnT. To each mL of magnetic streptavidin-coated dynabeads (Invitrogen) 10 µg biotinylated M11.7 was added. Additionally, beads were crosslinked three times with dimethyl pimelimidate. Subsequently, 200 µL of serum was precipitated with 50 µL of M11.7-coated beads for 1 hour at 4°C . Due to low cTnT concentrations in the GFC fractions, more sample was added to the beads, ranging from 200-5000 µL. In some cases, up to 4 fractions were merged to generate larger volumes. The average cTnT yield after immunoprecipitation was $>90\%$ (data not shown). After being washed with PBS containing 0.1% Tween-20 (PBST) the immunoprecipitates were eluted with 1 M glycine (18 µL), pH 3 for 15 min at 56°C . (SUPPLEMENTAL FIGURE 1)

As negative controls for immunoblotting of GFC samples and AMI patients' sera, running buffer or the serum pool of healthy volunteers was used, respectively. As positive controls, cTn T-I-C complex was spiked in running buffer and cTnT negative pooled serum, respectively. Only GFC fractions containing sufficiently high cTnT concentrations could be visualized on the immunoblot, corresponding to 9 out of 12 serum samples collected at ± 15 hours post admission.

Immunoprecipitates were diluted with XT Sample Buffer (6 µL) (Bio-Rad Laboratories), heated for 7 min. at 56°C , and separated on a 12% Criterion XT

SDS-PAGE gel (Bio-Rad) together with the Precision Plus Protein Standard (Bio-Rad). After stacking for 15 min at 100 V the proteins were resolved for 45 min at 200 V in XT MOPS electrophoresis buffer (Bio-Rad) and were transferred to the nitrocellulose membrane (Bio-Rad, 0.45 μ m) at 100 V for 1 h at 4°C. The membranes were treated with the vacuum SNAP i.d. protein detection system (Millipore) according to manufacturer instructions. Non-specific binding was blocked with 0.5% non-fat dry milk Blocking-Grade Blocker (Bio-Rad) in PBST. Primary and secondary antibodies were Roche M7 anti-cTnT antibody (epitope a.a.r. 125-131; 10 μ g/L) and goat anti-mouse peroxidase (0.4 μ g/mL; DAKO) in PBST, respectively. Subsequently, membranes were incubated for 5 minutes in Super Signal West Femto Substrate (Thermo Scientific) and bands were detected using the ChemiDoc XRS scanner (Bio-Rad) and Quantity One Software (Bio-Rad, Version 4.6.5.).

Statistical Analysis

Results are presented as means ($\pm$ SD) or when not normally distributed as medians (interquartile range (IQR)). The Wilcoxon signed rank test was applied to investigate differences in peak-to-peak ratios of the GFC elution profiles obtained 3 and 15 hours post-admission. All statistical analyses were performed using SPSS (Statistical Package for Social Sciences; version 18). A p-value <0.05 was regarded as statistically significant.

Results

Validation of cTnT degradation by GFC followed by Western blotting

Elution profiles of globular protein standards (MW varying from 16.9 to 75.0 kDa) and purified cTn standards (cTn T-I-C complex, free cTnT, cTnI and TnC) were determined. A schematic overview of the obtained retention volumes adjusted to V_0 (V_R) is given in FIGURE 1A.

FIGURE 1. Validation of GFC analysis. (A) Schematic overview of the retention volumes obtained for purified cTn standards (cTnT-I-C, cTnT, cTnI, TnC), globular proteins and internal serum standards (serum albumin and NT-proBNP). (B) Standard calibration curve obtained with globular proteins (●) and purified cTn standards (▲). The gel-phase distribution coefficient ($K_{av}=V_R/(V_c-V_0)$; V_c =column volume) was plotted against the log molecular weight (MW).

When spiking the intact cTnT standard in cTnT-negative serum (cTnT, 19.9 µg/L), cTnT elutes with V_R of 5 mL before the elution of the internal standard serum albumin (V_R = 11 mL) (SUPPLEMENTAL FIGURE 2A). Western blotting of these GFC fractions confirmed that this peak corresponded to intact cTnT (estimated MW, MW_{est} of 40 kDa) and for a small part to 29 kDa cTnT fragment, as shown in SUPPLEMENTAL FIGURE 2B. An identical elution profile and immunoblot analysis were obtained when the purified cTnT standard was spiked

in running buffer (data not shown). For the other purified cTn standards, V_R was determined to be 3 mL, 16 mL and 20 mL for the cTnT-I-C complex, intact cTnI and TnC, respectively. (FIGURE 1A) Moreover, the correlation between V_R and MW of the globular proteins can be represented by a linear calibration curve following the equation $y = -0.44x + 0.93$, as depicted by FIGURE 1B. In contrast, all cTn proteins (cTnT-I-C complex, free cTnT, cTnI and TnC) deviated clearly from the calibration curve.

Median (IQR) cTnT concentrations measured in serum of AMI patients, subjected to GFC, were 2.17 (5.07) $\mu\text{g/L}$ and 6.94 (6.46) $\mu\text{g/L}$ for 3 and 15 hours post-admission, respectively. After GFC separation, overall median (IQR) recovery of loaded cTnT (%) was 79 (27) % (SUPPLEMENTAL TABLE 1). The GFC elution profiles of each patient showed two cTnT peaks at an adjusted V_R of 5 and 22 mL, eluting before and after the internal standard serum albumin ($V_R = 11$ mL). As shown in FIGURE 2A and 2B this was observed at 3 as well as 15 hours post-admission, respectively. Western blot analysis using the antibodies of the clinical immunoassay identified the first cTnT peak at V_R of 5 mL as a degradation product of cTnT with a MW_{est} of 29 kDa (FIGURE 2C and D). The second peak ($V_R = 22$ mL) was allocated to smaller cTnT degradation products of MW_{est} between 14 and 18 kDa. Moreover, there was a significant relative increase in the second peak at 15 hours post admission as compared with 3 hours post-admission (median ratio of cTnT-peak at 22 mL/5 mL of 0.54 and 1.89 for 3 and 15 hours post-admission respectively; $P = 0.001$), suggesting a different cTnT degradation pattern that changed with time.

Time course of cTnT degradation at standardized time points

The release kinetics of cTnT, CK and LD determined in eighteen STEMI patients (66% males with mean(SD) age 60(9) years (see SUPPLEMENTAL TABLE 2) are shown in FIGURE 3A and SUPPLEMENTAL FIGURES 3 and 4, respectively. On average, peak cTnT concentrations were reached 8 hours after admission, with a median (IQR) concentration of 6.68 (4.54) $\mu\text{g/L}$. Furthermore, cTnT levels were still elevated 72 hours post-admission with a median (IQR) concentration of 2.65 (1.83) $\mu\text{g/L}$.

FIGURE 3B depicts a representative blot of an AMI patient showing that the antibodies of the clinical assay detected mainly cTnT degradation products in the circulation. As many as five different degradation products were reproducibly identified (MW_{est} of 14, 16, 18, 27 and 29 kDa). Moreover, a breakdown pattern of cTnT was observed over time. Western blot analysis of all patients showed that intact cTnT (MW_{est} of 40 kDa) was present only in three patients during the first 8 hours after admission (FIGURE 3C). Instead the 29 kDa fragment of cTnT was detected in highest abundance up to 24 hours post-admission. Afterwards, the larger fragments (MW_{est} of 27 and 29 kDa) disappeared and primarily low-molecular weight fragments between 14 and 18 kDa were identified. When blotting with an antibody directed against the C terminus of cTnT, the same degradation pattern for cTnT was visualized. (SUPPLEMENTAL FIGURE 5) On the contrary, when applying an antibody directed against the N terminus of cTnT, none of the cTnT fragments were detected.

FIGURE 3. Time course of cTnT degradation at standardized time points. (A) Release kinetics of cTnT in AMI patients represented by the median (black) and IQR (grey) cTnT-concentrations. (B) cTnT Western blot of serum samples obtained at standardized time points of a representative AMI patient P, positive control; M, marker; N, negative control. (C) Summary of the molecular forms of cTnT present after AMI at the standardized time points. Each dot represents a band on the Western blot of a patient.

Discussion

The present study shows for the first time that circulating cTnT is highly degraded in cases of AMI followed by rapid revascularization, as demonstrated by fractionation of serum from AMI patients with GFC and Western blotting, using the antibodies of the clinical cTnT assay.

GFC enables detailed characterization of non-covalent complexes present in the patient's serum, but also requires extensive and careful validation. Herein, we report similar elution profiles as previously found by Bates and colleagues [16], although they assigned their main peak to intact cTnT. As determined from the calibration curve, obtained with globular protein standards, the expected adjusted V_R for intact cTnT would be 20 mL. However, in this study we clearly demonstrate that purified cTnT, and also other cTn molecules, eluted much earlier and therefore deviated from the calibration curve. For this reason, molecular weight assignment of cTn elution profiles should not be based on the molecular weights of globular proteins. This is especially important for elongated shaped structures like cTnT [18, 19]. In the present study, we identified that the most abundant form of circulating cTnT detected by the cTnT immunoassay is actually a mixture of cTnT fragments. Previous findings have also postulated that ternary structures of the cTn complex are the most frequent form present in the patient's circulation [7, 16, 17, 20]. Using the same gel filtration technique [16, 17] we observed that this type of GFC column was not suitable for adequate separation of ternary bound cTn T-I-C complex ($V_R = 3$ mL) and intact cTnT ($V_R = 5$ mL) molecules. Therefore, the presence of ternary bound cTn molecules in the patient's circulation requires further study.

Furthermore, sera from AMI patients, drawn at standardized time points after admission to the emergency department, and analyzed directly with Western blot analysis revealed that cTnT is degraded in a time-dependent pattern. Intact cTnT was the largest cTnT form that could be detected and it was only present during the early hours (4 to 8 hours) after admission in three of the eighteen patients. Primarily 29 kDa cTnT fragments were visible up to 24 hours after AMI, corresponding to the peak concentrations of the cTnT release curve and the period of diagnostic testing. Later on, 29 kDa fragments seemed to diminish and degradation products with a smaller molecular mass (MW_{est} of 14 and 16 kDa)

dominated in peripheral blood. The immunoreactivity on the Western blot however seemed to differ between intact and low-molecular weight cTnT molecules, thereby limiting quantification of Western blot bands.

Since, Roche Diagnostics is the only manufacturer of clinical cTnT assays, cTnT degradation does not affect harmonization between different assays, as previously reported for the cTnI assays [11]. To ensure quality of the cTnT measurement in blood samples, Roche Diagnostics recommends the use of recombinant human cTnT spiked in a human serum matrix for monitoring the calibration and precision of the assay. However, as shown in the present study, this recombinant intact cTnT is not representative for AMI patient serum which contains mainly cTnT fragments. Furthermore, although studies have indicated the magnitude of cTnT measurements to be of prognostic value [21-23] and strongly correlated with infarct size [24, 25], whether the detection of different cTnT forms will affect the clinical utility of cTnT measurements has to be examined.

A limitation to this study is that the 4th generation cTnT assay was used. However, the epitopes of the capture and detection antibody are the same for the current hs-cTnT assay [26]. Subsequently, the same cTnT forms will be detected by both assays. As cTnT fragments were observed using two different techniques and cTnT degradation exhibited a time-dependent pattern, these fragments are unlikely to have been introduced by our methodology, and the time-dependent pattern suggests they are the products of an enzymatic process. Moreover, when the purified cTn T-I-C standard (NIST-SRM 2921) was spiked into serum or heparin plasma, cTnT was shown not to be susceptible to degradation when stored for 72 hours at 4°C or for 24 hours at 37°C [27, 28]. It should be noted that the NIST-SRM 2921 standard originally contained the 29 kDa fragment of cTnT before incubation. These finding further suggests that the cTnT degradation observed in this study is induced *in vivo*. In addition, several *in vitro* studies have demonstrated that the degradation of cTnT occurred upon ischemia, due to the activity of calpain-I [29-31] and caspase-3 [32] in the intracellular environment of cultured cardiomyocytes, possibly leading to the cleavage of the N terminus of cTnT. This all leads to the intriguing question of what modification patterns of cTn are present when myocardial necrosis is

thought to be absent, for instance after physical activity [33, 34], and the subsequent question of whether the diagnostic potential of cTnT can be further improved. Of note, the present study describes cTnT degradation in serum of AMI patients; the presence of cTnT fragments in heparin plasma samples has yet to be investigated.

In conclusion, this study demonstrates that circulating cTnT undergoes degradation in serum following AMI and the degradation pattern is dependent on time after symptom onset. Importantly, this is the first study to reveal that the cTnT immunoassay widely applied in the clinic binds both intact as degradation products of cTnT during the time frame of diagnostic testing.

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Disclosures

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SUPPLEMENTAL FIGURE 1. Optimization of the elution procedure during immunoprecipitation. Troponin T-I-C complex spiked in cTnT-negative serum was immunoprecipitated in triplo according to the methods described in the manuscript. Three different elution conditions were tested: (A) 1M glycine pH3 at 56°C (B) XT sample buffer (BioRad) at 95°C and (C) at 56°C.

SUPPLEMENTAL FIGURE 2. GFC analysis of purified cTnT (A) Chromatographic curve for purified free cTnT spiked in cTn-negative serum; serum albumin ($V_R = 11$ mL) and serum NT-proBNP ($V_R = 22$ mL) were used as internal standards. (B) Purification and detection of cTnT by means of the cTnT antibodies of the clinical assay in the obtained chromatographic fractions. P, positive control; M, marker; N, negative control.

SUPPLEMENTAL FIGURE 3. Release kinetics of creatine kinase (CK) after AMI (n=18), represented by the median (black) and IQR (grey) concentrations of CK. U/L indicates units per liter.

SUPPLEMENTAL FIGURE 4. Release kinetics of lactate dehydrogenase (LD) after AMI (n=18), represented by the median (black) and IQR (grey) concentrations of LD. U/L indicates units per liter.

SUPPLEMENTAL FIGURE 5. Western blot analysis using different cTnT antibodies. Two serum samples (S1 and S2) immunoprecipitated in triplo with and loaded on the SDS-PAGE gel according to the specifications given in the methods section. Each Western blot was subsequently stained with different cTnT antibodies, namely (A) Roche detection antibody (M7); (B) antibody directed against N terminus of cTnT (1 µg/mL, 9G6, Hytest Ltd) and (C) the C terminus of cTnT (1 µg/mL, 7A9, Hytest). The secondary antibody was for all Western blots goat-anti mouse peroxidase (0.4 µg/mL; DAKO). S1 indicates serum sample of patient 1 collected 12 hours after admission, [cTnT] = 14 µg/L; S2, serum sample of patient 2 collected 8 hours after admission, [cTnT] = 4.3 µg/L; P, positive control (troponin T-I-C complex spiked in cTnT-negative serum, [cTnT] = 2 µg/L; N, negative control (= cTnT-negative serum; [cTnT]<0.01 µg/L)

SUPPLEMENTAL TABLE 1. Recoveries (%) of cTnT and cTnI in gel filtration chromatography

Recovery (%) ^a	cTnT	cTnI
Troponin T-I-C	96%	79%
Purified cTnT	89%	-
Purified cTnI	-	79%
AMI patients	77% ^b	99% ^c

^a, all recoveries were calculated as follows:

$$recovery (\%) = \frac{\sum \left(\left(cTnT \text{ concentration } \left(\frac{pg}{mL} \right) \right)_{fractions} \times fraction \text{ volume } (1.25 \text{ mL}) \right)}{\left(\left(cTnT \text{ concentration } \left(\frac{pg}{mL} \right) \right)_{initial} \times injection \text{ volume } (1 \text{ mL}) \right)} \times 100$$

^b, mean recovery of all 24 GFC analyses; ^c, mean recovery based on 12 of the 24 GFC analyses

SUPPLEMENTAL TABLE 2. Baseline characteristics of AMI patients (n = 18) in which the molecular forms of cTnT were investigated at standardized time-points (0-72 hours after hospital admission).

N°	Sex	Age (yrs)	Lesion ^a	Δ time symptom onset and admission (h:min) ^b	ST max (mV) ^c	MRI (%) ^d
1	F	66	RCA prox	4:07	-3.8	18.3
2	M	56	RCA dist	2:35	6.9	27.6
3	M	78	RCA mid	2:42	7.9	n.a.
4	M	58	RCA prox	1:55	7.1	25.8
5	M	52	RCA mid	2:17	2.8	22.0
6	F	70	RCA prox	5:30	5.1	10.5
7	F	50	LAD mid	4:09	4.9	23.2
8	M	55	LAD prox	2:12	9.6	36.9
9	M	58	LAD mid	5:30	5.4	0.8
10	F	74	LAD mid	2:12	n.a.	24.5
11	F	66	RCA mid	2:00	2.0	5.0
12	M	54	RCA prox	3:30	3.3	10.3
13	M	64	LAD prox	3:45	14.4	n.a.
14	M	66	RCA prox	3:15	7.6	25.4
15	F	48	LAD mid	4:00	8.8	n.a.
16	M	44	RCA prox	4:30	n.a.	19.9
17	M	61	RCA mid	2:15	6.4	21.3
18	M	72	RCA prox	0:45	-1.9	2.5

a, indicates coronary artery in which stent was placed; b, time between symptom onset and hospital admission (hours:minutes), c, the maximum ST segment elevation during Holter electrocardiography; d, infarct size percentage measured with late gadolinium enhancement MRI; F, indicates female; M, male; RCA, right coronary artery; LAD, left anterior descending artery; mV, millivolt; U, units; n.a., not available

SEVEN

ACCUMULATION OF N-TERMINAL TRUNCATED CARDIAC TROPONIN T FRAGMENTS IN PATIENTS WITH END-STAGE RENAL DISEASE ON HEMODIALYSIS

Manuscript in preparation

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Abstract***Background***

Cardiac troponin T (cTnT) is known to be chronically elevated in patients with end-stage renal disease (ESRD), which is attributed to the presence of cardiac disease but also renal dysfunction. To date it remains unknown whether these chronically elevated cTnT concentrations reflect the accumulation of degraded or intact cTnT molecules.

Methods

By gel filtration chromatography (GFC), retention volumes for purified standards (cTnT-I-C complex and intact cTnT) and the clinical assay's quality control materials were investigated. Additionally, GFC profiles of acute myocardial infarction (AMI) patients' sera were determined. Eluates were subjected to immunoprecipitation and Western blotting, employing antibodies from the Roche immunoassay to mimic the clinical cTnT assay. Also, AMI patients sera were subjected directly to immunoblotting using either the detector antibody of the clinical assay or an antibody directed against the N-terminal end of cTnT. The validated GFC analysis was then applied to the sera from ESRD patients before and after hemodialysis (n= 10) and over 2 months follow-up (n=6) to establish the molecular forms of cTnT.

Results

Retention volumes (V_R) of cTn T-I-C complex and intact cTnT were found to be 20 and 27.5 mL, respectively. Also in quality control materials, cTnT eluted at 27.5 mL. AMI patients' sera revealed two cTnT peaks with retention volumes of 27.5 and 45 mL, and Western blotting illustrated that these peaks corresponded to N-terminal truncated cTnT fragments of 29 and <18 kDa, respectively. In contrast to AMI, ESRD patients demonstrated only one reproducible cTnT-peak at 45 mL in serum samples obtained before as well as after hemodialysis. Moreover, this cTnT-peak remained stable following 2 months in time. This cTnT-peak could be allocated to the low-molecular weight cTnT fragments (<18 kDa) in which the N-terminal end was found to be absent.

Conclusion

This is the first study to clearly demonstrate the presence of only degraded cTnT forms in the ESRD patients' sera. Moreover, these cTnT-fragments were observed before and after hemodialysis, and were found to be stable over time.

Introduction

The release of cardiac troponins (cTn) in the patient's circulation is considered to be a hallmark for myocardial damage because of their unique cardiospecificity. The diagnosis of an acute myocardial infarction (AMI) therefore relies on an increase or decrease in serial cTn measurements, preferably performed with high-sensitivity cTn (hs-cTn) assays [1]. Previously, we have demonstrated in serial AMI patients' sera that cTnT is degraded in a time-dependent pattern [2]. During the first hours after presentation at emergency department (ED), predominantly 29 kDa cTnT-fragments were present, while later on (>24 h after ED-presentation) only fragments <18 kDa were detected.

Chronically elevated cTnT concentrations are also common in patients suffering from renal dysfunction [3-5]. The presence of measurable cTnT concentrations in these patients have been associated with abnormal left ventricular morphology and function [6], myocardial stunning [7], and with a higher risk on future cardiovascular events and mortality [8, 9]. Apart from this, significant associations between cTnT and renal clearance have been demonstrated [10]. It has been shown that these chronically elevated cTn concentrations reflect the accumulation of cTnT-fragments, as illustrated with Western blotting employing different antibodies than those of the clinical cTnT assay [11]. However, these findings have been opposed by others, who found only intact cTnT when analyzing the serum of ESRD patients with gel filtration chromatography (GFC) [12]. To date it is therefore contradictory which cTnT forms circulate in the serum of ESRD patients and which are detected by the clinical (Roche) cTnT assay. Gaining insight into the molecular forms of cTnT following ESRD, will further improve our understanding on the nature of chronic versus acute cTnT elevations.

In this study, we therefore aim to elucidate the cTnT forms that are detected in the circulation of patients on hemodialysis using the clinical antibodies in a similar approach as previously performed in AMI patients [2], and as described in chapter 6 of this thesis.

Materials and Methods

Study populations

Molecular forms of circulating cTnT were assessed in the serum samples of two ESRD-populations, both on chronic hemodialysis therapy. In a first ESRD cohort of 10 patients, serum samples were drawn before and immediately after hemodialysis. In a second cohort of 6 patients, pre-dialysis serum samples were collected at baseline and 2 months later. All samples were immediately frozen at -80°C upon analysis. Both studies were conducted according to the principles of the Declaration of Helsinki and approved by the local ethics committees. Informed consent was obtained from all patients.

Biochemical testing

cTnT-concentrations were determined using the hs-cTnT assay (Roche Diagnostics) with a limit of detection at 5 ng/L and 10% CV cutoff at 13 ng/L. NT-proBNP was measured with a limit of detection at 5.08 ng/L (0.6 pmol/L) and CV 6.8% at 74.4 ng/L (8.78 pmol/L). Albumin was analyzed using the microalbumin assay with measuring range of 2.0-970 mg/L. All measurements were performed on the COBAS 6000 analyzer (Roche).

Fractionation by GFC

GFC was performed on the AKTA Prime Plus (GE Healthcare) equipped with a 1.6x60 cm Sephacryl-S300 column (GE Healthcare). The column was equilibrated with 0.26 mol/L NaCl, 2.5 mmol/L CaCl₂ · 2 H₂O, 0.02 mol/L Tris, 6 mmol/L NaN₃ and 1 g/L bovine serum albumin buffer, pH 7.4 [13] and operated at 0.5 mL/min. The void volume (V_0) determination and calibration was conducted using the Gel Filtration Calibration kit (GE Healthcare) containing Dextran Blue (molecular weight (MW), 2000 kDa), conalbumin (MW, 75.0 kDa), ovalbumin (MW, 43.0 kDa), carbonic anhydrase (MW, 29.0 kDa) and ribonuclease (MW, 13.7 kDa). Dextran Blue (1 mg/ml) and globular protein standards (3-4 mg/ml) were dissolved in running buffer, 0.5 mL was loaded on the column, and absorbance was detected at 280 nm, all in duplo. V_0 was determined to be 40 mL.

For GFC analysis, purified human cTn T-I-C complex (MW, 77 kDa; Hytest) and purified free cTnT (MW, 37 kDa, respectively; Hytest) were diluted in running buffer or spiked in serum pool of healthy volunteers (cTnT<14 ng/L). Calibration (level-2) and control (TN2) materials of the clinical Roche hs-cTnT assay were dissolved as prescribed by the manufacturer and immediately examined using GFC. Also residual serum of acute myocardial infarction patients were analyzed to validate previous results [2].

To obtain the standard calibration curve, the gel-phase distribution coefficient ($K_{av} = V_R / (V_c - V_0)$) was plotted against the logarithm of MW of globular proteins and purified cTn standards following the equation $y = -0.34x + 1.04$. V_R signifies the retention volume adjusted to void volume, while V_c indicated column volume and corresponded to 120 mL.

For serum samples, 1.0-2.0 mL was loaded on the column. Serum albumin (67 kDa) and serum NT-proBNP were used as internal standards in the patients' sera. For each sample loaded, fractions of 1.25 mL were collected, kept on ice until immunoassay measurements (within 1 hour) and subsequently stored at -80°C.

Purification and Western blot detection of cTnT

Purification and characterization of cTnT was performed using the antibodies from the Roche cTnT-assay (as kindly provided by Roche Diagnostics, www.roche.com). The capture cTnT antibody (M11.7, epitope a.a.r. 136-147) was used for immunoprecipitation of cTnT. To each mL of magnetic streptavidin-coated dynabeads (Invitrogen) 10 µg biotinylated M11.7 was added. Additionally, beads were crosslinked three times with dimethyl pimelimidate. Subsequently, 200-700 µL of serum was precipitated with 50 µL of M11.7-coated beads for 1 hour at 4°C. The average cTnT yield after immunoprecipitation was >90% (data not shown). After being washed with PBS containing 0.1% Tween-20 (PBST) the immunoprecipitates were eluted with 1 M glycine (18 µL), pH 3 for 15 min at 56°C.

As negative controls for immunoblotting of GFC samples and AMI patients' sera, running buffer or the serum pool of healthy volunteers was used, respectively. As positive controls, purified intact cTnT was spiked in running buffer and cTnT

negative pooled serum, respectively. Only GFC fractions containing sufficiently high cTnT concentrations (~ 500 ng/L) could be visualized on the immunoblot. Immunoprecipitates were diluted with XT Sample Buffer (6 μ L) (Bio-Rad Laboratories), reduced at 56°C, and separated on a 12% Criterion XT SDS-PAGE gel (Bio-Rad) together with the Precision Plus Protein Standard (Bio-Rad). After stacking for 15 min at 100 V the proteins were resolved for 45 min at 200 V in XT MOPS electrophoresis buffer (Bio-Rad) and were transferred to the nitrocellulose membrane (Bio-Rad, 0.45 μ m) at 100 V for 1 h at 4°C. The membranes were treated with the vacuum SNAP i.d. protein detection system (Millipore) according to manufacturer instructions. Non-specific binding was blocked with 0.5% non-fat dry milk Blocking-Grade Blocker (Bio-Rad) in PBST. Primary antibodies were Roche M7 anti-cTnT (epitope a.a.r. 125-131; 10 μ g/L), and cTnT-specific antibodies against the middle region (Hytest, 1C11, epitope a.a.r. 95-181; 7 μ g/L) and the N-terminal region (Hytest, 9G6; epitope a.a.r. 1-60; 1 μ g/L). As the secondary antibody, goat anti-mouse peroxidase (0.4 μ g/mL; DAKO) diluted in PBST were employed. Subsequently, membranes were incubated in Super Signal West Femto Substrate (Thermo Scientific) and bands were detected using the ChemiDoc XRS scanner (Bio-Rad) and Quantity One Software (Bio-Rad, Version 4.6.5.).

Results

GFC analysis of control and calibration material for clinical assay

GFC-analysis of purified cTn standards (cTn T-I-C complex, intact cTnT) spiked in cTnT-negative serum revealed adjusted retention volumes (V_R) of 20 and 27.5 mL, respectively (FIGURE 1A and B). Both standards eluted before the internal standard serum albumin ($V_R = 32.5$ mL) and NTproBNP ($V_R = 45$ mL). Western blotting of these GFC fractions using the clinical cTnT antibodies, confirmed that these two peaks both contain intact cTnT forms (estimated MW, MW_{est} of 40 kDa; data not shown) as previously described [2]. Furthermore, GFC-elution profiles of the control (TN-2; cTnT = 1921 ng/L) and calibrator (level 2; cTnT = 5586 ng/L) material of the clinical hs-cTnT assay both revealed a cTnT-peak at 27.5 mL (FIGURE 1 C and D respectively).

A. cTn-T-I-C complex in cTn-negative serum

B. intact cTnT in cTn-negative serum

C. Control TN2 (Roche Diagnostics)

D. Calibrator L 2 (Roche Diagnostics)

FIGURE 1. cTnT-peak profiles obtained by GFC analysis of purified cTn-T-I-C complex (A), purified intact cTnT (B), Roche clinical assay control (C) and calibration (D) material. In grey, retention volumes of the internal serum standards are indicated.

Validation of N-terminal cTnT fragments in AMI patients' sera.

As depicted in FIGURE 2A, the two-peak elution profiles of residual AMI serum samples was validated as previously described [2]. Retention volumes of 27.5 and 45 mL were established, eluting before and after the internal standard serum albumin ($V_R = 32.5$ mL), respectively. Western blot analysis assigned the first cTnT peak (V_R of 27.5 mL) as the 29 kDa (MW_{est})-degradation product of cTnT (FIGURE 2A, right panel). The second peak ($V_R=45$ mL) was allocated to even smaller cTnT degradation products of MW_{est} between 14 and 18 kDa.

FIGURE 2. GFC profile of a representative AMI patient (A), followed by Western blot analysis using the Roche cTnT antibodies (B), 1C11 antibodies for the detection of the middle region of cTnT (C) and an antibody directed against the N-terminal end of cTnT (D). P, indicates positive control of intact cTnT spiked GFC buffer; M, marker and N, negative control.

Furthermore, direct immunoprecipitation of residual AMI sera followed by Western blotting using the clinical cTnT antibodies assay (Roche M7 antibody) validated the presence of these 29 kDa and <18kDa cTnT-fragments that were also observed in the first and second GFC peak respectively (SUPPLEMENTAL FIGURE 1, left panel). However, none of these fragments demonstrated immunoreactivity with the antibody directed against the N-terminal end of cTnT (SUPPLEMENTAL FIGURE 1, right panel).

SUPPLEMENTAL FIGURE 1. Western blot analysis of AMI patients' serum samples using either Roche M7 (left) or the N-terminal cTnT (right) detection antibody. 1 – 3, indicates serum samples of 3 AMI patients; P, positive control containing intact cTnT spiked in serum; M, marker and N, negative control.

Presence and stability of N-terminal truncated cTnT fragments in ESRD patients

In the first ESRD population (N=10), median (IQR) hs-cTnT concentrations were 68.1 (52.2-80.4) ng/L and 62.5 (43.5-79.0) ng/L pre- versus post-dialysis, respectively (SUPPLEMENTAL FIGURE 2), demonstrating a minor and non-significant reduction in hs-cTnT concentrations following hemodialysis.

SUPPLEMENTAL FIGURE 2. Hs-cTnT concentrations measured in serum of ESRD patients (n=10) before and after hemodialysis

GFC-elution profiles of these serum samples indicated a reproducible peak at adjusted V_R of 45 mL before and after conventional hemodialysis (*Figure 3A and B*). Also in the GFC analysis, a non-significant reduction in cTnT-peak was observed post-hemodialysis.

In the second ESRD population (N=6), pre-dialysis samples at baseline (median (IQR) hs-cTnT concentrations of 151.8(134.7-159.6) ng/L) and 2 months later (median (IQR) hs-cTnT concentrations of 131.8 (115.7-158.2) ng/L) were analyzed (SUPPLEMENTAL FIGURE 3). Also here, GFC profiles could only distinguish a reproducible peak at 45 mL for both time-points (FIGURE 3C and D).

SUPPLEMENTAL FIGURE 3. Hs-cTnT concentrations measured in pre-hemodialysis serum samples of ESRD patients (n=6) at baseline and 2 months later.

FIGURE 3. GFC analysis for the different cTnT forms present in ESRD serum samples obtained either pre- and post-hemodialysis (A) or at baseline and 2 months later (B).

Discussion

In the present study, we clearly demonstrate that chronic cTnT elevations measured in ESRD patients' sera, only reflect degraded cTnT forms. Moreover, these cTnT-fragments were observed before and after hemodialysis, and were found to be stable over time.

By means of GFC analysis, we were able to distinguish ternary cTnT-I-C complexes from intact and degraded cTnT forms. Moreover, our technique could differentiate the 29 kDa cTnT fragment from low-molecular weight (<18 kDa) cTnT fragments. Only intact cTnT and the 29kDa cTnT form could not be completely separated, consequently requiring Western blotting analysis for further differentiation. Also, we confirmed earlier findings of a two peak cTnT profile following AMI, containing 29 kDa and <18 kDa cTnT fragments [2].

In cases of ESRD, we repeatedly distinguished one cTnT-peak at 45 mL, corresponding to the second peak in AMI patients, that was allocated to small N-terminal truncated cTnT forms. These results therefore indicate that cTnT elevations merely reflect completely degraded cTnT fragments in cases of ESRD as previously suggested [11] and not intact cTnT as also assumed [12]. The truncation of the N-terminal end of cTnT has been described to occur in the intracellular environment of cardiomyocytes [14-16]. However, whether observed cTnT fragments presented in this study are formed intracellularly or extracellularly goes beyond the purpose of this study and merits further research. Furthermore, whether these low-molecular weight cTnT fragments still contain their C-terminus has yet to be explored.

In a second ESRD cohort, similar cTnT fragments were found in serum samples drawn 2 months apart [4]. Unlike the time-dependent cTnT degradation pattern following AMI [2, 17], cTnT fragments in ESRD patients' sera therefore seem more stable in time. Moreover, the composition of cTnT forms clearly differs between AMI and ESRD patients, mainly during the time frame of clinical diagnosis of AMI (<12 hours) [2]. Consequently, it would be interesting to assess whether future assays, focusing only on the 27.5 ml peak, would increase the diagnostic performance of troponin assays in predicting acute coronary syndromes in patients with ESRD, which currently relies on serial hs-cTn measurements [18, 19].

Low-molecular weight cTnT forms were found predialysis and immediately after hemodialysis. A minor but non-significant decrease following hemodialysis in the cTnT GFC-peak was distinguished. It is therefore expected that these low-molecular weight cTnT-fragments are in all probability cleared by the kidneys or during renal replacement therapy.

Monitoring the calibration and precision of the assay is necessary to ensure quality hs-cTnT measurements. As shown in the present study, the materials used for this purpose seem to contain only intact cTnT molecules. Therefore, these quality controls are clearly not representative for ESRD and AMI patient's serum. However, the magnitude of hs-cTnT measurements has still been described to be of prognostic value in ESRD [8, 9] as well as other populations [20-22]. It is therefore of utmost importance to investigate whether immunoreactivity changes for different cTnT forms and subsequently the detection of a mixture of cTnT forms could have implications for the clinical assay [19, 23].

In conclusion, this is to the first study to conclusively reveal that low-molecular weight cTnT fragments (<18 kDa) are present in the serum of ESRD patients. Importantly, this is different from cTnT-forms are recognized by the clinical assay in the acute phase of AMI and in quality control materials.

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GENERAL DISCUSSION

There is a broad consensus regarding the use of cardiac troponins (cTn), either cTnT or cTnI, as the preferred biomarkers to identify acute myocardial infarction (AMI) [1]. Major analytical improvements to the cTn assays have resulted in more accurate and very sensitive information, giving rise to new opportunities. When measuring with high-sensitivity (hs-) assays, elevated hs-cTn concentrations were not solely detected in the context of AMI, but also in a whole range of other pathologies [2]. Due to this lack in disease-specificity, hs-cTn results can therefore be confusing for clinicians, especially in the low measuring range. In order for patients to fully profit from the benefits that these improved assays have to offer, it is of utmost importance to increase our understanding on how to interpret hs-cTn results.

The trouble with defining 99th percentile upper reference limits

The current definition of an AMI is based on the rise and/or fall in cTn concentrations with at least one value above the 99th percentile upper reference limit (URL) of normal [1]. In order to reliably assess the 99th percentile with a uncertainty of <5%, the guidelines recommend a minimum sample size of 300 healthy subjects [3-5]. Furthermore, the reference population should be screened for the presence of cardiac diseases, ideally using cardiac imaging techniques. Also, due to a lack of harmonisation between cTn assays, 99th percentile concentrations should be established for each assay [6].

Peer-reviewed literature on the real-life determinations of the 99th percentile cutoff has been reviewed in **chapter two**. This chapter illustrates that variable selection criteria are used to define the reference population, and subsequently a wide range of 99th percentile concentrations were found for each assay. Comparing these different reference populations, variations were identified in selection criteria, distribution of age and gender and the number of included individuals. Lack of attention regarding these issues can result in misleading decision cutoffs and can in this way influence clinical practice.

The definition of a healthy reference population

As described in **chapter two**, the concept of “healthy” varied between different publications, ranging from fully based on health questionnaires to extensive

screens using laboratory and imaging tests. Applying more stringent selection criteria can result in a significant reduction in the 99th percentile concentration [7-11]. However, applying rigorous screening tests such as echocardiography to a large number of presumably healthy subjects can be difficult and costly. Therefore, renal dysfunction combined with elevations in natriuretic peptides measurements [8], and left ventricular abnormalities assessed by echocardiography [9, 12] were reported to have the most pronounced effects on the 99th percentile URL. Importantly, in **chapter three** it has been emphasized that even decreases within a normal kidney function can affect hs-cTn concentrations. As to date, no clear definition on what constitutes a healthy reference population for the determination of hs-cTn cutoffs has been described [13].

The influence of age, gender and race

Numerous publications, as illustrated in **chapter two**, include variable age and gender distributions within their reference cohort. Previous studies have demonstrated that males have significantly higher hs-cTn concentrations than females [8-12, 14], which is also displayed in **chapter three**. The rationale behind this could be that males have more heart tissue than females [15]. In **chapter four**, we showed that elevated hs-cTn concentrations are commonly found in the elderly even when contributing comorbidities were excluded. These findings extend recent literature on the significant influence of age on hs-cTn concentrations [9, 16, 17] and further underline the need for gender and age-specific cutoffs in clinical practice as recently reviewed [18]. Although, large clinical trials investigating the diagnostic benefits of using these specific cutoffs are still lacking [19].

How many healthy individuals should be included?

When establishing the 99th percentile concentrations, it appeared that many studies include less than 300 subjects (**chapter two**). Noticeably, in these small reference cohorts, subjects were more likely to be highly screened for the absence of cardiovascular diseases, whereas large reference populations were less rigorously screened (by means of health questionnaires). However, to ensure an accurate cutoff, enough patients should be included as the 99th

percentile concentration is extremely sensitive to outliers [5]. Importantly, when identifying gender and age specific cutoffs, at least 300 subjects should also be included in each subcategory [15].

Association of renal clearance and high-sensitivity cardiac troponins

One of the most common pathologies, besides AMI, for elevated cTn concentrations includes chronic kidney disease (CKD) and end-stage renal disease (ESRD) [2, 20]. In contrast to AMI, ESRD patients exhibit elevated hs-cTn levels that are more stable over time [21, 22]. The explanation of these chronic cTn elevations is still incompletely understood, and subclinical cardiac diseases are the most obvious explanation. However, a recent study including patients with renal dysfunction but without acute coronary syndromes, hs-cTn concentrations were still elevated and highly associated to renal clearance [23]. This renal influence also becomes evident in the study described in **chapter five**, where the effect of hemodialysis and the more promising hemodiafiltration treatments on hs-cTn concentrations are investigated. In this chapter a significant reduction in hs-cTnT as well as hs-cTnI concentrations following hemodiafiltration is described, as measured in serum samples drawn before and after hemodiafiltration. When patients received conventional hemodialysis, however no significant reduction in both hs-cTn concentrations could be established. This difference can be attributed to the presence of convection in hemodiafiltration and not hemodialysis, which is known to induce more uremic toxin removal from the patients' circulation [24]. Together these data validate the hypothesis that cTn molecules are cleared by the kidneys. In principle, both cTn molecules are small enough to be filtered by the kidneys (37 and 24 kDa, respectively). For final elucidation, hs-cTn concentrations should however be measured in urine or dialysate samples, as reported for NTproBNP [25, 26]. Up till now, this remains difficult due to matrix effects and dilution of already very low serum cTn concentrations by urine or dialysate. Importantly, in mild CKD to ESRD patients, hs-cTn elevations should not be discarded as irrelevant, since they remain highly associated with increased risk of death and cardiovascular disease, even when adjusted for renal dysfunction [6, 27, 28].

As to date, the magnitude of the impact of decreased renal function on hs-cTn concentrations is unknown. In **chapter three** we increased our understanding on the cardiac and renal contribution to higher hs-cTnT and hs-cTnI concentrations in patients with chest pain, without any signs of renal disease. Herein, a significant inverse association between eGFR and hs-cTn was shown, which has also been established in other study populations [27, 29, 30]. Moreover, in **chapter three**, we revealed that the association with renal function was independent and stronger than with any measured CT parameters, such as CAD severity and coronary calcium score. Similar analyses were performed in an elderly population of nursing home residents (**chapter four**), with prevalent heart failure (~40%) and renal dysfunction (~54%). Also here, hs-cTn concentrations were highly associated with renal dysfunction even beyond the presence of heart failure. Together, these studies demonstrate that hs-cTn concentrations, especially hs-cTnT, reflect both renal and cardiac disease and subsequently both pathologies can greatly affect the 99th percentile URL. Furthermore, these findings could offer an explanation why diagnostic cutoffs established in cardiac and renal healthy subjects have a reduced diagnostic performance in patients with renal dysfunction [31]. Therefore, further research should focus on the influence of renal dysfunction on cTn cutoff values and investigate whether adjusting cTn cutoffs for renal function is of clinical value or baseline hs-cTn results should be accompanied with renal function assessment.

Proteolysis of cardiac troponin T

Injury to the myocardium can result in the activation and release of proteolytic enzymes. For cTnI it is generally acknowledged that proteolysis following myocardial infarction results in the presence of different cTnI fragments in human serum [32-34]. This diversity in molecular forms of cTnI had serious implications for the harmonisation between different cTnI assays [34, 35]. Since all cTnI assays employed different antibodies that recognize different cTnI forms, up to 10-fold variation between cTnI-results assays could be observed. For this reason it is necessary that antibodies should recognize epitopes in the most stable parts of cTnI [34, 35], resulting in considerably less variations in cTnI nowadays.

In contrast to cTnI, degradation of cTnT upon myocardial infarction remained controversial. By means of Western blotting, but employing other antibodies as the clinical cTnT assay, the presence of degraded cTnT forms has been shown in serum of AMI patients [36, 37]. Also, in serum of ESRD patients, only cTnT fragments were found [23]. However, these results were disputed in studies using gel filtration chromatography (GFC), who found cTnT in serum of AMI [38] and ESRD patients [39] to be mainly in the intact or complexed form with cTnI or TnC. Therefore, a combination of GFC and Western blotting, using the same antibodies as the clinical assay, was utilized in **chapter six** and **chapter seven** to finally elucidate the cTnT forms that circulate in AMI and ESRD patients' sera, respectively.

Time-dependent degradation of cTnT

In **chapter six** we demonstrate that a heterogeneous mixture of cTn fragments was found in the blood of AMI patients and these fragments are identified with the clinical cTnT assay antibodies. It was shown that not intact cTnT, but the N-terminal truncated form of cTnT, corresponding to a molecular weight of ~29 kDa, was most abundantly present during the critical hours of diagnostic testing (<8 hours). Thereafter, smaller fragments ranging in size from 14-18 kDa appeared to circulate in AMI patients' sera, validating the time-dependent degradation pattern that was previously described [36].

In **chapter seven**, we found in ESRD patients with chronic cTnT elevations that exclusively low-molecular weight fragments (<18 kDa) were present in the circulation. The same fragments were found for all ESRD patients (total of 17 patients) in samples drawn at different timepoints (at 2 months of follow up and pre- versus post-dialysis). It should be noted that these ESRD patients were treated with conventional hemodialysis, which was shown in **chapter five** to not significantly influence hs-cTn concentrations pre- versus post dialysis. In contrast, a significant intradialytic decrease in hs-cTnT concentrations was shown following hemodiafiltration (**chapter five**). It would therefore be interesting to also examine cTnT-fragments that are present pre- versus post-hemodiafiltration.

Implications for the clinical assay results

Since, Roche Diagnostics is the only manufacturer of clinical cTnT assays, cTnT degradation does not affect harmonization between different assays. To ensure quality of the cTnT measurement in blood samples, the manufacturer recommends the use of recombinant human cTnT spiked in a human serum matrix for monitoring the calibration and precision of the assay. However, as shown in this thesis, recombinant intact cTnT is not representative for AMI and ESRD patients' serum, which was clearly shown to mainly contain cTnT fragments.

It is currently unknown whether immunoreactivity of the assay is equal for different cTnT forms. It seems very likely that the assay response to the various troponin forms might differ and, consequently, will affect the performance of serial measurements [40]. Whether the time-dependent degradation pattern influences the diagnostic performance of cTnT, still needs further clinical evaluation [41].

Nevertheless, in this thesis it was clearly shown that the most prevalent molecular forms differed between AMI (~29 kDa) and ESRD (<18 kDa) patients. These findings have led to the hypothesis that the cTnT assay could be improved by recognizing specific molecular cTnT forms, and thereby make a better differentiation between acute and chronic cTnT elevations.

Intracellular or extracellular degradation of cTnT

It still remains to be elucidated whether the observed degradation of cTnT occurs intracellular or extracellular of ischemic cardiomyocytes. Several *in vitro* studies have demonstrated that the degradation of cTnT occurred upon ischemia, due to the activity of either calpain-I [42-44] and caspase-3 [45] in the intracellular environment of cultured cardiomyocytes. This intracellular proteolytic activity is shown to result in a N-terminal truncated form of cTnT, that does not impair the function of cTnT but reflects an intracellular adaption to stress conditions [43]. In contrast, *in vitro* studies on metabolic deprived cardiomyocytes of primary cell cultures [46] and a contracting cell-line [47] have demonstrated that after cell death mostly intact cTnT and only minor fragments are detected in the medium of the cell culture. Current literature therefore

suggests not only a role for intracellular but also extracellular proteolytic activity. This all leads to the intriguing question of which modification patterns of cTn are present when myocardial necrosis is thought to be absent, for instance after physical activity. Moreover, understanding the mechanisms that result in these post-translational modifications of cTn can give further insights into the release of cTn from cardiomyocytes.

Differences between cTnT and cTnI

Throughout this thesis, hs-cTnT and hs-cTnI were measured simultaneously in different patient cohorts (TABLE 1). Interestingly, in each cohort we observed that median hs-cTnT concentrations were noticeably higher than hs-cTnI concentrations. This was consistent with data published in peer-reviewed articles who also investigated hs-cTnT and hs-cTnI concentrations in ACS and non-ACS patients (TABLE 2). On the other hand, in AMI patients hs-cTnI concentrations were found to be markedly higher than hs-cTnT and were only moderately associated with each other (Pearson $R \sim 0.60$) (TABLE 2) [48-51]. Despite the fact that differences between hs-cTnT and hs-cTnI have been described, no detailed analysis regarding the differences in chronic versus acute diseases is present to date. Moreover, according to the current guidelines, cTnT and cTnI results are considered interchangeably from each other [4].

TABLE 1. Summary of hs-cTnT versus hs-cTnI concentrations measured in different study populations throughout this thesis. *, indicates eGFR was calculated according to the CKD-EPI formula using serum creatinine concentrations

Population	Baseline characteristics			Roche Hs-cTnT assay		Abbott hs-cTnI assay		hs-cTnI T> hs-cTnI, n(%)	Pearson R correlation factor (p-value)
	n	Age (years), mean±SD	Males, n(%)	eGFR* (mL/min/1.73m ²) Median(IQR)	Hs-cTnT (ng/L) Median(IQR)	>99 th centile (14 ng/L), n(%)	Hs-cTnI (ng/L) Median(IQR)	>99 th centile (26.2 ng/L), n(%)	
Stable CAD patients (Chapter three)	1864	55.8±11.0	1043 (56%)	91.7 (80.2-101.1)	7.2 (5.8-9.2)	123 (7%)	2.6 (1.8-4.1)	39 (2%)	0.64 (p<0.001)
All aged nursing home residents (Chapter four)	495	82.4±6.8	178 (36%)	64.8 (49.6-80.0)	20.6 (17.8-30.6)	372 (77%)	6.8 (4.1-12.5)	37 (8%)	0.58 (p<0.001)
Aged nursing home residents with heart failure (Chapter four)	156	84.3±6.2	51 (33%)	54.4 (44.0-71.3)	26.4 (18.7-43.8)	147 (94%)	11.4 (7.2-20.3)	27 (17%)	0.59 (p<0.001)
Hemodialysis patients (pre-dialysis) (Chapter six)	13	53.6±20.4	10 (77%)	-	43.1 (36.1-62.8)	13 (100%)	11.4 (8.2-17.7)	2 (15%)	0.69 (p=0.012)

TABLE 2. Review of peer-reviewed literature in which hs-cTnT and hs-cTnI concentrations were measured within the same study population.

	Reference	Baseline characteristics				Hs-cTnT, ng/L		Hs-cTnI, ng/L		
		Population	n	Age (years)	Males, n(%)	eGFR (mL/min/1.73m ²)	Roche Diagnostics	Abbott Diagnostics	Siemens	Beckman Coulter
Acute hscTn elevations	[51]	AMI ^A	443	71 (59-79)	72.7%	66 (51-83)	65.6 (28.0-173.0)	176.6 (27.9-948.1)	268.2 (42-1587.7)	167.4 (28.1-955.7)
	[52]	STEMI ^B	302	63.4 ± 12.5	75.5%	86.7 ± 29.5	803 ± 1476	-	8457 ± 15418 ^x	-
		NSTEMI ^A	1027	67.5 ± 11.7	70.8%	80.1 ± 31.7	262 ± 578	-	3288 ± 8465 ^x	-
Chronic hscTn elevations	Klinkenberg et al (unpublished)	Type 2 Diabetes	23	62.7±6.6	100%	89.2 (81.0-93.5)	11.8 (9.3-15.2)	3.2 (2.4-4.0)	-	-
	[53]	Stable CAD	24	68 (64-72)	64%	N/A	12.7±2.0	7.8±10.5		
	[22]	Hemodialysis ^C	18	71 (35-84) [#]	96%	-	86.1 † (19.2-227.3)	41.5 † (5.5-160.1)	-	-
	[54]	Chronic Kidney Disease ^D	148	63 (56-71)	68.9	37.3 ±16.7	17.0 (11.2-31.9)	-	6.3 (3.4-14.4)	-

Values are presented as median (interquartile range) or mean ± SD. *, indicates range; #, Median (range); †, Mean (range); N/A, not available; ^AAMI was diagnosed when there was evidence of myocardial necrosis (at least one cTn value exceeding the 99th percentile together with a significant rising or falling) in association with a clinical setting consistent with myocardial ischemia; ^BSTEMI was defined by the presence of ECG evidence of ST-segment elevation ≥ 0.1 mV in unipolar leads or elevation ≥ 0.2 mV in anterior wall leads; ^CCKD patients who had been receiving hemodialysis at least twice weekly for at least 2 months; ^DCKD patients not requiring renal replacement therapy and without a history of acute myocardial infarction or coronary artery bypass grafting.

^XcTnI Siemens; LoD: 0.006 µg/L, 99th percentile: 0.04 µg/L, 10% CV 0.03 µg/L; Hs-cTnT (Roche Diagnostics) LoD: 5 ng/L, 99th percentile: 14 ng/L, 10% CV 13 ng/L; Hs-cTnI (Abbott Diagnostics) LoD: 1.9 ng/L, 99th percentile: 26.2 ng/L, 10% CV 4.7 ng/L; Hs-cTnI (Siemens) LoD: 0.5 ng/L, 99th percentile: 9 ng/L, 10% CV 3 ng/L; Hs-cTnI (Beckman Coulter) LoD: 2 ng/L, 99th percentile: 9 ng/L, 10% CV 9 ng/L

Release kinetics of cTnT versus cTnI molecules

The release kinetics of cTnI have been described to deviate from cTnT in both earlier release [52] and decline [53] following myocardial damage. Namely, cTnT exhibits a second peak that has been allocated to a slower dissociation of cTnT molecules due to its structural bound to myofibrils, which is absent for cTnI [53, 54]. Whether also more cTnI molecules are therefore released upon myocardial damage is currently unknown.

In **chapter three** and **chapter four** we observed that hs-cTnT was more strongly associated to renal dysfunction in comparison to hs-cTnI. In line with this finding, excluding patients with renal dysfunction had the most effects on hs-cTnT in comparison to hs-cTnI (**chapter four**). More robust associations of eGFR with hs-cTnT in comparison to hs-cTnI have also been reported by others [8, 55] and generally there are more elevations reported for cTnT than cTnI in ESRD patients [21, 56]. Together, these findings evidence that hs-cTnT results are more susceptible to changes in renal function than hs-cTnI results. To our opinion, the second peak in the release curve of cTnT might therefore be attributed to the accumulation of cTnT molecules following a slower clearance from the system in comparison to cTnI.

Additionally, in **chapter five**, it was found that the influence of hemodialysis differed between hs-cTnT and hs-cTnI concentrations. Although, it remains difficult to separate the effects of release and clearance on higher hs-cTn concentrations, hemodiafiltration was shown to have more effect on hs-cTnT in comparison to hs-cTnI. Structural differences between cTnT and cTnI can contribute to these dissimilarities as the molecular size of cTnI (24 kDa) is smaller than cTnT (37 kDa). Additionally, it is known that both proteins are degraded in the patients serum, resulting in a heterogeneous mixture of cTnT (<29 kDa, **chapter six** and **chapter seven**) and cTnI (<20 kDa; [32, 34]) molecules. Due to these structural differences, release from damaged myocardium [53] and transfer of cTnT and cTnI across the viable cell membrane may not be equal.

Prognostic value of hs-cTnT versus hs-cTnI concentrations

Several large clinical trials have demonstrated that low hs-cTnT and hs-cTnI concentrations, even below the diagnostic cutoff, are associated with a higher risk on developing cardiovascular events and all-cause mortality, and even on top of traditional risk factors [30, 55, 57]. In **chapter four**, hs-cTnT was found to be a superior predictor for all-cause mortality in comparison to hs-cTnI. These findings have also been observed in the APACE trial, enrolling patients at the emergency department with symptoms suggestive for AMI [58]. In these patients, risk on mortality was more accurately predicted by hs-cTnT than by hs-cTnI. Similarly, in other populations such as ESRD [6], heart failure [59] and ACS [60] patients, hs-cTnT was found to be a better predictor for all-cause mortality than cTnI assays. In line with these results, Omland and colleagues found that hs-cTnT and hs-cTnI can hold independent prognostic information to each other [55], questioning whether cTnT and cTnI are truly interchangeable risk predictors. The reasoning behind these observed differences is still speculative and could involve the stronger association of hs-cTnT with renal disease. Remarkably, for the prediction of cardiovascular disease these differences were less apparent than for all-cause mortality, as demonstrated in **chapter three** [58]. More studies involving both hs-cTnT as hs-cTnI assays should therefore be performed for final elucidation of these differences.

Cardiospecificity of cTnT versus cTnI proteins

Since the discovery of cTn proteins as cardiac biomarkers, it has been shown that the cardiac isoform of cTn is specifically expressed in cardiac muscle [61, 62]. However, the cardiospecificity of cTnT, but not cTnI, has been questioned especially in patients with neuromuscular diseases [63]. In these patients, cTnT results were elevated whereas normal (<URL) cTnI concentrations were detected. In these studies, the rationale for this phenomenon was the re-expression of the cardiac cTnT isoform in the skeletal muscles, resulting in false-positive cTnT elevations [64, 65]. In this thesis, we demonstrate that hs-cTnT results are in general higher than hs-cTnI results. Moreover, elevated cTn concentrations are defined by the 99th percentile upper reference limit, which is

highly dependent on the reference population and mostly reported to be lower for cTnT as for cTnI [15].

Using western blotting it was shown that the Roche capture and detector antibodies are each capable of detecting cTnT in skeletal biopsies of patients with neuromuscular disorders [64]. However, to ensure minimal aspecific binding of the assay, the clinical cTnT assay encompasses the combination of the two antibodies. Due to the a kind collaboration with Roche Diagnostics, we did not found any aspecific binding towards skeletal forms of troponin T using Western blotting incorporating both clinical (capture and detection) antibodies. Also, hs-cTnT and hs-cTnI were shown to correlate equally as well to cardiac diseases such as AMI and heart failure. Therefore, no hard evidence is currently given to question the cardiospecificity of the cTnT assay.

Conclusion

It is apparent from data in the present thesis that the 99th percentile is not a perfect diagnostic cutoff and has its limitations concerning the influences of age, gender and renal function of the cardio-healthy reference population. Noteworthy is that hs-cTn concentrations are no stand-alone tests and should always be interpreted in the clinical context together with clinical symptoms and additional diagnostic testing [1].

Although the interpretation of hs-cTn results is complex, in this thesis we observed that both hs-cTnT and hs-cTnI concentrations are still highly associated with cardiac diseases and even very low levels were valuable risk stratifying tools. Our results however also show that next to cardiovascular disease, hs-cTn concentrations, especially hs-cTnT, are highly associated with renal function as well.

Furthermore, we clearly demonstrate that upon its release from the damaged myocardium, cTnT is degraded in a time-dependent manner, ranging from intact to low-molecular weight fragments, which are all recognized by the antibodies of the clinical assay (FIGURE 1). The smallest degradation products also accumulate in the circulation of ESRD patients, and in this form, cTnT molecules are presumably cleared from the circulation (FIGURE 1).

FIGURE 1. Following myocardial damage, cTn molecules are released into the blood stream. In the patients' circulation, degraded cTnT (chapter seven and eight) and cTnI [33] forms have been detected. We hypothesize that these low-molecular weight cTnT and cTnI fragments will eventually be cleared from the circulation via the kidneys. However, these influences are complicated by the fact that release and clearance of cTn are simultaneous processes and cardiac and renal disease can aggravate each other.

Finally, we observed remarkable differences between hs-cTnT and hs-cTnI concentrations in all our clinical populations, seriously questioning whether these results are truly interchangeable from each other. Future work should illustrate in more detail if a combination of both cTnT and cTnI testing can have added diagnostic value in more complex cases.

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SUMMARY

SAMENVATTING

Summary

The diagnosis of acute myocardial infarction (AMI) is based on the release of cardiac troponins (cTn) in the bloodstream. Major analytical improvements to the clinical assays have led to more accurate and very sensitive information regarding cTn. However, the current high-sensitivity tests for cTnT (hs-cTnT) and cTnI (hs-cTnI) also resulted in the detection of elevated concentrations in patients with diseases other than AMI, such as heart failure, and renal dysfunction. This complicates the application of the assay and it is therefore of utmost importance to increase our understanding on how to interpret hs-cTn results, especially in the low-measuring range.

The diagnostic cTn cutoff for myocardial infarction is set at the 99th percentile upper reference limit (URL) of a healthy reference population. An overview of peer-reviewed literature on the real-life determinations of the 99th percentile cutoff is given in [chapter two](#). This chapter illustrates that variable selection criteria are used to define the reference population, and subsequently a wide range of 99th percentile concentrations were found for each assay. Lack of attention regarding these issues can result in misleading decision cutoffs and can in this way influence clinical practice.

The association between kidney function and cTn concentrations has been described, although it is currently unclear whether chronic cTn elevations reflect chronic release due to cardiac damage or they are also the result of a decreased renal clearance. In [chapter three](#), we therefore examined the extent that renal function contributes to higher cTn concentrations in patients with chest discomfort, in whom circulating troponins are mainly attributed to the presence and severity of atherosclerotic plaques or echocardiographic abnormalities. Remarkably, we revealed that the association with renal function was independent and stronger than with any measured CT parameters, such as CAD severity and coronary calcium score. Similarly, in an elderly population of nursing home residents ([chapter four](#)), with prevalent heart failure (~40%) and renal dysfunction (~54%), hs-cTn concentrations, especially hs-cTnT, were highly associated with renal dysfunction even beyond the presence of heart failure. Together, these studies demonstrate that hs-cTn concentrations are highly and independently associated with both renal as cardiac diseases and

subsequently both pathologies can greatly affect the 99th percentile URL. Consequently, the interpretation of baseline hs-cTn concentrations might be improved when accompanied with the assessment of renal clearance.

End-stage renal disease (ESRD) patients often exhibit elevated cTn levels that are more stable over time. To date, it remains unclear how dialysis affects cTn results, as both intradialytic increases and decreases have been reported.

Chapter five is the first study to reveal the effects of extended hemodialysis and hemodiafiltration on cardiac markers and describes a significant reduction in hs-cTnT as well as hs-cTnI concentrations following hemodiafiltration.

Furthermore, there is evidence that cTn proteins are also subjected to posttranslational modifications, such as degradation. Which circulating cTnT forms are detected by the clinical assay in the blood of AMI and ESRD patients is investigated in **chapter six** and **chapter seven**, respectively. In chapter six, we show that, upon its release from the damaged myocardium, cTnT is degraded in a time-dependent manner, ranging from intact to low-molecular weight fragments. The smallest degradation products also accumulate in the circulation of ESRD patients (chapter seven). In this thesis, it was therefore clearly shown that the most prevalent molecular forms differed between AMI (~29 kDa) and ESRD (<18 kDa) patients. It would therefore be interesting to investigate whether the clinical assay can be improved by detecting specific molecular cTnT forms.

Finally, throughout this thesis, hs-cTnT and hs-cTnI concentrations have been measured in various acute and non-acute settings, and we observed remarkable differences between hs-cTnT and hs-cTnI concentrations in all clinical populations, discussed in **chapter eight**. This seriously questions whether hs-cTnT and hs-cTnI results are truly interchangeable from each other. Future work should illustrate in more detail if a combination of both cTnT and cTnI testing can have added diagnostic value in more complex cases.

Samenvatting

De diagnose van een acuut hartinfarct berust op de vrijzetting van cardiale troponines (cTn) in de bloedbaan. Door de ontwikkeling van meer accurate en gevoeligere cTn testen, zijn er steeds meer mensen waarbij cTn-verhogingen worden gedetecteerd. De huidige hoge gevoeligheidstesten voor cTnT (hs-cTnT) en cTnI (hs-cTnI) tonen aan dat beide testen ook verhoogd zijn bij patiënten met een andere pathologie als een myocardinfarct, zoals bijvoorbeeld bij hartfalen en renale dysfunctie. Dit alles bemoeilijkt het gebruik van de assay en roept vragen op over de interpretatie van cTnT en cTnI resultaten, vooral in het lage meetbereik.

De diagnostische afkapwaarde voor een myocardinfarct wordt gedefinieerd als de 99ste percentiel concentratie, gemeten in een gezonde referentiepopulatie. Een overzicht van de gepubliceerde literatuur over de werkelijke bepaling van deze 99ste percentiel afkapwaarde is weergegeven in hoofdstuk twee. Dit hoofdstuk illustreert dat in elke studie andere selectiecriteria gebruikt werden om de referentiepopulatie te definiëren. Dit heeft als gevolg dat steeds verschillende afkapwaarden gevonden werden voor éénzelfde assay. Gebrek aan aandacht voor deze verschillen kan resulteren in misleidende afkapwaarden en kan op deze manier de klinische diagnose beïnvloeden.

Dat er een associatie is tussen de nierfunctie en cTn-concentraties is reeds beschreven. Toch is momenteel niet geweten of deze chronische verhogingen het gevolg zijn van een ophoping van cTn moleculen door een gedaalde nierklaring of dat het om cardiale schade gaat. In hoofdstuk drie is daarom onderzocht in welke mate nierfunctie bijdraagt tot hogere cTn-concentraties bij patiënten met pijn op de borst. In deze populatie worden gedetecteerde cTn-waarden immers toegeschreven aan de aanwezigheid en ernst van coronaire atherosclerose of echocardiografische afwijkingen. Opmerkelijk is dat in deze studie wordt aangetoond dat de associatie met de nierfunctie onafhankelijk en sterker was dan de associatie met CT-parameters, zoals de ernst van de atherosclerose en een hoge coronaire calcificatie score. In een populatie van fragiele verpleeghuisbewoners (hoofdstuk vier), met een hoge prevalentie van hartfalen (~40%) en nierfunctiestoornissen (~54%), wordt dit ook duidelijk. Hier tonen we aan dat hs-cTn concentraties, met name hs-cTnT, zeer sterk

geassocieerd zijn met een gedaalde nierfunctie, onafhankelijk van de aanwezigheid van hartfalen. Samen laten deze studies zien dat hs-cTn concentraties niet enkel hart- maar ook nieraandoeningen kunnen weerspiegelen en dat vervolgens beide pathologieën een grote invloed hebben op de 99^{ste} percentiel afkapwaarde. Bijgevolg zou de interpretatie van hs-cTn concentraties kunnen worden verbeterd wanneer zij gecombineerd worden met een schatting van de renale klaring.

Patiënten aan dialyse vertonen vaak verhoogde cTn resultaten die meer stabiel zijn over de tijd. Tot op heden blijft onduidelijk hoe dialyse de cTn resultaten kan beïnvloeden, gezien zowel cTn stijgingen als dalingen gemeten worden na dialyse. Hoofdstuk vijf is de eerste studie die de effecten van langere hemodialyse- en hemodiafiltratie-behandeling onderzoekt. Deze studie laat zien dat een significante daling in hs-cTnT en hs-cTnI concentraties aanwezig is na hemodiafiltratie.

Tevens is er bewijs dat cTn eiwitten ook post-translationele modificaties, zoals degradatie, kunnen ondergaan. De verschillende cTnT vormen, in het bloed van hartinfarct en dialyse patiënten, die gedetecteerd kunnen worden door de huidige klinische assay worden onderzocht in respectievelijk hoofdstuk zes en hoofdstuk zeven. In hoofdstuk zes tonen we aan dat na een myocardinfarct, cTnT gedegradieerd voorkomt in het bloed. Deze degradatie gebeurt in een tijdsafhankelijke patroon, waarbij eerst intact en vervolgens laag moleculaire cTnT-fragmenten gedetecteerd worden. In hoofdstuk zeven wordt echter bewezen dat in de bloedcirculatie van dialyse-patiënten enkel deze kleine afbraakproducten aanwezig zijn. In dit proefschrift wordt daarom duidelijk dat de gedetecteerde moleculaire cTnT-vormen gedurende de eerste uren na een hartinfarct (~ 29 kDa) verschillen van dialyse-patiënten (<18 kDa). Het zou daarom zeer interessant zijn om te onderzoeken of de huidige cTnT-test verbeterd kan worden door specifiekere cTnT-vormen te detecteren, zoals het 29-kDa fragment.

Tot slot werd in dit proefschrift hs-cTnT en hs-cTnI gemeten in diverse populaties met acute en niet-acute pathologieën. Hierbij zagen we opmerkelijke verschillen tussen hs-cTnT en hs-cTnI, zoals bediscussieerd in hoofdstuk acht. Deze bevindingen stellen daarom in vraag of de resultaten van de cTnT en cTnI

assays daadwerkelijk uitwisselbaar zijn van elkaar. Verdere studies moeten in meer detail uitwijzen of een combinatie van beide testen een diagnostische meerwaarde kunnen betekenen in patiënten met meer complexere ziektebeelden.

APPENDIX 1

VALORISATION ABBREVIATIONS

Valorisation

Economic and Social Relevance

Cardiovascular diseases (CVD) are a major cause of death with a mortality rate of approximately 30% across Europe and America [1, 2]. CVD covers a wide range of disorders, including diseases of the cardiac muscle and of the vascular system. The majority of CVD that lead to mortality are due to acute myocardial infarction (AMI) and stroke. Cardiovascular research has already resulted into more interventions with significant reductions in morbidity and mortality [3, 4]. Regardless of this decline in mortality, more patients are at cardiovascular risk due to increased life expectancy and comorbidities related to an unhealthy lifestyle. Clinical care of these patients is already costly and is expected to further increase in the future. Additionally, treatments may also tremendously impact the quality of life of these patients. Future research should therefore focus on prevention and earlier detection of heart diseases.

Cardiac troponins are an essential component of the diagnostic criteria for myocardial infarction, especially when electrocardiographic results are inconclusive. According to the current guidelines a rise and/or fall in cardiac troponin (cTn) T or I concentrations, with at least one value above the 99th percentile of a healthy reference population, signifies myocardial damage and must be accompanied with also clinical indications such as ischemic symptoms or imaging evidence of myocardial damage [5].

Increasingly sensitive cTn assays enable an earlier diagnosis but severely impair the specificity towards AMI. When measuring with high-sensitivity (hs-) assays, elevated hs-cTn concentrations were also detected in a whole range of other pathologies [6]. Due to this lack in disease-specificity, unravelling the causes of hs-cTn positive results can therefore be challenging, especially in patients affected by more than one disease.

On the contrary, the increases in analytical sensitivity also gave rise to new opportunities for these assays. Even below the diagnostic cutoff, higher hs-cTn values are strongly associated with an increased risk on cardiovascular diseases and mortality, making them promising risk stratifying tools [7, 8].

Target groups

Cardiac troponins are important and highly used cardiac markers and their results are mainly interpreted by clinicians and laboratory specialists. Even before the publication of the current guidelines [5], a European survey demonstrated that 59% of the inquired laboratories were using cTnT or cTnI concentrations to diagnose AMI [9]. Results presented in this thesis can guide clinicians and laboratory specialists to a better understanding of hs-cTn results, by gaining insight into important clinical questions such as changes in renal clearance can affect hs-cTn results. Eventually a better interpretation of hs-cTn results can lead to an earlier diagnosis or rule out of patients. These advancements all have the potential to lead to more efficient, better and cost effective diagnostic process.

Activities and/or products

The results described in this thesis can be used for the development of various products or tools.

In this thesis we show that elevated cTn, especially hs-cTnT, concentrations were detected in elderly people without any evidence of heart and kidney diseases. These results support the concept that also other clinical indicators, besides acute myocardial damage, can greatly influence baseline hs-cTn results. As such, when considering individuals with non-acute symptoms, a young female individual without any cardiac history and a normal renal clearance will have lower baseline cTn concentrations as compared to an older male individual with decreases in renal clearance. For this reason, applying generalized cutoffs can result in under- or over-diagnosis within different patient cohorts [10-12], already implying that it is of great importance to interpret hs-cTn results to a more individual level. The development of a clinical algorithm might therefore be helpful to give direction of which hs-cTn result to expect in a patient, taken into account important factors (such as age, gender,

cardiovascular history, renal clearance, timing of blood collection, precision of the assay, etc.) Current guidelines also indicate the advantage of measuring more than one cTn concentration in time, although it remains unclear which delta to use for a safe rule out of a patient [5, 13-15]. Also here, a clinical algorithm might be helpful for a better indication what constitutes a significantly increased value that requires further clinical examinations.

Although cTn results are currently not used in the clinic to indicate the risk on future cardiovascular diseases and mortality, all current literature undeniably shows that higher troponin concentrations are significantly associated with the incidence of such a risk [16]. It seems therefore quite logic that hs-cTn results should be implemented in clinical risk scores that can be used in patients at risk for cardiovascular diseases. Potentially these clinical risk scores can serve guidance for preventive therapies in patients at high risk.

Furthermore, measuring multiple biomarkers have been proposed in the management of acute coronary syndromes. The combination of cTn measurements with more traditional biomarkers (C-reactive proteins, NTproBNP) and newly developed biomarkers (such as copeptin, heart-type fatty acid-binding protein) are widely investigated [17-19]. In this thesis, preliminary data shows that combining hs-cTnT and hs-cTnI assays might also be promising to further enhance the diagnostic potential of cardiac troponins.

We are the first to clearly show that the most prevalent molecular cTnT forms detected by the clinical cTnT assay are fragments. Moreover, different cTnT forms were observed during the early acute cTnT release following AMI (~29 kDa) as compared to chronic elevations seen in ESRD (<18 kDa) patients. New and unpublished results show that larger cTnT forms (≥ 29 kDa) still contain their C-terminal end, while the smaller fragments (<18 kDa) do not. This thesis therefore indicates that targeting the larger cTnT fragments have the potential to enhance the disease-specificity of the clinical assay. Adding a third antibody, directed against the C-terminal region of cTnT, to the current clinical ELISA

method can detect the proportion of these larger cTnT fragments, as illustrated in FIGURE 1. Future research has to indicate whether such a test can be used in addition to the current clinical hs-cTnT test, and might lead to a better differentiation between acute and chronic cTnT elevations.

FIGURE 1. A. Conventional cTnT testing according to the ELISA method of the clinical assay. **B.** Incorporation of a third cTnT antibody directed against the C-terminal end.

Implementation in clinical practice

Cardiac troponins are already incorporated in clinical practice. Results obtained in this thesis show promising new opportunities for this biomarker in the clinic. Over the past years, increasingly more research is performed on the determinants and risk-stratification of hs-cTn results. However, specific algorithms incorporating more clinical indicators for high hs-cTn results are currently not available and still need to be developed. Also, many clinical scores have already been developed for the prediction of adverse events in high-risk

patients with ACS [20, 21], low-risk patients with symptoms of chest pain [22, 23] and even more general populations [24, 25]. However, most of these risk scores do not include hs-cTn results. Up till now, only one risk algorithm incorporates cTn concentrations when determining the risk in patients presenting with chest pain at the emergency department [23], which is still validated in the clinic trials. Whether hs-cTn concentrations can further improve these risk scores, also in more general populations, still merits further research.

Furthermore, the development of a new assay entails careful validation of the quantification, linearity and specificity of the results. To ensure the detection of cardiospecific cTnT isoforms, we propose the inclusion of a third antibody to the current hs-cTnT assay. For this reason, this new method must be developed in close collaboration with the manufacturer of the current hs-cTnT test (Roche Diagnostics).

Once these advancements are developed, the added value should be monitored in prospectively randomized control studies. Eventually, correct implementation of these tools requires that specialists should be educated on how to interpret the results through scientific exposure (peer-reviewed publications or international congresses) and guideline documents that are published by national and international societies in cardiology and biochemistry.

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Abbreviations

AMI	acute myocardial infarction
CAD	coronary artery disease
cTn	cardiac troponin
cTnT	cardiac troponin T
cTnI	cardiac troponin I
TnC	troponin C
hs-cTn	high-sensitivity cardiac troponin
hs-cTnT	high-sensitivity cardiac troponin T
hs-cTnI	high-sensitivity cardiac troponin I
ECG	electrocardiography
STEMI	ST-elevation myocardial infarction
NSTEMI	non-ST elevation myocardial infarction
URL	upper reference limit
CV	coefficient of variation
CKD	chronic kidney disease
ESRD	end-stage renal disease
ACS	acute coronary syndromes
LoB	limit of blank
LoD	limit of detection
RCV	reference change value
CVD	cardiovascular disease
CCTA	cardiac computed tomographic angiography
CCS	coronary calcium score
eGFR	estimated glomerular filtration rate
LVH	left ventricular hypertrophy
LVEF	left ventricular ejection fraction
IQR	interquartile range
ROC	receiver-operating curve
AUC	area-under-the-curve
CI	confidence interval
CABG	coronary artery bypass graft

PCI	percutaneous coronary intervention
NTproBNP	N-terminal fragment of the prohormone brain-type natriuretic peptide
OR	odds ratio
HR	hazard ratio
HD	hemodialysis
HDF	hemodiafiltration
RR	reduction ratio
GFC	gel filtration chromatography
CK	creatinine kinase
LD	lactate dehydrogenase
MW	molecular weight
V0	dead volume
VR	retention volume
Kav	gel-phase distribution coefficient
ED	emergency department
PBS	phosphate buffered saline

APPENDIX 2

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CURRICULUM VITAE

Name	Eline Cardinaels
Date of Birth	June 18th 1986
Place of Birth	Bree, Belgium
Nationality	Belgian

PROFESSIONAL EXPERIENCE

2010-2015 PhD at the department of Clinical Chemistry
Central Diagnostic Laboratory - Cardiovascular Research Institute
Maastricht (CARIM) - Maastricht University Medical Center+ (MUMC+)

2008-2009 Master Internships

- Department of Pathology, Maastricht University Medical Center+ (MUMC+)
Project: Determination of mechanisms leading to trastuzumab resistance in breast tumor patients
- Department of Neurosurgery and Radiology - Ziekenhuis Oost-Limburg (Belgium)
Project: Peroneal Neuropathy after weight loss: histological and ultrasonographic characterization of the n. fibularis communis

EDUCATION

2009-2010	Pharmaceutical Sciences Katholieke Universiteit Leuven (KULeuven), Belgium
2008-2009	Master in Clinical and Molecular Sciences Hasselt University (UHasselt), Belgium (<i>graduated with distinction</i>)
2004-2008	Bachelor in Biomedical Sciences Hasselt University (UHasselt), Belgium
1998-2004	Sciences and Mathematics (8 h/week) Onze-Lieve-Vrouw Lyceum Genk, Belgium

GRANTS

- **Winner of the Noyons research grant 2014** (€15.000)- Spring congress of the *Dutch association of Clinical Chemistry (NVKC) - Veldhoven*
- **Congress Bursary winner 2013** (€300) - Euromedlab congress of the *International Federation of Clinical Chemistry (IFCC) , Milan, Italy*

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