High-sensitivity cardiac troponin assays : laboratory and clinical aspects

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High-sensitivity cardiac troponin assays

Laboratory and clinical aspects

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High-sensitivity cardiac troponin assays

Laboratory and clinical aspects

PROEFSCHRIFT

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aan de Universiteit Maastricht,

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volgens het besluit van het College van Decanen,

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Contents

	page
List of abbreviations	6
Chapter 1 General introduction	9
Chapter 2 A comprehensive review of upper reference limits reported for (high-) sensitivity cardiac troponin assays and the challenges that lie ahead	23
Chapter 3 Reference population and marathon runner sera assessed by high-sensitivity cardiac troponin T and commercial cardiac troponin T and I assays	47
Chapter 4 Cardiac troponin T elevations, using highly sensitive assay, in recreational running depend on running distance	65
Chapter 5 Cystatin C, a marker for renal function after exercise	81
Chapter 6 Haemodialysis patients longitudinally assessed by high-sensitivity cardiac troponin T and commercial cardiac troponin T and cardiac troponin I assays	91
Chapter 7 The extent of coronary atherosclerosis is associated with increasing circulating levels of high-sensitivity cardiac troponin T	105
Chapter 8 High-sensitivity cardiac troponin T: risk stratification tool in patients with stable chest-pain	123
Chapter 9 Time- and temperature-dependent stability of NIST standard reference material 2921 in serum and plasma	137
Chapter 10 Circulating cardiac troponin T is degraded after acute myocardial infarction	149
Chapter 11 General discussion	165
Samenvatting	179
Summary	183
Dankwoord / Acknowledgment	187
Curriculum vitae	190
List of publications	191

List of abbreviations

a.a.r.	amino acid residue
AKI	acute kidney injury
AMI	acute myocardial infarction
ACS	acute coronary syndrome
AUC	area-under-curve determined with ROC analysis
BMI	body mass index
BNP	B-type natriuretic peptide
BP	blood pressure
CABG	coronary artery bypass grafting
CAD	coronary artery disease
CCS	coronary calcium or Agatston score
CCTA	coronary / cardiac computed tomographic angiography
C-G	Cockcroft-Gault equation
CI	confidence interval
CK	creatinine kinase
CKD	chronic kidney disease
CKMB	creatinine kinase muscle-brain type
CLSI	clinical and laboratory standards institute
CT	computed tomography
cTn	cardiac troponin
cTnC	cardiac troponin C (similar to slow skeletal muscle)
cTnI	cardiac troponin I
cTnT	cardiac troponin T
CV	coefficient of variation, SD / mean
CV _I	within subject variation
CV _G	between subjects variation
CV _A	analytical variation
CVD	cardiovascular disease
ECG	electrocardiogram / electrocardiography
EDTA	ethylenediaminetetraacetic acid
eGFR	estimated GFR
ESRD	end-stage renal disease
EQA	external quality assessment
FRS	risk score estimated in the Framingham study that predicts the 10-year risk on cardiovascular disease
GFC	gel filtration chromatography
GFR	glomerular filtration rate
HAPPY	healthy lifestyle program organized by our hospital: www.happyazm.nl
HDL	high-density lipoprotein
HPLC	high performance liquid chromatography

HR	hazard ratio
hs	high-sensitivity (assay)
hsCRP	high sensitivity C-reactive protein
hs-cTn	high-sensitivity cardiac troponin (assay)
IC	cTn complex consisting of cTnI and cTnC
IDI	integrated discrimination improvement
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
II	index of individuality
IT	cTn complex consisting of cTnI and cTnT
IQR	interquartile range
LCMS	liquid chromatography online coupled to mass spectrometry
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LOB	limit of blank
LOD	limit of detection
MCQE	Mayo Clinic Quadratic Equation
MDRD	modification of diet in renal disease equation
MI	myocardial infarction
MW	molecular weight
NACB	National Academy of Clinical Biochemistry
NIST	National Institute of Standards and Technology
NRI	net reclassification improvement
NT-proBNP	N-terminal pro-BNP
PBS	phosphate buffered saline
PBST	PBS with 0.1% Tween-20
PCI	percutaneous coronary intervention
PROCAM	risk score estimated in the Prospective Cardiovascular Münster study that predicts the 10-year risk on AMI
Q4	fourth quartile
RCV	reference change value
ROC	receiver-operating-characteristic curves
SD	standard deviation
SKML	Dutch foundation for quality assessment in clinical laboratories
SPPS	Statistical Package for Social Sciences
SRM	standard reference material
TIC	cTn complex consisting of cTnT, cTnI, and cTnC
URL	upper reference limit
V ₀	void volume

General introduction

Acute coronary syndrome

– Incidence of acute coronary syndromes

A total of 40,868 subjects deceased in 2008 in the Netherlands because of cardiac or vascular disease. This is 30% of all-cause mortality,¹ one of the two leading causes of death, and similar to other developed regions.² The highest cardiac mortality rate accounted for ischemic diseases (28%), like an acute myocardial infarction (AMI), and the relative incidence increased with age.¹ A total of 345,830 subjects with cardiovascular disease were hospitalized in 2008, which is almost twice as much as in 1980. In the meanwhile, the mean length of hospitalization was drastically reduced, as were the number of subjects suffering from AMI that died while being hospitalized. Despite the impressive increase in hospitalizations, this went along with a decreasing trend in the absolute number of subjects that died from cardiovascular disease. In 2008, it was for the first that mortality rates for cardiovascular disease and malignant carcinoma were in the same order.

– Definition of an acute coronary syndrome

An acute coronary syndrome (ACS) starts with the slow development of atherosclerotic plaque in the coronary arteries (coronary artery disease, CAD) that suddenly is characterized by the rupture of the plaque and the subsequent formation of thrombus.^{3,4} In case the vessel becomes occluded, either partially or completely, this results in ischemia in the surrounding cardiac muscle and is diagnosed as an acute myocardial infarction (MI).^{3,5} For a successful survival of patients presenting at the emergency department with such life-threatening signs, it is crucial to recognize this cardiac syndrome from other non-cardiac complications.

The diagnosis for an AMI was recently redefined by international guidelines of clinicians^{4,6} and laboratory scientists^{7,8} in: the detection of a rise and/or fall of preferably

cardiac troponin (cTn) - (either T, cTnT or I, cTnI) - with at least one value exceeding the upper reference limit (99th percentile) measured in a cardio-healthy group together with clinical (history, physical exam) and imaging (electrocardiogram, echo) findings. Blood should be obtained at hospital presentation and 6-9 hours later, as can be interpreted from the peak profile in Figure 1. If previous measurements were not elevated but the suspicion for AMI is high, serial sampling should be continued. Moreover, the upper reference limit should be measured with optimal precision defined by a coefficient of variation (CV) <10%.

Figure 1: Release curve of cardiac troponin T (cTnT) and I (cTnI) after onset of symptoms of an acute myocardial infarction (AMI). In general, cTnT and cTnI concentrations follow a bi- and monophasic release curve, respectively, although the distinction is often not that obvious.

– Treatment of an acute coronary syndrome

Treatment of CAD aims to repair the flow in the coronary arteries and is accomplished by percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG). Details on revascularization are out of the scope of this thesis and are described elsewhere.⁹⁻¹²

Troponin's release in the bloodstream, the hypothesis

– Troponins: contractile regulatory proteins

The contractile apparatus of cardiac and skeletal muscle cells consists of thick and thin filaments organized in sarcomeres that slide along each other upon muscle contraction and relaxation, as shown in Figure 2. Thick filaments are built up from myosin, thin filaments

from a double helix of actin, with tropomyosin wrapped around and at regular sites a troponin complex. The troponin complex is comprised of troponin C (TnC, calcium binding), troponin I (TnI, inhibitory function by blocking the myosin-actin binding site), and troponin T (TnT, location on tropomyosin). Both cTnI and cTnT isoforms are characterized by a cardiac specific N-terminal extension (Swiss-Prot, P19429 and P45379, respectively), while cTnC in cardiac and slow skeletal muscle is similar (Swiss-Prot, P63316). In addition, around 5-10% of the total cTnT and cTnI content is estimated to be present free and unbound in the cytosol, as indicated in Figure 3A, though the procedure of estimation^{13,14} has been criticized.⁶

Figure 2: Structural characteristics of human myocytes with a focus on their contractile apparatus (sarcomeres) and the regulatory function of cTnT, cTnI, and cTnC upon calcium stimulation.

Upon electrical stimulation of the myocytes, a pool of calcium ions is released from mainly the sarcoplasmic reticulum into the cytosol. This change from sub-micromolar to micromolar calcium concentrations results in the adenosine-triphosphate-driven process that is illustrated in Figure 2: calcium binds to cTnC, subsequently there is a conformational change in the cTn complex and the tropomyosin location, resulting in accessible binding sites between actin and myosin, and consequently contraction of the muscles.

The crystal structure of the core domain of the calcium-saturated human cTn complex (Protein Data Bank, 1JE1) demonstrates two distinct subdomains, the regulatory head and the cTn I-T arm, connected via flexible linkers¹⁵ as reviewed before.¹⁶ High resolution data have not been reported for the flexible and cardio-specific N-terminal sites of the cTn and for the complex in the low calcium environment. Nevertheless, experimental hydrogen/deuterium exchange in the cTn complex has added insight in the calcium-dependent intra-cTn switch that regulates muscle contraction.¹⁷

– Irreversible troponin release

Serum concentrations of cTnT and cTnI in AMI patients typically show a main peak within 1 day after onset of symptoms and remain elevated for approximately 1 week,^{13,18,19} as depicted in Figure 1. Katus et al found that AMI patients with permanent coronary occlusion are characterized by only the second cTnT peak, while AMI patients with early reperfusion show the typical biphasic release.¹³ It was hypothesized that the first peak could be the relatively fast release of cytosolic cTn, while the second broader peak or plateau could be the slower dissociation of cTn from the sarcomeres (Figure 3C). It is quite remarkable, though, that this hypothesis is not given for cTnI, for which there is consensus that it is mainly present in blood in the cTnI-cTnC complex.^{19,20}

Apart from this, we previously showed time-dependent degradation of cTnT in 20 AMI patients with no intact cTnT present anymore 12 hours after onset of symptoms.¹⁸ Langendorf-reperfusion studies in rat hearts also described the intracellular cTn degradation upon prolonged ischemia.²¹ This would suggest that the biphasic release kinetics might also be attributed to the different half-lives of the cTnT forms. However, since the antibodies from the Roche immunoassay measurements differed from the Western Blots, these results have been criticized and cTnT degradation has been disputed.^{20,22}

– Reversible troponin release

Measurable cTn concentrations have occasionally been noticed without the clear evidence of AMI, among others in subjects with myocarditis or after prolonged strenuous exercise, as summarized before.^{4,23,24} Since the commercial cTn assays appear to be absolutely specific for myocardial tissue,²⁵⁻²⁷ this raises the possibility that cTn can be released from damaged myocytes but without typical myocardial necrosis (Figure 3B). Proposed mechanisms for this

phenomenon of so called reversibly damaged myocytes are the formation of radicals²⁸ or blebs (bubbles)²⁹ in the plasma membrane, among others,³⁰ possibly via a stretch-related mechanism mediated by integrins.³¹

Exercise-induced cTn elevations remain an active topic of discussion,^{32,33} as regular exercise belongs to a healthy life-style but endurance exercise is now and then frightened by a sudden collapse, often due to CAD, hypertrophic cardiomyopathy, or left ventricular hypertrophy.³⁴⁻³⁶ In prolonged strenuous exercise like marathon running or triathlon, cTnT concentrations are elevated in around half of the subjects but in the absence of clinical symptoms.^{24,37} Comparison of cTnI studies is limited, though, as discussed later on in this chapter. More research is therefore required to further understand the pathophysiology of exercise-induced cTn elevations.

Figure 3: Distribution of cTn in myocytes and the blood circulation **(A)** in healthy condition with cTn present in cytosol and at sarcomeres; **(B)** after reversible cardiac injury with leakage of cytosolic cTn, as might be after early or minor ischemia as seen post exercise; **(C)** after irreversible cardiac injury with break down of the whole myocyte, as seen with necrosis in AMI patients.

The recent introduction of high-sensitivity troponin assays

Improvements have been advocated for the cTn assays for several reasons. As mentioned before, the diagnostic cut-off concentration for cTn is defined at the 99th percentile measured in a population of cardio-healthy individuals.^{4,6-8} However, at the time this universal definition was published (in the year 2007), most cTn immunoassays that were commercially available were unable to detect cTn concentrations in the blood circulation of healthy individuals (Figure 4A, decision limit 'b').⁷ If not, these assays lacked analytical performance to measure the 99th percentile concentration with sufficient precision (CV <10%), as explained in Figure 4B.³⁸ Apart from this, more than 20 cTnI immunoassays are commercially available in contrast to the patented cTnT assay. Comparison of cTnI results among different laboratories remains therefore limited.³⁸⁻⁴⁰

Figure 4: (A) Biomarkers within the blood circulation follow a bell-shaped gaussian distribution within a group of subjects. The diagnostic cut-off concentration for cTn is defined at the 99th percentile as measured in a healthy reference control group, so 1% of these subjects with the highest cTn concentrations are defined unhealthy.^{4,7,8} At the time of definition, cTn concentrations were undetectable in all healthy individuals, as illustrated by decision limit 'b'. Improvements in the lower measuring range have lately resulted in cTn reference concentrations and thus also a true 99th percentile concentration, as indicated by 'a'. However, this improvement in sensitivity=TP/(TP+FN) goes along with a worsening of the specificity=TN/(TN+FP). TP, number of subjects that were truly assigned positive; FN, number of subjects that were falsely assigned negative; TN, number of subjects that were truly assigned negative; FP, number of subjects that were falsely assigned positive; (B) A typical precision profile of an immunoassay. The diagnostic cutoff concentration should be measured with sufficient precision (coefficient of variation, CV=SD/mean, <10%).³⁸

– Specifications of the high-sensitivity troponin assays

Lately, improved sensitivity and accuracy in the lower measuring range have been achieved, resulting in a new generation cTn immunoassays. These so-called high-sensitivity (hs) cTn immunoassays are characterized by measurable cTn concentrations in the blood circulation of healthy individuals. They enable the determination of the true upper reference limit (99th percentile), and, as a result, the decision limit lowers from 'b' to 'a' (Figure 4A). Cardiologists are quite suspicious about the introduction of the hs assays, as they are afraid to become overloaded by an increasing number of AMI patients as well as for insurance and employment issues.^{41,42}

The high-sensitivity assays are characterized by a ratio of the 10% CV cut-off to the 99th percentile upper reference concentration of ≤ 1.0 and detection of cTn concentrations in >50% of healthy individuals.^{43,44} To date, most assays achieve a 10% CV cut-off concentration somewhere between the median and 99th percentile concentration measured in healthy individuals. The research Singulex Erenna assay is the only cTnI assay today that achieves CV <10% across most of the reference distribution.⁴⁵⁻⁴⁷

Apple proposed a scorecard to judge which cTn assays are suitable to use in the clinic and classified them into guideline acceptable, clinically usable, and non-acceptable assays.⁴⁸ Such an objective score system would be of great help to see the wood for the trees, except that his classification system does not include the clinical performance of the assays.⁴⁹

– High-sensitivity troponin assays for diagnostic use

Improved sensitivity of the cTn immunoassays is expected to facilitate rapid decision making and treatment of patients presenting with symptoms of acute chest pain at the emergency department. Recent results indeed showed that for such patients (n = 718) at presentation (<3 hours after onset of symptoms) the diagnostic performance significantly increased from an area under the curve (AUC) of 0.76 (95% CI 0.64-0.88) to 0.92 (95% CI 0.87-0.97) when using the cTnT as compared to the hs-cTnT assay (P=0.01), respectively.⁵⁰ Comparable results have been confirmed by others,⁵¹ also for hs-cTnI assays.⁵²⁻⁵⁴ Remarkably, the clinical performance of the Abbott Architect-cTnI assay, which does not fulfil the precision requirements of the guidelines, was comparable to the hs-cTnT and Siemens Ultra-cTnI assays.⁵⁰ All of them achieved an AUC >0.90, being significantly higher than the current cTnT

assay ($P < 0.05$) with an AUC of 0.71 and 0.85 within 2 and 10 hours after onset of symptoms, respectively.

An increase in sensitivity goes along with a decrease in specificity, as illustrated in Figure 4A by using a lower decision limit 'a' instead of 'b'. In the clinical example above, the sensitivity increased from 83% (95% CI 76-90) to 95% (95% CI 90-98), while the specificity decreased from 93% (95% CI 91-95) to 80% (95% CI 77-83).⁵⁰ Comparable trends were obtained in the other studies.⁵¹⁻⁵⁴ Still, specificity could be further improved by serial sampling.^{55,56} The guidelines estimated that a delta change in cTn concentration of 20% could be of clinical value.^{4,6-8} More research is however required to validate the optimal delta cut-off for diagnosis of AMI.

– High-sensitivity troponin assays for prognostic use

The identification of patients at coronary risk remains an ongoing challenge. Traditional risk factors for cardiac events are among others age, gender, smoking, and the cholesterol concentration. Algorithms of prediction models combine all these factors and estimate the total risk for the occurrence of a cardiac event, like the cardiovascular Munster (PROCAM) risk score that estimates the 10-year risk on AMI and the Framingham risk score that estimates the 10-year risk on cardiovascular disease in the general population.^{57,58} However, these well known models turn out to perform only moderately well in the clinic.

With the development of the hs-cTn assays, new possibilities seem available to screen patients with a potential cardiovascular risk. A first prognostic hs-cTnT study indeed showed a significant association between increased cTnT concentrations and the incidence of cardiovascular events in patients with stable chronic heart failure.⁵⁹ As biomarkers are a relatively easy and low-cost screening method, the use of cardiac biomarkers to identify high risk subjects has been proposed before, like for N-terminal pro-B-type natriuretic peptide⁶⁰ and high sensitivity C-reactive protein.⁶¹ Unfortunately, so far, cardiac markers added only moderately to cardiac risk assessment, even in a multi-marker approach.^{62,63} The next step is now to validate whether hs-cTnT has incremental value on the clinical work-up of mediate or high risk patients.⁶⁴

– Harmonization and standardization of troponin assays

Among the more than 20 cTnI immunoassays available on the market, assay outcome deviates largely, up to 20-fold.³⁸ This is not the case for cTnT, as the cTnT assay is highly patented by the company Roche Diagnostics.^{65,66} Each cTnI assay uses different antibodies, calibrators, and control materials, limiting comparison of laboratory and study results. Especially the N- and C-terminus of cTnI turned out to be instable and most manufacturers nowadays choose for antibodies against epitopes in the stable region between amino acid 30-110.^{67,68}

To validate cTnI assay harmonization, Panteghini emphasized the need for primary and secondary reference material, as depicted in Figure 5.^{67,69} The American Association for Clinical Chemistry in collaboration with the National Institute of Standards and Technology (NIST) formed a cTnI Standardization Committee^{39,70} and, as a result, the NIST standard reference material (SRM) 2921 became recently available as primary reference material.⁷¹ However, previous stability studies of NIST SRM 2921 spiked in pooled heparin plasma showed time-dependent reduction in assay results.⁴⁰ In addition, instability of purified cTnT (Jart Diris, PhD thesis, Maastricht University, 2003) or recombinant cTnI⁷² when spiked in serum and incubated at 37°C was proven with Western Blot detection. We expect that the reduction in assay outcome could be attributable to the susceptibility of cTn to degradation that hampers the antibody recognition, thereby limiting the application of NIST SRM 2921 for harmonization and standardization purposes.

Figure 5: Suggested approach for the standardization of cardiac troponin I (cTnI) measurements. Figure adapted from Panteghini.⁷³

Outline

Both cTnT and cTnI are nowadays the golden standard for the diagnosis of a myocardial infarction. Nevertheless, the diagnosis and risk assessment of coronary syndromes are expected to be further improved with the recent development of high-sensitivity cTn immunoassays. This might already be true for subjects that suffer from only minor coronary artery burden. With use of especially the new hs-cTnT assay, we investigated the distribution of cTn concentrations in healthy subjects, in subjects after prolonged strenuous exercise, in renal disease patients, and in patients with stable chest-pain. Moreover, we expected that complete insight in the structural aspects of cTn in the blood circulation might assist in unraveling the pathophysiology of cTn release, but also in cTnI immunoassay harmonization and standardization, and in the clinical interpretation of assay outcome in general.

Chapter 2 reviews the analytical characteristics of the hs-cTnT and hs-cTnI immunoassays currently available. *Chapter 3* describes our validation of the hs-cTnT assay and the Architect-cTnI assay in a healthy reference population and in marathon runners. *Chapter 4* compares the exercise-induced hs-cTnT elevations in recreational runners among different running distances. *Chapter 5* validates whether renal function after marathon running is reduced, as a possible clarification for the exercise-induced cTn elevations. *Chapter 6* describes the longitudinal distribution of hs-cTnT and Architect-cTnI concentrations in haemodialysis patients suffering from end-stage renal disease. *Chapter 7* investigates the extent of coronary atherosclerosis in stable chest-pain patients in relation to hs-cTnT concentrations in the blood circulation. *Chapter 8* shows with survival analysis the incremental value of hs-cTnT as a risk stratification tool in chest-pain patients visiting the cardiology outpatient department. *Chapter 9* describes the instability of purified cTn complex NIST SRM 2921 when spiked in serum and questions thereby its application for assay harmonization and standardization. *Chapter 10* illustrates the structures and conformation of cTn in the blood circulation of AMI patients. *Chapter 11* discusses the results and conclusions from the studies mentioned above with directions for future research.

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A comprehensive review of upper reference limits reported for (high-)sensitivity cardiac troponin assays and the challenges that lie ahead

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 sex- 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.

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 clinical³ 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 (coefficient of variation, CV) of 10% at the 99th percentile reference limit.⁵ In a recent overview, Apple classified the available assays⁷ 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, the 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 sex 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, sex/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 1, 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 3 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 samples¹⁷ 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 found²² and similar 99th percentile cutoffs were also reported by others, using smaller reference populations,²⁶ comprised of 66% males.¹⁹

Sex related differences in cTnT levels have been documented using the hs-cTnT assay.^{18,21,22,24,25,27} All studies except one²⁷ 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 sex 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 except for one, this cutoff was measured with an imprecision $\leq 10\%$.

ADVIA Centaur TnI-Ultra Assay – Siemens Healthcare Diagnostics

Analytical sensitivity. Four studies with the new generation ADVIA centaur reported a LOD of 6 ng/L,²⁹⁻³² and one study found a slightly higher LOD of 8.5 ng/L.³³ The package insert (Siemens Healthcare Diagnostics, Frimley, Camberley, UK, version 2009) stated that the LOD of the ADVIA Centaur TnI-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,^{31,32,34,35} but higher 10% CV cutoffs ranging from 45 to 67 ng/L were also reported (Table 1B).^{29,30,33,36,37}

A *cardio-healthy reference population* was selected by laboratory tests,^{29,36} sometimes in combination with echocardiography.^{30,34,37,38} Three studies excluded cardiac diseases and their possible risk factors by means of medical history examinations and/or health questionnaires.^{32,33,35} The majority of the studies included an acceptable sample size. Only two studies included less than the recommended 300 subjects^{33,37} from which one study did not meet the minimum inclusion of 120 subjects.³⁷

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^{30,31} but 1.5 to 2 fold higher 99th percentile concentrations have also been reported for lithium heparin plasma and serum samples.^{29,33-36,38} 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.³⁴ 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.³⁷ Even lower concentrations for the 99th percentile (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.³²

Sex dependent differences were also reported, with significantly higher cTnI concentrations in men than in women ($P < 0.05$).^{29,32,36,38} A statistically significant correlation between age and cTnI concentrations was reported ($R\ 0.268$; $P < 0.0001$),^{29,36} and in line with this observation older subjects had higher cTnI levels than the younger subjects ($P \leq 0.0002$).^{34,38} No significant differences were found among multi-ethnic Asian populations.³⁵

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^{30,32,37} 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.³⁹⁻⁴² 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.⁴⁰⁻⁴²

The inclusion criteria of reference populations were not specified in the majority of the publications, including the package insert and the only study including >300 subjects.⁴¹ Others screened either history of cardiac disease, hematochemical alterations and hyperpyrexia⁴² or by means of history check, ECG, treadmill tests, stress echocardiography or angiogram,³⁹ using both sample sizes <200 subjects.

The 99th percentile concentration provided by the manufacturer is 70 ng/L. This value was reproduced by Christenson,⁴¹ but lower cutoff concentrations of 30 ng/L⁴² and 50 ng/L⁴⁰ were also reported with an accompanying 10% CV to 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.⁴¹ 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.⁴³

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.⁴³

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 to 99th percentile ratio of 1.64. No

significant *sex and age related differences* in cTnI levels are currently described for this assay. However, caution is required in interpretation of the limited available evidence.⁴³

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.⁴³

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,³⁷ but other studies reported 10% CV cutoffs of 40 ng/L⁴⁴ and 93 ng/L.³²

The reference populations used by Tate et al and La'ulu et al have already been described above (section on the TnI-Ultra assay).^{32,37} 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.⁴⁵

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.⁴⁵ 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.⁴⁵ Other studies by Tate et al, and La'ulu et al calculated much lower 99th percentile concentrations in populations that were extensively screened,³⁷ or comprised relatively young subjects.³² 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. Consequently, all studies but one⁴⁵ reported a 10% CV to 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.

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. The lowest cTnT concentration which can be measured with a 10% CV was shown to be 60 ng/L according to the package insert (version 2006). In several publications a similar value was mentioned⁴⁶⁻⁴⁸ although higher concentrations measured with a 10% CV were also found: 104,³⁷ 101,³² and 75 ng/L⁴⁹. Also, 20% CVs for these publications were reported to be 43, 34, and 15 ng/L, respectively. Recently, the AccuTnl assay was optimized leading to a lower 10% CV value of 14 ng/L.^{34,50}

As described previously, *the reference populations* consisted mostly of apparently healthy subjects, with no details on how the subjects were screened.^{45,47,48,51-53} In the various studies health questionnaires were applied, either alone,^{32,54} or in combination with ECG and routine blood analysis.^{34,37,49,55,56} 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).⁵⁰

Similar 99th *percentile cutoff* as indicated by the package insert (40 ng/L) were mostly described using a wide variety of reference populations.^{37,45,46,48,52,53,55,56} Lower cutoffs were found when less than 300 subjects were included^{47,51,57} or in a relatively young population.³² On the other hand, higher 99th percentile concentrations (60-80 ng/L) were also measured.^{34,49} A sample matrix effect was observed between serum and plasma,⁴⁹ although others opposed this finding.⁵⁵⁻⁵⁷

Sex-related differences in the 99th percentile cutoffs were reported to be statistically significant in three studies.^{50,52,54} Ethnicity was also reported to affect the 99th percentile cutoff,⁵⁴ although later on this fact was contradicted.⁵²

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 publications report cutoffs that were not in agreement with the manufacturer's statements.

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.^{22,56,58,59} In the remaining publications, the 10% CV values were indicated to be higher, ranging from 40 to 75 ng/L.^{32,37,60,61} Among these studies, two authors described also higher 10% CV values for Access AccuTnl assay and Vitros ECI Troponin I ES Assay.^{32,37}

The screening methods for the *reference populations* were primarily not specified.^{45,58-60} For this assay, only three reference populations were screened by means of medical history check, ECG and blood laboratory tests.^{32,37,56} One study included less than the advised minimum of 300 subjects.³⁷ 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.^{45,58,59,61} Six independent studies, report cutoffs lower than 20 ng/L.^{22,32,37,45,60} 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.⁵⁶

Age and sex related differences in 99th percentile cutoffs were not statistically significant.

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.

Table 1 (next page): Analytical characteristics, 99th percentile cutoffs, and baseline characteristics of the reference study populations of seven cTn assays frequently used in clinical practice. Included are the five high-sensitivity assays: high sensitivity cTnT (hs-cTnT, Roche Diagnostics), ADVIA Centaur Tnl-Ultra (Siemens Healthcare Diagnostics), Stratus CS Acute Care cTnl (Siemens Healthcare Diagnostics), Dimension Vista cTnl (Siemens Healthcare Diagnostics) and Vitros ECI cTnl ES (Ortho Clinical Diagnostics) assays. Also included are two “clinically usable” assays: Access AccuTnl (Beckman Coulter) and Architect cTnl assay (Abbott Diagnostics). Distinction is made between populations with sufficient subjects (n ≥ 300) and smaller populations (n < 300).^{8*}

Table 1. (continued)

Publication	Sample matrix	Platform	LOB (ng/L)	LOD (ng/L)	10% CV (ng/L)	99 th perc. (ng/L)	Ratio 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)													
n ≥300													
Latini et al 2007 ¹⁷	EDTA plasma	Elecsys 2010	-	1	^a	12	-	1061 (-)	-	1. Laboratory tests: NT-proBNP <125 pg/mL	-	-	-
Vasile et al 2010 ²⁰	Serum	Elecsys 170	2	4.5	^b	13.5	< 1.00	2992 (-)	-	-	-	-	-
Giannitsis et al 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	<0.01
Roche Diagnostics 2010	-	-	3	5	13	14	0.92	533 (-)	-	Apparently healthy subjects	-	-	-
Beyrau et al 2009 ¹⁸	-	-	-	2	12	14	0.86	546 (-)	20-71	-	-	-	<0.05 ^d
Saenger et al 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	<0.001
Aw et al 2010 ²³	Serum	Cobas 6000	-	-	11.5	15	0.77	380 (47%)	31-60	Apparently healthy subjects	-	-	-
Mingels et al 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	<0.01
n <300													
Koerbin et al 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	<0.01
Collinson et al 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.

Table 1. (continued)

Publication	Sample matrix	Platform	LOB (ng/L)	LOD (ng/L)	10% CV (ng/L)	99 th perc. (ng/L)	Ratio		Subjects (%male)	Age range (years)	Screening methods/Inclusion criteria	99 th perc. Male (ng/L)	99 th perc. Female (ng/L)	P value
							CV/ 99 th perc.	10% CV/ 99 th perc.						
Chenevier-Gobeaux et al 2011 ²⁶	Li heparin plasma	Elecsys 2010	-	-	9.2	16.9	0.54	0.54	213 (36%)	21-90; 52±15 ^c	1. Medical history check; 2. clinical examination 3. routine blood analysis	-	-	0.0001
Vasatova et al 2010 ³³	Serum	Elecsys 2010	-	3	j	17	-	-	73 (66%)	56 ± 13 ^c	Cardio-healthy blood donors	-	-	-
b. ADVIA Centaur Tnl-Ultra (Siemens Healthcare Diagnostics)														
<i>n ≥300</i>														
La'ulu et al 2010 ³²	Li heparin plasma	ADVIA Centaur	-	6 ^h	39	18	2.17	2.17	400 (40%)	18-65 (30) ^j	1. Medical history examinations to exclude CVD, cardiovascular medications, cancer, diabetes mellitus, hypertension, hypercholesterolemia and hyperlipidaemia.	-	-	0.028
Collinson et al 2009 ³⁰	Serum	ADVIA Centaur	-	6	45	39	3.00	3.00	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	0.75	648 (-)	17-91	Apparently healthy subjects	-	-	-
Keller et al 2009 ³¹	-	-	-	6	30	40	0.75	0.75	5000 (51%)	35-74 55.5 ±10.9 ^c	General population	-	-	n.s.
Shaneshwar et al 2010 ³⁵	Serum	ADVIA Centaur	-	-	36	61	0.59	0.59	442 ^k (53%)	18-73	1. Health questionnaires	-	-	n.s.
Clerico et al 2008 ³⁸	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	<0.0001
Prontera et al 2008 ²⁹	Li heparin Plasma	ADVIA Centaur - ADVIA Centaur CP	-	6	57	72	0.79	0.79	645 (43%)	15-89; 47.4 ±16.2 ^c	1. Clinical Examinations 2. Laboratory tests	12 ^f	8 ^f	<0.0001
Venge et al 2009 ³⁴	Serum	ADVIA Centaur	-	-	30	80	0.38	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 et al 2007 ³⁶	Li heparin plasma	ADVIA Centaur - ADVIA Centaur CP	6	-	67 ^l	87	0.77	0.77	418 (49%)	16-89 50.7 ±16.6 ^c	1. Clinical Examinations 2. Laboratory tests	15±18 ^d	9±14 ^d	<0.0001

Table 1. (continued)

Publication	Sample matrix	Platform	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
n <300													
Tate et al 2008 ³⁷	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 cardiovascular treatment 2. Stress echocardiography 3. Routine Laboratory tests on blood and urine.	22	19	n.s.
	Serum					21	2.14				18	27	n.s.
Van de Kerkhof et al 2008 ³³	Li heparin plasma	ADVIA Centaur	-	8.5	50	60	0.83	221 (50%)	14-86 58 ^l	1. Medical history questionnaires to exclude myocardial disease	-	-	-
n ≥300													
Christenson et al 2004 ⁴¹	Li heparin whole blood	Stratus CS	-	10	60	70	0.86	345 (-)	-	Apparently healthy subjects	-	-	-
n <300													
Di Serio et al 2005 ⁴²	Li heparin whole blood	Stratus CS	-	15	70	30	2.33	105 (59%)	18-77 50 ± 16 ^c	1. Medical history investigations to exclude ACS 2. Hematochemical alterations 3. Exclusion of hyperpyrexia.	-	-	-
Altinier et al 2000 ⁴⁰	Li heparin whole blood	Stratus CS	-	20	60	50	1.20	85 (57%)	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 et al 1999 ³⁹	Li heparin whole blood	Stratus CS	-	10	30 ^m	80 ⁿ	<1.00	161(-)	-	1. Medical history examinations to exclude CVD; 2. ECG; 3. Treadmill tests 4. Stress echocardiography or angiogram.	-	-	-

c. Stratus CS Acute Care cTnI assay (Siemens Healthcare Diagnostics)

Table 1. (continued)

Publication	Sample matrix	Platform	LOB (ng/L)	LOD (ng/L)	10% CV (ng/L)	Ratio		Subjects (%male)	Age range (years)	Screening methods/Inclusion criteria	99 th perc. Male (ng/L)	99 th perc. Female (ng/L)	P value
						10% CV/ 99 th perc.	99 th perc.						

d. Dimension Vista cTnI assay (Siemens Healthcare Diagnostics)

n ≥300													
Arrebola et al 2010 ⁴³	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.
n <300													
Siemens Healthcare Diagnostics 2008	-	Dimension Vista	15	-	<40	45	<0.89	199 (-)	-	Apparently healthy subjects	-	-	-

e. Vitros ECI cTnI ES assay (Ortho Clinical Diagnostics)

n ≥300													
La'lulu et al 2010 ⁴²	Li heparin plasma Serum	Vitros ECI	-	12 ^h	93	18 13	5.17 7.15	400 (40%)	18-65 30 ¹	1. Medical history examinations to exclude CVD, cardiovascular medications, cancer, diabetes mellitus, hypertension, hypercholesterolemia and hyperlipidaemia	17	26	n.s.
Ortho Clinical Diagnostics 2007-2009	Li heparin / EDTA plasma / serum	Vitros ECI	7	12	34	34	1.00	>10 000 (-)	-	-	-	-	-
Apple et al 2007 ⁴⁵	Li heparin plasma Serum	Vitros ECI	-	12 ^h	34 ^h	31 34	1.10 1.00	2000 (75%) 2992 (-)	18-66	Apparently healthy subjects	-	-	-
n <300													
Tate et al 2009 ³⁷	Li heparin plasma Serum	Vitros ECI	-	12 ^h	34	16	2.13	108 (57%)	25-74	1. Medical history examinations to exclude diabetes mellitus, hypertension, CVD, hyperlipidaemia and cardiovascular treatment 2. Stress echocardiography 3. Routine Laboratory tests on blood and urine	15	14	n.s.
Saw et al 2009 ⁴⁴	-	Vitros ECI	-	-	40	39	1.03	311 (-)	20-72	Blood donors	15	15	n.s.
											-	-	-

Table 1. (continued)

Publication	Sample matrix	Platform	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
f. AccuTnl assay (Beckman Coulter)													
n ≥300													
Zaninotto et al 2009 ⁴⁸	serum	Access 2	-	-	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	1.5	-	-	-	-	-	n.s.
James et al 2006 ⁵⁶	EDTA plasma	-	-	-	-	40	-	442 (67%)	-	1. Health questionnaire; 2. Clinical examination; 3. ECG; 4. routine blood analysis	-	-	-
Venge et al 2003 ⁵⁵	EDTA plasma	-	-	-	-	40	-	436 (67%)	-	1. Health questionnaire; 2. Clinical examination; 3. ECG; 4. routine blood analysis	-	-	-
Eggers et al 2009 ⁵⁰	EDTA plasma	Access	-	6	14	28	0.31	1005 (50%)	≥ 70	General population older than 70 years	72	28	0.03
Apple et al 2004 ⁵²	Serum	Access	-	-	-	50	-	374 (51%)	30-84 (49) ⁱ	General population older than 70 years screened for cardiovascular disease, left ventricular hypertrophy, ECG abnormalities, or elevated NT-proBNP	33	25	n.s.
Apple et al 2003 ⁵⁴	Heparin plasma	Access	-	-	-	80	-	696 (45%)	18-84	1. Health questionnaires	100	34	0.034
Apple et al 2007 ⁴⁵	Serum	Access	-	-	-	40	-	2992 (-)	-	Apparently healthy subjects	-	-	-
Venge et al 2009 ³⁴	EDTA plasma	Access	-	-	14	80	0.175	442 (67%)	-	1. Health questionnaire; 2. Clinical examination; 3. ECG; 4. routine blood analysis	-	-	-
n <300													
Eriksson et al 2005 ⁴⁷	Serum	Access	-	-	60	15	4.0	144 (-)	-	Patients without cardiac-related symptoms	-	-	-

Table 1. (continued)

Publication	Sample matrix	Platform	LOB (ng/L)	LOD (ng/L)	10% CV (ng/L)	99 th perc. (ng/L)	Ratio		Subjects (%male)	Age range (years)	Screening methods/Inclusion criteria	99 th perc. Male (ng/L)	99 th perc. Female (ng/L)	P value
							CV/	99 th perc.						
Beyne et al 2004 ³⁷	Li heparin plasma / serum	Access	-	-	-	16	-	-	52 (73%)	60 ± 16	Non-ACS patients	-	-	-
La'ulu et al 2010 ³²	Li heparin plasma	Access 2	-	10 ^h	101	18	5.61	18-65	400 (40%)	30 ⁱ	1. Medical history examinations to exclude CVD, cardiovascular medications, cancer, diabetes mellitus, hypertension, hypercholesterolemia and hyperlipidaemia	33	52	n.s.
	Serum		-			13	7.77	30 ⁱ				-	-	-
Venge et al 2001 ⁵¹	serum	Access	-	-	30	20	1.5	26-73 (41) ⁱ	122 (43%)		Apparently healthy subjects	-	-	-
Tate et al 2008 ³⁷	Li heparin plasma	Access2	-	-	104	30	3.47	25-74	105 (57%)		1. Medical history examinations to exclude diabetes mellitus, hypertension, CVD, hyperlipidaemia and cardiovascular treatment 2. Stress echocardiography 3. Routine Laboratory tests on blood and urine	40	30	n.s.
	Serum		-			40	2.6					30	30	n.s.
Le Moal et al 2007 ⁵³	EDTA plasma	-	-	-	-	40	-	18-60 (42±10) ⁿ	130 (57%)		Healthy donors with no history of heart disease	-	-	-
Uettwiller-Geiger et al 2002 ⁴⁶	Serum?	Access	-	4	60	40	1.5	19-88	254 (35%)		14% non-cardiac related surgery patients; 20% healthy volunteers; 66% outpatients.	-	-	-
Apple et al 2007 ⁴⁵	Heparin plasma	Access	-	-	-	40	-	18-66	254 (75%)		Apparently healthy subjects	-	-	-
Song et al 2008 ⁴⁹	Heparin plasma	Access 2	-	0	75	61	1.23	30-68	259 (51%)		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	0.9					-	-	n.s.
	Heparin plasma	Dxl 800	-	8	58	50	1.16					-	-	n.s.
	serum					63	0.92					-	-	n.s.

g. Architect cTnI assay (Abbott Diagnostics)

n ≥300

Mingels et al 2009 ²²	serum	i2000SR	-	-	32	13	2.46	51 (26-71) ^g	479 (55%)		1. Laboratory tests: Cardiac biomarkers < mean + 3 SD	13	12	n.s.
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Table 1. (continued)

Publication	Sample matrix	Platform	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
	Li Heparin Plasma	i1000SR	-	<10	49	18	2.72				35	17	n.s.
La'ulu et al 2010 ³²	Serum		-			13	3.79	400 (40%)	18-65 30 ¹	1. Medical history examinations to exclude CVD, cardiovascular medications, cancer, diabetes mellitus, hypertension, hypercholesterolemia and hyperlipidaemia	-	-	-
	Li Heparin Plasma	i2000SR	-	<10	76	20	3.8				40	17	n.s.
	Serum		-			16	4.75				-	-	-
Apple et al 2007 ⁴⁵	Serum	i2000 system	-	-	-	25	-	2992 (-)	-	Apparently healthy subjects	-	-	-
Dupuy et al 2009 ⁵⁹	-	-	-	-	32	27	1.19	-	-	-	-	-	-
Abbott Diagnostics (2004)	-	-	-	-	32	28	1.14	449 (50%)	18-63	Apparently healthy subjects	33	13	-
Lam et al 2006 ⁵⁸	Serum plasma	CI8200	-	4	30	30	1	475 (66%)	16-82	Healthy blood donors	-	-	-
			-	4	30	30	1	480 (-)			-	-	-
James et al 2006 ⁵⁶	EDTA plasma	-	-	-	32	38	0.84	442 (67%)	-	1. Health questionnaire; 2. Clinical examination; 3. ECG; 4. routine blood analysis	-	-	-
								<i>n</i> <300					
Apple et al 2007 ⁴⁵	Heparin plasma	i2000 systm	-	-	-	12	-	224 (75%)	18-66	Apparently healthy subjects	-	-	-
Hubi et al ⁶⁰	serum	CI8200	-	4	40	20	2.00	130 (-)	-	1. Exclusion of pregnancy, cardiac, pulmonary, gastrointestinal, endocrinological, renal, hepatic or metabolic disease	-	-	-
	Li heparin plasma		-	-		20	2.75	108 (57%)	25-74	1. Medical history examinations to exclude diabetes mellitus, hypertension, CVD, hyperlipidaemia and cardiovascular treatment 2. Stress echocardiography 3. Routine Laboratory tests on blood and urine	15	21	-
Tate et al 2008 ³⁷	Serum	CI8200	-	-	55	21	2.62				14	18	-
Tovey et al 2010 ⁶¹	-	-	-	-	50	30	1.67	153 (52%)	30-94	1. ECG; 2. Routine laboratory tests	-	-	-

* 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 manufacturer¹, median age; ⁱ, 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.

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 described by the manufacturer, due to variations in sample size, health condition, age, sex 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, suspecting 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 guidelines⁶ However, four studies are eligible, reporting a reference population comprised of more than 300 individuals and screened by means of non-invasive imaging.^{30,34,38,56} It must be noted that in two of these studies the same reference population was used (SWISCH study)^{34,56} Importantly, studies that most extensively screened their reference population, reported lower 99th percentile cutoff concentrations.^{25,30,37,50,55,56} Evidently, in smaller reference populations more stringent screening methods can be performed, improving the composition of the reference population.^{25,27,37,42} This is counterbalanced by the fact that a small sample size makes the 99th percentile cutoff more prone to outliers.

Ideally, the distribution of sex 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^{32,43} or older subjects.^{30,34} Only two publications use a group of reference individuals in the same age category as AMI patients (>45 years).^{30,34} Although, we estimate that collecting a younger reference population should be better for prognostic purposes. The 99th percentile cutoff concentration had a tendency to increase with age.^{22,25,30,34,36} This supports recent ROC analysis from Reiter et al who reported that optimal diagnosis for AMI was achieved at higher cutoff concentrations in elderly as compared to younger subjects (cutoff, 70 years).⁶³ Moreover, sex 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, with mean/median concentrations statistically higher in males.^{21,22,24,25,29,36-38} Also ethnicity was reported to influence the 99th percentile cutoff,⁵⁴ although others contradict this finding.^{19,52} Taken together, these findings add to the existing body of evidence that sex and age influence cTn levels.⁶⁴ Future research should reveal whether sex- 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 an 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 perspectives

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.^{30,34,38,56} 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 sex 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.^{22,25,30,34,36}

There is thus need for a large and highly screened population of cardio-healthy subjects that are preferably sex-and age-matched for AMI patients, although nowadays the value of sex-and age-specific cutoffs remain unclear. 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 sex/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.

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Reference population and marathon runner sera assessed by high-sensitivity cardiac troponin T* and commercial cardiac troponin T and I assays

Background: Endurance exercise can increase cardiac troponin (cTn) concentrations as high as those seen in cases of minor myocardial infarction. The inability of most cTn assays to reliably quantify cTn at very low concentrations complicates a thorough data analysis, and the clinical implications of such increases remain unclear. The application of recently developed high-sensitivity cTn immunoassays may help resolve these problems.

Methods: We evaluated the pre-commercial high-sensitivity cardiac troponin T (hs-cTnT) assay from Roche Diagnostics and the Architect cardiac troponin I (cTnI) assay from Abbott Diagnostics by testing samples from a reference population of 546 individuals and a cohort of 85 marathon runners. We also measured the samples with the current commercial cTnT assay for comparison.

Results: Although the hs-cTnT and Architect cTnI assays were capable of measuring cTn concentrations at low concentrations ($<0.01 \mu\text{g/L}$), only the hs-cTnT assay demonstrated a CV of $<10\%$ at the 99th percentile of the reference population and a near-gaussian distribution of the measurements. After a marathon, 86% of the runners had cTnT concentrations greater than the 99th percentile with the hs-cTnT assay, whereas only 45% of the runners showed increased concentrations with the current cTnT assay. cTn concentrations remained significantly increased the day after the marathon. A multiple regression analysis demonstrated marathon experience and age to be significant predictors of post-marathon cTn concentrations ($P < 0.05$).

Conclusions: The hs-cTnT assay was the only assay tested with a performance capability sufficient to detect cTn concentrations in healthy individuals. The number of runners with increased cTn concentrations after a marathon depends highly on an assay's limit of detection. The assay in this study with the lowest detection limit, the hs-cTnT assay, showed that almost all runners had increased cTn concentrations. The clinical implications of these findings require further investigation.

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* At the time of investigation, the high-sensitivity cardiac troponin T (hs-cTnT) assay was only pre-commercially available.

Regular exercise is part of a healthy lifestyle and aids in the prevention of cardiovascular disease.¹ In endurance exercise such as marathon running, however, physical collapse is frequently observed during and after races, and such collapses are often associated with coronary artery disease or left ventricular hypertrophy.¹⁻³ The risk for such a cardiac event has been suggested to be comparable with that encountered in other daily activities and thus seems relatively low.²⁻⁴ Nevertheless, the concentrations of highly specific cardiac markers such as the cardiac troponins (cTn) are known to increase after prolonged exercise to concentrations similar to those seen after a minor myocardial infarction,⁵⁻⁹ as we have recently reviewed for cardiac troponin T (cTnT).¹⁰ Because the consequences of cTn release are still unclear, the phenomenon of exercise-induced cTn release is an active topic of discussion and requires further study.

The recent development of more sensitive cTnT and cardiac troponin I (cTnI) immunoassays and their evaluation in different clinical settings¹¹⁻¹³ have prompted a redefinition of the diagnosis of acute myocardial infarction (AMI) as follows: an increase and/or decrease in the concentrations of cardiac markers, preferably cTnT or cTnI, should be documented by at least one observation above the 99th percentile value of the reference population, and such results should be accompanied by clinical, electrocardiographic, or imaging findings.^{14,15} Until recently, however, most cTn assays lacked an analytical performance sufficient to detect cTn concentrations in a reference population or to distinguish reference values from the analytical noise. The inadequacy of cTn assays can be attributed either to the limit of detection (LOD) of the cTn assay being higher than reference values or to assay imprecision (coefficient of variation, CV) being >10% at the 99th percentile value of the reference population.¹⁶⁻¹⁸

Because of the wide variation in cTn assays, comparisons of previous studies of exercise-induced cTn release make sense only for studies that have used the same cTn immunoassay. Such comparisons are especially difficult for cTnI studies because, in contrast to the patented cTnT assay, the approximately 20 cTnI immunoassays that have been developed use different antibodies directed against different epitopes.^{14,16,17,19} In addition, the various assays use different calibrator and control materials. We previously demonstrated that the cTnT concentration increases after prolonged exercise by 59% on average.¹⁰ In brief, exercise-induced cTn release is characterized by a peak after the event is finished and a return to baseline concentrations within 1 day.¹⁰ In the presence of clinical symptoms,

exercise-induced cTn release would be indicative of AMI and would require further investigation. The use of recently developed high-sensitivity cTn assays may provide new insights into the exercise-induced release of cTn.

We studied the analytical performance of 2 recently introduced cTn assays, the pre-commercial high-sensitivity cTnT (hs-cTnT) assay from Roche Diagnostics and the Architect cTnI assay from Abbott Diagnostics. cTn concentrations were investigated both in a reference population and in a cohort of marathon runners. We included the current commercially available cTnT assay (fourth generation) in the study for comparison.

Methods

Reference population

The reference population consisted of 546 apparently healthy persons from a health-check program at our hospital (www.happyazm.nl), and all individuals provided informed consent. To rule out individuals with cardiac syndromes, we included individuals in the study only when the following cardiac biomarker concentrations were all available: creatine isoenzyme MB, N-terminal pro-B-type natriuretic peptide, cTnT and cTnI. Consequently, we excluded 45 individuals from the reference population. We also excluded 22 individuals because the concentration of one of these 4 biomarkers exceeded the mean + 3*SDs: creatine kinase isoenzyme MB mass (male cutoff, >10 µg/L; female cutoff, >7.9 µg/L; maximum, 13.37 µg/L) in 10 individuals, N-terminal pro-B-type natriuretic peptide concentration (cutoff, >41 pmol/L; maximum, 166 pmol/L) in 8 individuals, and cTn concentration (cTnT maximum, 0.134 µg/L; cTnI maximum, 0.217 µg/L) in 4 individuals.

Marathon population

Of the 836 runners who participated in the 2007 Maas Marathon (42.2 km), 85 runners were enrolled in the present study. This study was approved by the ethics committee (Maastricht University Medical Center, the Netherlands), and all participants signed informed consent forms. The maximum temperature on the day of the marathon was 23.4°C with a south wind <14 m/s. We collected serum samples 0-2 h before the race, <1 h after the race, and on the day after the race in a subgroup of 23 runners whom we selected for logistical reasons.

Biomarker measurement

The serum samples were clotted, centrifuged, and stored at -80°C until analysis. cTnI was measured with the Architect i2000SR (Abbott Diagnostics), with the LOD of 0.009 µg/L, a CV of ≤10% at 0.032 µg/L, and the 99th percentile cutoff of 0.012 µg/L, as provided by the manufacturer. We measured cTnT on the Elecsys 2010 instrument (Roche Diagnostics) with the current commercially available cTnT immunoassay (fourth generation), with an LOD of <0.01 µg/L, a CV ≤10% at 0.03 µg/L, and a 99th percentile cutoff at <0.01 µg/L. cTnT was also measured with the pre-commercial hs-cTnT assay. Complete validation of the hs-cTnT assay (same lot number) was performed in the research and development department of Roche Diagnostics. Intra-assay CVs were 5.7 and 0.5% at 0.022 and 2.98 µg/L, respectively; inter-assay CVs were 3.0 and 1.4% at 0.021 and 3.03 µg/L, respectively. The linearity of the hs-cTnT assay was evaluated by serial dilution, from 1 part serum plus 9 parts diluent to 9 parts serum plus 1 part diluent (initial cTnT concentration in serum, 9.5 µg/L; Diluent Universal, Roche Diagnostics). The measured cTnT concentrations deviated from the expected concentrations by factors of 0.99 to 1.05. A comparison of the current cTnT assay and the hs-cTnT assay (cTnT up to 8 µg/L, n=160) yielded the following regression equation: $y = 0.996x + 0.003$ µg/L, where x represents results obtained for the current commercial cTnT assay and y represents results for the hs-cTnT assay [$r=0.9926$; 95th percentile of the residual distribution from the median is 0.331 (Passing-Bablok regression analysis)]. We also measured creatine kinase and albumin concentrations with the Synchron LX 20 instrument (Beckman Coulter).

Statistical analysis

Data were analyzed with SPSS, Version 13.0. A nonparametric approach was used to calculate the upper reference limits (97.5th and 99th percentiles), and the Kolmogorov–Smirnov test was used to evaluate whether biomarker data deviated from a gaussian distribution. The LOD was defined by the sum of the mean of 10 measurements of cTn-negative serum + 3 SDs, but, hindsight, more accurate would be to describe this as the LOB. For variables with a gaussian distribution, we used the paired-samples *t*-test to evaluate differences between pre- and post-exercise samples and used the independent-samples *t*-test to test sex differences. Multiple regression analysis was used to evaluate possible associations of sex, age, body mass index, and experience (number of prior completed marathons) with post-marathon cTn concentration. We log-transformed the data with non-

gaussian distributions and analyzed the data as described above if the transformed data approximated a gaussian distribution. For variables with a non-gaussian distribution, we analyzed the original data with the nonparametric Wilcoxon signed rank test and the Mann–Whitney U-test. For statistical calculations, cTnT concentrations less than the LOD were set equal to the LOD. Unless otherwise stated, the threshold for statistical significance was set at a P level of 0.05.

Figure 1: Precision profiles using a panel of serum samples as established for **(A)** the hs-cTnT assay with CV = 10% at 0.009 µg/L and for **(B)** the Architect cTnI assay with CV = 10% at 0.032 µg/L.

Results

We used the NCCLS (now CLSI) EP5 guideline to establish precision profiles for the hs-cTnT and Architect cTnI assays. As shown in Figure 1, the assay profiles produced 10% CV cutoff concentrations at 0.009 and 0.032 µg/L, respectively. We used 10 measurements of cTn-negative serum (mean + 3*SD) to establish LODs for the hs-cTnT and Architect cTnI assays. The LOD was <0.001 µg/L for the hs-cTnT assay and 0.009 µg/L for the Architect cTnI assay.

Figure 2 shows cTn concentrations obtained for the reference population. With the Architect cTnI assay, almost all measurements (97%) were below the LOD. In contrast, the hs-cTnT assay yielded measurable concentrations for most of the samples in the reference population. For the Architect cTnI assay, the 99th percentile value (0.013 µg/L) was less than the 10% CV value. In contrast, the hs-cTnT assay had a CV of <10% at the 99th percentile

Figure 2: cTn concentrations in the reference population (n=479) as measured with the **(A)** hs-cTnT and **(B)** Architect cTnI assay. According to the 4th generation cTnT assay, for all samples the cTn concentration was below LOD (<0.01 µg/L). See also comments in methods section regarding LOD.

Table 1: cTn reference values as measured with the hs-cTnT and Architect cTnI assays.^a

	hs-cTnT assay	Architect cTnI assay
Study population	µg/L	µg/L ^b
Total n=479		
mean	0.004	0.001
median	0.004	<0.001
97.5 th percentile	0.011	0.008
99 th percentile	0.016	0.013
Females n=215		
mean	0.003	0.001
median	0.003	0.000
97.5 th percentile	0.007	0.007
99 th percentile	0.008	0.012
Males n=264		
mean	0.005	0.001
median	0.005	<0.001
97.5 th percentile	0.014	0.008
99 th percentile	0.018	0.013

^a Data for both assays were not normally distributed (P <0.001), including after log transformation. Differences between males and females in mean cTn concentration: hs-cTnT assay, P <0.001; Architect cTnI assay, P 0.788.

^b Ninety-seven percent of the measurements were below the LOD (0.009 µg/L).

value (0.016 µg/L). The current commercially available cTnT assay (fourth generation) produced values that were all below the LOD (<0.01 µg/L). Finally, analysis of the hs-cTnT data revealed cTn reference values that were higher for males than for females (P <0.001; Table 1).

Table 2 summarizes the baseline characteristics of the marathon population (85 runners). These data are comparable with those of the total marathon population of 836 runners (88% men; mean age, 45 years; mean running time, 3.76 h). The participants in our study seem to be highly experienced runners. Fortyfour percent had previously completed 1-10 marathons, and 36% had completed >10 marathons.

Figure 3 shows that only the hs-cTnT and Architect cTnI assays were able to measure prerace cTn concentrations. All prerace concentrations obtained with the current commercially available cTnT assay were below the assay's LOD. Table 3 shows that prerace concentrations obtained with the hs-cTnT assay were within the reference interval (P 0.282). Pre-race concentrations were significantly higher than the reference values (P <0.001) when the Architect cTnI assay was used; however, pre-exercise concentrations obtained with the Architect cTnI assay should be considered with care, because 82% were below the LOD of the assay (<0.009 µg/L).

Immediately after the marathon, all runners in the study showed an approximately 10-fold increase in cTnT and cTnI concentrations in the hs-cTnT and Architect cTnI assays (Table 3). Albumin concentrations increased only slightly after the marathon; hence, we did not correct cTn concentrations for the effect of dehydration. The hs-cTnT assay showed that the

Table 2: Baseline characteristics of the reference and marathon study population.

	age ^a years	weight ^a kg	height ^a m	marathons completed ^b n	running time ^a h
Reference population					
Total (n=479)	51 (26-71)				
Females (n=215)	49 (26-68)				
Males (n=264)	53 (32-70)				
Marathon runners					
Total (n=85)	47 (27-67)	70 (53-89)	1.77 (1.58-1.92)	7 (191)	3.80 (2.90-5.00)
Females (n=15)	46 (27-60)	57 (50-65)	1.66 (1.58-1.75)	5 (57) ^c	4.18 (3.49-5.34)
Males (n=70)	47 (30-68)	73 (61-90)	1.79 (1.68-1.93)	8 (255)	3.71 (2.87-4.69)

^a The data are consistent with a gaussian distribution and are presented as the mean (95% CI).

^b The data did not fit a gaussian distribution and are presented as the median (97.5th percentile).

^c The 92nd percentile is shown because of the small sample size (n=15).

Figure 3: cTn concentrations in the reference and marathon study populations as measured with the current commercially available cTnT assay, the hs-cTnT assay, and the Architect cTnI assay. Boxes represent the interquartile range (IQR), whiskers represent 1.5× the IQR values, and horizontal lines represent medians. Extreme values (o) represent values between 1.5× IQR and 3× IQR; outliers (*) represent values >3× IQR.

Table 3: Measurement statistics for the marathon study population for serum samples taken before, immediately after, and the day after the race.

	cTnT µg/L	hs-cTnT µg/L	Architect cTnI µg/L	CK U/L	albumin g/L
Pre-race					
mean	<LOD	0.004	0.007	141	43.8
median	<LOD	0.004	0.003	117	43.8
97.5 th percentile	<LOD	0.010	0.022	293	48.0
normality test, P value	<0.001	0.200 ^{a,b}	<0.001	0.200 ^{a,b}	0.200 ^b
gender difference, P value	1.000	0.008 ^a	0.021	0.002 ^a	0.136
Post-race 0 h					
mean	0.026	0.042	0.057	508	47.8
median	<LOD	0.033	0.029	378	48
97.5 th percentile	0.119	0.133	0.231	2249	52.7
normality test, P value	<0.001	0.200 ^{a,b}	0.200 ^{a,b}	0.021 ^a	0.200 ^b
pre/post difference, P value	<0.001	<0.001 ^a	<0.001 ^a	<0.001 ^a	<0.001
gender difference, P value	0.411	0.451 ^a	0.156 ^a	0.793 ^a	0.031
Post-race 24 h					
mean	<LOD	0.012	0.031	2183	42.6
median	<LOD	0.010	0.011	1458	42.8
97.5 th percentile	<LOD	0.023 ^c	0.145 ^c	6240 ^c	47.0 ^c
normality test, P value	<0.001	0.200 ^{a,b}	0.200 ^{a,b}	0.200 ^{a,b}	0.200 ^b
pre/post difference, P value	1.000	<0.001 ^a	<0.001 ^a	<0.001 ^a	0.359
gender difference, P value	1.000	0.214 ^a	0.967 ^a	0.596 ^a	0.170

^a Data normalized by log-transformation prior to testing
^b Highest possible P value given by the Kolmogorov-Smirnov test of normality
^c Because of the small sample size (n=23), values for the 96th percentile are shown

cTnT concentration had increased to above the 99th percentile (0.016 µg/L) in almost all of the runners (86%). In contrast, only about half of the runners (45%) were above the 99th-percentile value (0.01 µg/L) when the current commercially available cTnT assay was used. Results obtained with the hs-cTnT assay were highly correlated with values obtained with the current cTnT assay (Spearman rank correlation coefficient 0.955, for values above the 10% CV cutoff concentration; $P < 0.001$). The Architect cTnI assay yielded cTnI concentrations that were increased to greater than the 99th percentile in 81% of the runners. This percentage appeared comparable to that of the hs-cTnT assay; however, the 10% CV cutoff (0.032 µg/L) was exceeded in only 47% of the runners when the Architect cTnI assay was used. For the hs-cTnT assay, nearly all of the post-exercise concentrations (98%) were greater than the 10% CV concentration (0.009 µg/L).

Multiple regression analysis (Table 4) revealed running experience (the number of previously completed marathons) and age to be significant predictors of post-marathon cTn concentration as detected with the hs-cTnT assay (experience, $P 0.005$; age, $P 0.017$) and the Architect cTnI assay (experience, $P 0.001$; age, $P 0.008$). Indeed, a comparison of the 2 outer quartiles of these cTn results showed that the runners with the lowest cTn concentrations had significantly more experience in marathon running than those with the highest cTn concentrations (hs-cTnT assay, $P 0.005$; Architect cTnI assay, $P 0.036$). With respect to age, we found no significant difference between the 2 outer cTn quartiles for either the hs-cTnT assay ($P 0.254$) or the Architect cTnI assay ($P 0.689$). We also noted no significant interaction between experience and age in the regression model (hs-cTnT assay, $P 0.542$; Architect cTnI assay, $P 0.696$). Furthermore, gender and body mass index showed no significant association with post-exercise cTn concentration (Table 4). A regression analysis of the change in cTn concentration (post-exercise concentration minus pre-exercise concentration) showed experience and age to be significant predictors of the change in cTn concentration (Table 5). Finally, in contrast to pre-exercise cTn concentrations, post-exercise concentrations appeared to be higher in women than in men, but this gender difference was not statistically significant (Table 3).

The day after the marathon, cTn concentrations returned to below the LOD when the current commercially available cTnT assay was used (Figure 3). When hs-cTnT and Architect cTnI assays were used, cTn concentrations measured 1 day after the race remained significantly increased compared with pre-race cTn concentrations ($P < 0.001$ for both the hs-

cTnT and Architect cTnI assays). The cTn concentration was still greater than the 99th percentile value in 17% of the runners measured with the hs-cTnT assay and in 43% of the runners measured with the Architect cTnI assay. The selected group of 23 runners who were studied the day after the marathon was compared with the total marathon study population (85 runners). The 2 groups showed no significant differences in pre-race and post-race cTn concentrations ($P > 0.1$) measured with any of the cTn assays used in this study.

Discussion

cTnT and cTnI immunoassays that have lower LODs, such as the hs-cTnT and cTnI- Architect assays, are better able to delineate the upper reference limits for cTn because of analytical improvements made in the lower portion of measurement interval ($< 0.01 \mu\text{g/L}$). The hs-cTnT assay was the only assay tested in this study that achieved sufficient precision,^{14,15} because the 10% CV cutoff concentration ($0.009 \mu\text{g/L}$) was lower than the 99th percentile value of the reference population ($0.016 \mu\text{g/L}$, diagnostic cutoff). The hs-cTnT assay had the lowest LOD in this study and in this respect appears superior to the other cTn assays currently available.²⁰ Our analytical results for the hs-cTnT assay are in agreement with those of Latini et al¹¹ and Kurz et al,²¹ the only other reports to have described the use of this pre-commercial assay. Latini et al obtained an LOD of $0.001 \mu\text{g/L}$, an inter-assay CV of 5% at $0.01 \mu\text{g/L}$, and an inter-assay CV of 8%. In the reference population ($n=1061$, with a concurrent N-terminal pro-B-type natriuretic peptide concentration of $< 125 \text{ ng/L}$), these investigators obtained a 99th percentile cTnT cutoff value of $0.012 \mu\text{g/L}$. We obtained significantly higher cTnT concentrations in males than in females with the hs-cTnT assay, a difference that has not been reported previously.^{19,22} Given that the mean heart size is larger for males than for females,^{23,24} it is reasonable to expect cTn reference values of males and females to differ. More accurate methods are required to study this possible sex difference, because the 10% CV cutoff value of the hs-cTnT assay ($0.009 \mu\text{g/L}$) was higher than the mean and median cTnT concentrations in the reference population studied (males, $0.005 \mu\text{g/L}$; females, $0.003 \mu\text{g/L}$). In addition, it is noteworthy that the 99th percentile value of the reference population is slightly higher with the hs-cTnT assay ($0.016 \mu\text{g/L}$) than with the current commercially available assay ($< 0.01 \mu\text{g/L}$), but measurements with the current assay are not reliable for concentrations $< 0.03 \mu\text{g/L}$ (with CVs $> 10\%$). Recently, it was indeed confirmed that cTnT concentrations with the current assay at $0.03 \mu\text{g/L}$ were approximately 75% higher when

Table 4: Multiple linear regression models for log-transformed cTn concentrations directly after the race as measured with the **(A)** hs-cTnT and **(B)** Architect cTnI assay.**A**

	log(hs-cTnT)	B ^a	SE ^b	P value	power	R ^c
Model 1				0.061	0.469	0.242
	intercept	1.63	0.07	<0.001		
	log(experience)	-0.11	0.06	0.061		
Model 2				0.010	0.792	0.383
	intercept	1.20	0.19	<0.001		
	log(experience)	-0.19	0.06	0.005		
	age	0.01	0.00	0.017		
Model 3				0.025	0.730	0.387
	intercept	1.21	0.19	<0.001		
	log(experience)	-0.19	0.06	0.005		
	age	0.01	0.00	0.021		
	gender	-0.04	0.09	0.657		
Model 4				0.055	0.669	0.387
	intercept	1.30	0.56	0.025		
	log(experience)	-0.19	0.06	0.005		
	age	0.01	0.00	0.024		
	gender	-0.05	0.11	0.641		
	BMI	0.00	0.02	0.871		

B

	log(Architect cTnI)	B ^a	SE ^b	P value	power	R ^c
Model 1				0.023	0.629	0.290
	intercept	1.74	0.10	<0.001		
	log(experience)	-0.20	0.09	0.023		
Model 2				0.002	0.907	0.437
	intercept	1.01	0.28	0.001		
	log(experience)	-0.33	0.09	0.001		
	age	0.02	0.01	0.008		
Model 3				0.006	0.864	0.440
	intercept	0.98	0.29	0.001		
	log(experience)	-0.33	0.10	0.001		
	age	0.02	0.01	0.007		
	gender	0.07	0.15	0.657		
Model 4				0.015	0.816	0.440
	intercept	1.12	0.82	0.178		
	log(experience)	-0.33	0.10	0.001		
	age	0.02	0.01	0.009		
	gender	0.05	0.16	0.746		
	BMI	-0.01	0.03	0.857		

^a unstandardized coefficient in regression equation^b Standard Error^c Pearson's correlation coefficient

Table 5: Multiple linear regression models for log-transformed delta (post- minus pre-race) cTn concentrations as measured with the **(A)** hs-cTnT and **(B)** Architect cTnI assay.**A**

	log(delta hs-cTnT)	B ^a	SE ^b	P value	power	R ^c
Model 1				0.048	0.510	0.254
	intercept	-1.42	0.08	<0.001		
	log(experience)	-0.13	0.07	0.048		
Model 2				0.031	0.656	0.336
	intercept	-1.78	0.22	<0.001		
	log(experience)	-0.19	0.07	0.010		
	age	0.01	0.01	0.081		
Model 3				0.070	0.587	0.340
	intercept	-1.77	0.22	<0.001		
	log(experience)	-0.19	0.07	0.011		
	age	0.01	0.01	0.091		
	gender	-0.05	0.11	0.673		
Model 4				0.133	0.526	0.341
	intercept	-1.64	0.65	0.014		
	log(experience)	-0.19	0.07	0.011		
	age	0.01	0.01	0.103		
	gender	-0.06	0.12	0.636		
	BMI	-0.01	0.03	0.826		

B

	log(delta Architect cTnI)	B ^a	SE ^b	P value	power	R ^c
Model 1				0.081	0.416	0.227
	intercept	-1.38	0.12	<0.001		
	log(experience)	-0.19	0.10	0.081		
Model 2				0.013	0.765	0.376
	intercept	-2.17	0.34	<0.001		
	log(experience)	-0.32	0.12	0.007		
	age	0.02	0.01	0.018		
Model 3				0.024	0.738	0.393
	intercept	-2.24	0.35	<0.001		
	log(experience)	-0.34	0.02	0.005		
	age	0.02	0.01	0.013		
	gender	0.17	0.18	0.356		
Model 4				0.050	0.682	0.395
	intercept	-2.56	1.00	0.014		
	log(experience)	-0.33	0.12	0.006		
	age	0.02	0.01	0.013		
	gender	0.19	0.20	0.331		
	BMI	0.01	0.04	0.734		

^a unstandardized coefficient in regression equation^b Standard Error^c Pearson's correlation coefficient

measured with the hs-cTnT assay.²⁵ With the Architect cTnI assay, we did not obtain sufficient precision in the lower part of the measurement interval (a CV >10% at the 99th percentile value of the reference population). Recently, Tate et al reported a comparison study of 9 cTn assays.²⁶ Individuals were excluded from the reference population in cases of diabetes mellitus, hypertension, cardiac disease, hyperlipidemia, and patients taking cardiac medications. The 99th percentile value in this reference population (n=111) with the Architect cTnI assay was 0.021 µg/L, which is even higher than the cutoff we reported (0.013 µg/L). In addition, Wu et al recently showed that cTnI concentrations assayed in a reference population with a prototype assay based on single-photon fluorescence detection fit a gaussian distribution.^{27,28} The introduction of more sensitive and accurate cTn assays affects the number of AMI patients who are detected.²⁹ Serial cTn testing with the use of high-sensitivity assays will be necessary to determine the clinical significance of cTn concentrations at the lower end of the measurement interval.³⁰

In most studies that have investigated prolonged exercise, cTn concentrations became detectable immediately after exercise.^{10,31,32} Furthermore, pre-exercise concentrations were below the LOD,¹⁶⁻¹⁸ as was seen in the present study for the current commercially available cTnT assay. In addition, assay imprecision was too high to differentiate pre-exercise values from noise,¹⁶⁻¹⁸ an observation that held true for the Architect cTnI assay in our study. The hs-cTnT assay showed increases in cTnT in almost all of the marathon runners (86%). In contrast, a meta-analysis of 26 studies (1120 individuals) that used second- and third-generation cTnT assays showed cTnT increases in only 47% of the individuals.³³ When a third generation cTnT assay was used, about half of the runners (59%) also showed increased cTnT concentrations.¹⁰ It is still questionable whether cardiovascular insufficiency is the underlying mechanism of collapse during or after prolonged exercise. With a fourth-generation cTnT assay, Siegel et al reported that only 18% of collapsed marathon runners (n=99) showed increased cTn concentrations.³⁴

The Architect cTnI assay produced a broader cTn distribution than the hs-cTnT assay, both immediately after the race and a day later. This finding might be explained by the higher imprecision of the Architect cTnI assay. Nevertheless, both the hs-cTnT and Architect cTnI assays showed that cTnT and cTnI concentrations remained significantly increased the day after the marathon. This finding is in contrast with the results obtained with the majority of cTn assays, in which cTn seems to return to baseline concentrations within a day.

The cTn concentrations in runners after a marathon were higher than the cutoff used for diagnosing AMI.^{14,15} The concentration difference was minor, however, and the increases occurred in the absence of any clinical symptoms. Two opposing theories attempt to explain the link between exercise-induced cTn release and (acute) cardiac events.³⁵ First, the reversibility concept proposes that exercise increases the number of radicals and thereby membrane permeability, causing cTn leakage from the cytosolic cellular pool.^{36,37} This release has been suggested to be relatively fast and may correspond to the first cTn peak seen in AMI patients (<1 day).^{31,38,39} Subsequently, however, there would be an influx and efflux of cytoplasmic constituents up to toxic levels. The second theory, the irreversibility concept, suggests that the cTn released after prolonged exercise is due to the breakdown of myocytes. This release would require the dissociation of cTn from the cTn complex (on actin molecules) and is thought to be much slower (>1 day). It therefore could correspond to the later cTn release that is seen as a second peak in AMI patients.^{31,38,39} Whether prolonged exercise has any long-term consequences remains to be clarified. To address this issue, Hessel et al studied cTnI release from cultures of rat cardiomyocytes and demonstrated the release of intact cTnI from viable cardiomyocytes,⁴⁰ which would imply that reversible cell damage must take place after prolonged exercise. Further research is required to reveal whether troponin release after AMI is similar to the release occurring after prolonged exercise, both from a structural and from a kinetic point of view.

In the largest marathon population studied thus far (482 runners), less marathon experience and an older age appeared to be associated with increases in cTn, whereas race duration and the presence of traditional cardiovascular risk factors were not.⁸ Neilan et al used both echocardiography and serum biomarkers to study non-elite marathon runners specifically³² and found that cTnT concentrations were significantly higher in runners who trained ≤ 56 km/week than in runners who trained > 72 km/week. In a meta-analysis of 1120 individuals, Shave et al³³ found exercise duration to affect post-exercise cTn concentration but found the effect of age to be non-significant. When we used more sensitive assays, we also found a significant negative correlation between post-marathon cTn concentration and experience and found a non-significant positive relationship with age.

The clinical impact of exercise-induced increases in cTn concentration has not yet been fully clarified. Herrmann et al advised that until the phenomenon is better understood, affected athletes should undergo further cardiologic investigation, including a stress test.⁴¹

Whyte et al suggested that serial measurements should be made after a marathon to evaluate a patient for an AMI.⁴² In the present study, the use of cTn assays with lower LODs showed 86% of the athletes to have increased cTnT concentrations after a marathon and 81% to have increased cTnI concentrations. There seems to be no rationale for examining all athletes with positive cTn concentrations in the absence of clinical symptoms. Further research is required to investigate whether a diagnostic cTn cutoff higher than the 99th percentile value is more realistic for well-trained athletes.

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Cardiac troponin T elevations, using highly sensitive assay, in recreational running depend on running distance

Background: Endurance exercise is frequently associated with cardiac troponin (cTn) concentrations, otherwise corresponding to minor myocardial infarction. However, research on the underlying mechanisms has been limited because of assay restraints in the low concentration range.

Methods and results: Using the pre-commercial, highly sensitive hs-cTnT assay, cTnT concentrations were measured in samples from recreational runners obtained before and after running 5 km (trained, n=43 / untrained, n=122), 15 km (n=38), 21 km (n=10), and 42 km (n=85) (all trained). The percentage of runners with elevated hs-cTnT concentrations after the run increased with running distance (0, 11, 13, 40, and 86%), in contrast to NT-proBNP (2, 7, 0, 0, and 5%). Median (IQR) hs-cTnT post-run concentrations were 0.004 µg/L (0.003), 0.006 µg/L (0.008), 0.010 µg/L (0.006), 0.014 µg/L (0.019), and 0.030 µg/L (0.029), respectively.

Conclusions: We found, using a novel hs-cTnT assay, the distance of recreational competitive running to be positively related to asymptomatic increases in cTnT post-run concentrations. In contrast, NT-proBNP showed no increase. In addition, the data indicated that a relatively short running distance of 5 km resulted in cTnT release of untrained participants, in contrast to trained participants, which underlines the necessity of sufficient training. Further effort is needed to clarify the significance of exercise induced cardiac biomarker elevations.

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* At the time of investigation, the high-sensitivity cardiac troponin T (hs-cTnT) assay was only pre-commercially available.

Recreational running and other types of regular exercise are performed by millions of people worldwide and the cardiovascular benefits are commonly accepted. However, prolonged strenuous exercise is often associated with cardiac complications ranging from elevations of cardiac biomarkers^{1,2} to infrequent physical collapse or even sudden death.^{3,4} It is for this reason that the American Heart Association and European Society of Cardiology recommend pre-participation screening protocols for young competitive athletes to check for cardiovascular abnormalities.^{5,6} Nevertheless, it remains an active topic of discussion whether the asymptomatic exercise-induced increases of cardiac troponin (cTn) and N-terminal pro-B-type natriuretic peptide (NT-proBNP), the preferred markers for diagnosing acute myocardial infarction and heart failure, respectively, are associated with cardiac cell death.^{7,8}

Research on the underlying mechanisms behind exercise-induced cTn elevations has been limited because of cTn assay restraints.^{9,10} First, comparison of cTnI studies is complicated, as approximately 20 cTnI immunoassays have been developed, each using different antibodies, calibrators and control materials.^{10,11} This is in contrast to the patented cTnT assay, as we previously reviewed.¹² Moreover, until recently, cTn immunoassays were unable to measure detectable concentrations in healthy individuals or assays lacked analytical performance (coefficient of variation, CV >10% at the upper reference limit, URL, of a reference population) to measure these concentrations with sufficient precision.¹⁰ Table 1 summarizes our literature research in subjects after prolonged strenuous exercise when using the contemporary cTnI assays, to further indicate the difficulty in study comparison.

Lately, analytical improvements have been made in the lower part of the cTn measurement interval. When using the pre-commercial highly sensitive hs-cTnT assay, we recently reported an almost Gaussian distribution in a reference population (n=479),¹³ while Kavsak et al¹⁴ showed a similar distribution for hs-cTnI when using the adapted AccuTnI assay from Beckman Coulter. With the new generation hs-cTnT and Architect cTnI assay, we showed that cTn concentrations were elevated after running a marathon race in almost all runners (cTnT, 86%; cTnI, 80%), but in the absence of clinical symptoms.¹³ However, the level of release of cTnT in people running shorter than marathon distances and how this relates to the level of preparedness is unknown.

Table 1: Literature overview of cTnI elevations after prolonged strenuous exercise when using contemporary cTnI assays.

Reference	year	exercise	n	cut-off µg/L	1 st post time hours	elev. part. %	range µg/L	2 nd post time hours	elev. part. %	range µg/L	3 rd post time days	elev. part. %	range µg/L
Access cTnI assay													
Koller et al ¹⁶	1995	marathon	19	0.1	0	5	<0.7				2 d	0	0 g
Herrmann et al ¹⁷	2003	marathon	45	0.04	0	60	0.01-0.36	3	73	0.020-0.93	1 d	11	
Urhausen et al ¹⁸	2005	exercise	21 ^a	0.04	0	95		3	95	<1.725 ^b			
Scharhag et al ²	2005	marathon	46	0.04	0	73 ^c	0.05 ^d	3		0.07 ^d	1 d	7	<0.14 ^e
		ultramarathon	14	0.04	0	57 ^c	0.04 ^d	3		0.05 ^d	1 d	7	<0.14 ^e
		mountainbike 100 km	45	0.04	0	86 ^c	0.09 ^d	3		0.10 ^d	1 d	7	<0.14 ^e
Ashley et al ¹⁹	2006	exercise 300 miles	54	0.01		41	<0.36						
Scharhag et al ²⁰	2006	exercise 1h	20 ^a	0.04	0.5			3	25	0.01-0.06			
		exercise 3h	20 ^a	0.04	0.5			3	25	0.01-0.14			
Tian et al ²¹	2006	half marathon	10	0.1	2	60	0.01-0.27 ^f	4	60	0.01-0.51 ^f	1 d	10	0.01-0.11 ^f
Ortega et al ²²	2006	mountainbike 95 km	11	0.5	0	0 ^g	0.02-0.39						
Stratus cTnI assay													
Bhagat et al ²³	1997	half marathon		1.7	0	0	<0.5-0.9				1 d	0	<0.5-0.9
Davies et al ²⁴	1997	marathon	46					4	0	<0.13	1 d	0	<0.13
Davila et al ²⁵	1997	ultramarathon	14	3.1	0	7	<5.0				0.5 d	0	<1.8
Ver Elst et al ²⁶	1999	exercise	6	0.4			0.015-0.023 ^h	4	0	<0.3	0.5 d	0	0 ^d
Schulz et al ²⁷	2002	bicycle exercise	10	0.1	0	0							
Schulz et al ²⁸	2007	bicycle exercise	10	0.02	0	0	<0.031 ^b						
AxSYM cTnI assay													
Ver Elst et al ²⁶	1999	exercise	6	0.4				4	0	<0.3	0.5 d	0	0 ^b
Cleave et al ²⁹	2001	triathlon	64	2		8	0.0-4.8						
Neumayr et al ³⁰	2001	cycling 230 km	38	0.5	0	34	0.9-4.9				1 d	3	<4.0
Neumayr et al ³¹	2002	cycling 509 km	16	0.5	0	38	0.0-5.1				1 d	0	
La Gerche et al ³²	2004	triathlon	15	0.5							4.7 d	7	<0.3-0.9
La Gerche et al ³³	2007	triathlon	26	0.15	0	58	<0.15-3.5 ^b				1 wk		
Koller et al ³⁴	2007	marathon	13	0.04	0.5	54					0.5 d	70	

^a subjects selected from Scharhag et al² when cTnI concentrations were above 0.04 µg/L; ^b mean ± 3*SD; ^c from Urhausen et al¹⁸; ^d median; ^e out of three events n=14; ^f interpreted from figure; ^g in case 0.04 µg/L is used as cut-off; cTnI is elevated for 36% of the subjects as interpreted from figure; ^h 95% CI

Table 1: (continued)

Reference	year	exercise	n	assay	cut-off µg/L	1 st post time hours	elev. part. %	range µg/L	2 nd post time hours	elev. part. %	range µg/L	3 rd post time days	elev. part. %	range µg/L
Remaining cTnI assays														
Cummins et al ³⁵	1987	marathon	11	In-house made	20	0	9	<10-23	6.5	0	3.0-19.2 ^b	1 d	0	<10 ^b
Adams et al ³⁶	1993	marathon	9	In-house made	3.1							1 d	0	<2.2
Siegel et al ³⁷	1997	marathon	43		2				4	2.3				
					1.5				4	2		1 d	0	
Davies et al ²⁴	1997	marathon	46	BIAcore COBAS	0.2				4	0	<0.13	1 d	0	<0.13
Sorichter et al ³⁸	1997	exercise	30		0.1							4 d	0	<0.1
Koller et al ³⁹	1998	marathon	28	Sanofi Diagnostics	0.1	0	0	<0.1	2	0	<0.1	1 d	0	<0.1
				Pasteur ERIA ⁱ										
Rifai et al ⁴⁰	1999	triathlon	23	Dade Behring	2.0	0	9	<4.44						
				Opus Plus ^j										
Lucia et al ⁴¹	1999	marathon	10	Sanofi Diagnostics	0.1	0	0	<0.1				1 d	0	0.02 ^d
				Pasteur ERIA ⁱ										
Shave et al ⁴²	2001	exercise	8	DPC Immulite ^j	0.1	0	0	<0.02				2 d	0	<0.02
Siegel et al ⁴³	2001	marathon	41	Bayer ACS:180 ^j	0.2				4	5		1 d	8	
		marathon	51	STATus POCT	1.5				4	0	0.072-0.216 ^b	1 d	0	0.031-0.253 ^b
		marathon	11	Triage cardiac panel	0.2				4	0	<0.108 ^b	1 d	0	<0.004 ^b
Apple et al ⁴⁴	2002	marathon	19	Dade Behring Dimension ^l	0.1	0	32	<0.3				1-2 d	5	<0.55
Kratz et al ⁴⁵	2002	marathon	37	Triage cardiac panel	a.c. ^k				4	8	<0.14 ^b	1 d	0	0 ^b
Smith et al ⁴⁶	2004	marathon	34	Triage cardiac panel	0.3	0	0	<0.16 ^b						
Vidotto et al ⁴⁷	2005	half marathon	25	Dade Behring Dimension ^l	0.1	0	12	<0.15 ^b	2	32	0.243 ^b			
Fortescue et al ¹	2007	marathon	482	Bayer ACS Centaur ^l	0.1	0	68	0.05-4.17						

^{a-h} see previous page; ⁱ currently hold by Beckman Coulter; ^j currently hold by Siemens Diagnostics; ^k a.c. according to company

Another evolving cardiac biomarker is NT-proBNP with a release different from cTn, namely a response to increases in ventricular wall stress and the subsequent increase in synthesis of the BNP hormone.¹⁵ Echocardiographic research in recreational marathon runners indeed indicated that exercise affected systolic and diastolic ventricular functions due to mechanical or volume overload.⁴⁸ Nevertheless, exercise-induced NT-proBNP elevations were shown to be correlated only with a (temporary) reduction in left ventricular early diastolic filling.⁴⁹ For NT-proBNP measurements, the four available immunoassays used antibodies from Roche Diagnostics, simplifying study comparison.⁵⁰ Up till now, with the older generation cTn immunoassays, correlations were not found between exercise-induced elevations of cTn and NT-proBNP.^{2,47}

In the present study, we compared cardiac cTnT (using the pre-commercial highly sensitive hs-cTnT immunoassay) and NT-proBNP concentrations in pre- and post-run serum samples of recreational runners participating in different running events (5, 15, 21, and 42 km).

Methods

Study populations

The study population consisted of recreational runners who participated in Maastrichts Mooiste 2007 (distance 5 km, n=43; distance 15 km, n=38) or in the Maas Marathon 2007 (distance 21 km, n=10; distance 42 km, n=85, see also chapter 3). We also included participants of HAPPY Run 2006 (5_{HAPPY}, distance 5.4 km, n=122), who either walked or ran. HAPPY is an activity organized by our hospital to promote healthy lifestyle and physical activity in the general population (www.happyazm.nl). For comparison with other studies, we included runner characteristics in Table 2, as gathered from personal communication prior to the running event. The present study was approved by the ethical committee (Maastricht University Medical Center, the Netherlands) and all participants signed informed consent forms. The maximal temperatures were 23°C, 23°C, and 20°C at Maastrichts Mooiste, the Maas Marathon and HAPPY Run, respectively. The wind was <9 m/s from no specific direction, a south wind of <14 m/s, and a west wind of <7 m/s, respectively. Serum samples were collected 0-2 h before the run and <1 h immediately after the run. After clotting and subsequent centrifugation, serum was stored at -80°C until analysis. Post-run

concentrations were corrected for the effect of dehydration using the ratio of pre- versus post-run albumin serum concentrations.

Biomarkers

cTnT was measured on the Elecsys 2010 (Roche Diagnostics) with the pre-commercial, highly sensitive cTnT (hs-cTnT) assay with LOD at $<0.001 \mu\text{g/L}$, $\text{CV} \leq 10\%$ at $0.009 \mu\text{g/L}$ and the URL (99th percentile) at $0.016 \mu\text{g/L}$, as validated previously in chapter 3. Recently, Giannitsis et al⁵¹ reported 75% higher values for cTnT $<0.1 \mu\text{g/L}$ for the hs-cTnT assay as compared to the fourth generation cTnT assay. Furthermore, intra-assay CVs were 5.7 and 0.5% at 0.022 and $2.98 \mu\text{g/L}$, respectively; inter-assay CVs were 3.0 and 1.4% at 0.021 and $3.03 \mu\text{g/L}$, respectively. NT-proBNP was also measured on the Elecsys 2010 with LOD at 0.6 pmol/L , intra-assay CV 2.5% at 8.98 pmol/L and inter-assay CV 6.8% at 8.78 pmol/L as given by the manufacturer. In our reference population defined in chapter 3, we measured the 99th percentile at 36 pmol/L and the 97.5th percentile at 28 pmol/L . In addition, albumin (reference range $32.0\text{--}47.0 \text{ g/L}$) was measured on the Synchron LX20 (Beckman Coulter).

Statistics

Data were analyzed with SPSS, Version 15.0. The nonparametric Wilcoxon signed-rank test as used for comparison of post-run with pre-run concentrations within each study population, since the biomarker concentrations were not normally distributed. With the Pearson chi-square test, we tested differences in ratio male/female between the groups. Linear regression analysis was used for comparison of the other variables between the

Table 2: Baseline characteristics of the runner study populations.

	5 _{HAPPY} km ^a	5 km	15 km	21 km	42 km	P value
n	122	43	38	10	85	
sex (male/female)	59/63	24/19	31/7	8/2	70/15	<0.001
age (years) ^b	54 (11)	45 (9)	47 (9)	43 (13)	47 (9)	<0.001
BMI (kg/m^2) ^b	n.a. ^c	24.4 (3.5)	23.8 (2.1)	24.0 (1.3)	22.4 (1.7)	<0.001
Experience ^d	n.a.	0.8 (2.6) ^e	10 (31) ^e	n.a.	7 (21) ^f	n.a.
netto run time (min) ^b	n.a.	28 (4)	78 (9)	118 (13)	228 (30)	<0.001
velocity (km/h) ^b	n.a.	10.9 (1.3)	11.6 (1.3)	10.9 (1.1)	11.3 (1.5)	0.181

^a non-competitive event of our hospital (HAPPY); ^b mean (SD); ^c not available; ^d median (IQR); ^e total kilometers trained ($\times 1000$) = $\text{km/week} \times 52 \times \text{years of running}$; ^f number of marathons completed, available for $n=77$

groups. When applied on the hs-cTnT and NT-proBNP concentrations, data were first normalized by log transformation. Regression analysis on the post-run concentrations was corrected for pre-run concentration, age, and sex. Correlations between hs-cTnT and NT-proBNP concentrations were calculated using Spearman's correlation coefficient and tested with the one-sample *t*-test for correlations. Runners from different populations were independent to each other. The threshold for statistical significance was set at $P < 0.05$.

Results

Table 2 summarizes the baseline characteristics of the runner populations in the present study. All individuals were recreational runners participating in a competition, except for the individuals participating in the 2006 HAPPY, an activity of our hospital to promote healthy lifestyle and physical activity, who either walked or ran 5 km (5_{HAPPY}). For the shortest distances, about half of the participants were male, while for the longer distances most participants were male ($P < 0.001$). As shown in Table 2, age was higher in the 5_{HAPPY} km population compared to the other populations ($P \leq 0.002$). BMI was lower in the 42 km population compared to the 5 and 15 km runners ($P \leq 0.003$) and marginally lower compared to the 21 km runners ($P 0.054$). The 5_{HAPPY} km participants had not been stimulated in training, while the 5 km runners were trained. In the latter population, 26% runners had experience for only 3 months and participated in the training program 'Start to Run', while 53% had running experience for ≥ 1 year. As expected, runners trained on average more kilometers for the 15 km than for the 5 km run ($P 0.001$). The marathon runners seemed to be highly trained, as 44% had completed one to ten marathons and 36% more than 10 marathons previously.

Pre-run concentrations of hs-cTnT and NT-proBNP were within the reference range in most of the runners, as shown in Figure 1. Pre-run hs-cTnT concentrations were significantly higher with increasing running distance ($P < 0.001$). However, these low hs-cTnT concentrations should be considered with care, as they were not measured with adequate precision (91% of the pre-run concentrations were below 0.009 $\mu\text{g/L}$, the 10% CV cutoff value of the hs-cTnT assay). Pre-run NT-proBNP concentrations were only marginally different between the groups ($P 0.046$).

After running, hs-cTnT concentrations were significantly elevated ($P \leq 0.005$), as depicted in Figure 1A, except in the 5 km runners ($P = 0.522$). Median (IQR) post-run hs-cTnT concentrations were 0.006 $\mu\text{g/L}$ (0.008) in the 5_{HAPPY} km population and 0.004 $\mu\text{g/L}$ (0.003), 0.010 $\mu\text{g/L}$ (0.006), 0.014 $\mu\text{g/L}$ (0.019) and 0.030 $\mu\text{g/L}$ (0.029) in the 5, 15, 21, and 42 km runners, respectively. After correction for the effect of pre-run concentration, age and sex, linear regression analysis showed that hs-cTnT concentrations were significantly higher with increasing running distance ($P < 0.001$), except between the 15 and 21 km runners ($P = 0.347$). Also, the percentage of runners with elevated hs-cTnT concentrations after the run increased with running distance, as shown in Figure 1A.

Figure 1B shows that NT-proBNP post-run concentrations were also significantly elevated compared to the prerun concentrations ($P < 0.001$), while it was marginally elevated in the 21 km runners ($P = 0.051$). Median (IQR) post-run NT-proBNP concentrations were 8.41 pmol/L (11.39) in the 5_{HAPPY} km population and 7.32 pmol/L (8.69), 9.14 pmol/L (10.54), 5.06 pmol/L (7.23) and 9.67 pmol/L (8.98) in the 5, 15, 21, and 42 km runners, respectively. Regression analysis showed that post-run NT-proBNP concentrations were significantly higher in the 15 and 42 km runners compared to the other groups ($P < 0.001$) after correction for the pre-run NT-proBNP concentration, sex and age. Furthermore, post-run concentrations in the 5 km runners were significantly higher than in the 5_{HAPPY} group ($P < 0.001$), but not significantly different from the 21 km runners ($P = 0.187$). Nevertheless, in contrast to the hs-cTnT post-run concentrations, NT-proBNP post-run concentrations mainly remained below the URL (Figure 1B, overall in 96% of the runners). For comparison, when the URL is defined at the 97.5th percentile as proposed in the National Academy Clinical Biochemistry guideline,¹ NT-proBNP post-run concentrations were above the URL in 12% of the 5_{HAPPY} km population and in 7, 0, 10, and 11% of the runners, respectively.

No clear correlations were identified between pre-run concentrations of hs-cTnT and NT-proBNP ($r = 0.13$, $P = 0.025$) and the same held true for the post-run concentrations ($r = 0.21$, $P < 0.001$).

Discussion

Distance effect on hs-cTnT post-run concentrations

When using the new generation hs-cTnT assay, we found that the distance of competitive running was positively related to the increase in cTnT post-run concentrations, as shown in

Figure 1: (A) hs-cTnT and (B) NT-proBNP concentrations in recreational runners before and after running 5 km, 15 km, 21 km, and 42 km. Serum samples were measured pre-run (white boxplot) and immediately after the run (grey boxplot). Post-run concentrations were corrected for the effect of dehydration. All runners participated in a competition, except for the participants of the 5 km HAPPY (5_{HAPPY}), who either walked or ran. The dotted lines represent the upper reference limit (URL, 99th percentile measured in a reference population as reported previously in chapter 3). The boxplots represent the median (line), the interquartile range (IQR, box), and the range (1.5 × IQR). Outliers are not depicted. Indicated are the comparison of post-run with pre-run concentrations per runner population (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) and the percentage of runners above URL (between brackets).

Figure 1A for running 5, 15, 21, and 42 km. After marathon running for the largest distance studied, hs-cTnT was elevated in most of the runners (86%, median 0.030 µg/L). Also, we showed that post-run hs-cTnT concentrations were significantly increased after 5 km of exercise in the untrained and non-competitive HAPPY participants (5_{HAPPY}), while not in the trained population after 5 km of competitive running.

Such a distance effect on hs-cTnT post-run concentrations has not been reported so far. As mentioned in the introduction of this chapter, other exercise studies that used older generation cTn assays were restricted because of immunoassay limitations and cTn

concentrations only became detectable immediately after exercise.^{8,12} For example, when using the third generation cTnT assay (hs-cTnT assay is the fifth generation), Scharhag et al² could not find significantly lower cTnT elevations after marathon running compared to ultra-marathon running (100 km). We showed that in untrained individuals, even running a short distance as 5 km could lead to significant increases in hs-cTnT concentrations, in contrast to trained runners. In fact, in the marathon runners experience turns out to be a significant predictor of the cTnT post-run concentrations when using the hs-cTnT assay, as previously reported in chapter 3 and as also suggested by the data reported by Fortescue et al.¹ Neilan et al used both echocardiography and serum biomarkers to study non-elite marathon runners specifically and found that both median cTnT and median NT-proBNP concentrations were significantly higher in runners who trained ≤ 56 km/week (cTnT, 0.09 $\mu\text{g/L}$; NT-proBNP 21.5 pmol/L) than in those who trained > 72 km/week (cTnT, < 0.01 $\mu\text{g/L}$; NT-proBNP 12.5 pmol/L).⁴⁹ Others investigated recreational runners,^{47,52} but did not investigate the effect of training and, to the best of our knowledge, no studies have shown cTn elevations in professional runners.

Distance effect absent on NT-proBNP post-run concentrations

In contrast to hs-cTnT, running distance was not found to be related to post-run NT-proBNP elevations, as shown in Figure 1B. Nevertheless, NT-proBNP concentrations were significantly elevated after running for all studied distances, although the number of individuals with elevated post-run concentrations greatly depended on the URL definition. As suggested in the NACB guideline,⁵⁰ the URL in the present study was defined by the 97.5th percentile value measured in a reference population (28 pmol/L, n=479, chapter 3). In literature, though, often 125 pg/mL (14.8 pmol/L) is used as the diagnostic cutoff for heart failure^{53,54} as given in the package insert. But even with this lower cut-off, we could not identify increased NT-proBNP post-run elevations with increasing running distances as we did for hs-cTnT (post-run NT-proBNP concentrations were elevated in 29, 14, 26, 10, and 24% of the runners, respectively). Lippi et al⁵⁵ found that NT-proBNP concentrations did not exceed 14.8 pmol/L in 17 male runners after running half a marathon. However, in even more intense duration events, Scharhag et al² did show significant higher post-run NT-proBNP concentrations in ultra-marathon runners (median, 28 pmol/L as interpolated from figure) compared to marathon runners (median, 14 pmol/L) and marathon mountain bikers

(median, 11 pmol/L). In addition, Serrano-Ostariz et al⁵⁶ reported in 95 male cyclists (206 km) that exercise-induced NT-proBNP elevations were significantly correlated with training status, race time and exercise load (training impulse values), but not with age and exercise intensity.

Effect of exercise intensity on hs-cTnT and NT-proBNP

We show here that post-run hs-cTnT release increases with running distance and thereby with exercise duration, in contrast to post-run NT-proBNP. However, a meta-analysis of 26 exercise studies (1,120 individuals) that used second and third generation cTnT assays showed a slightly negative relationship with exercise duration (total studies, P 0.022; 15 running studies only, P 0.016).⁵⁷ It was thought that the shorter endurance exercise events were performed with higher exercise intensities than the longer endurance exercise events.^{7,57} One way to express exercise intensity is to estimate running velocity.⁵⁸ As shown in Table 2, we did not notice different mean running velocities in our populations of competitive running. Next, Jassal et al⁵² reported a modest correlation between post-run cTnT and the time required to complete a marathon race (median cTnT, 0.036 $\mu\text{g/L}$; mean time, 310 min; SD, 30 min; $r=0.40$, $P<0.001$). In the present study, we could not confirm a significant association of running time with the post-run hs-cTnT and NT-proBNP elevations (hs-cTnT, P 0.558; NT-proBNP, P 0.893) by using linear regression analysis with correction for the effect of running distance, pre-run concentration, age and gender.

Clarifications for exercise-induced biomarker increases

To explain the link between exercise-induced cTn release and (acute) cardiac events, two opposing theories exist, namely the reversibility and irreversibility concept of cardiomyocyte damage,⁸ as discussed previously in chapter 3. Recently, Sabatine et al⁵⁹ indicated by using the ultra-sensitive Singulex assay that hs-cTnI was released in patients due to myocyte ischemia without necrosis. Hessel et al⁶⁰ studied cTnI release from cultures of rat cardiomyocytes and demonstrated the release of intact cTnI from viable cardiomyocytes. Another clarification was made by Middleton et al⁶¹ who proposed that the cTn elevation could be due to an increased turnover of cardiac cTn, as a physiologic response to endurance exercise and the associated increased myocardial demand. Apart from this, a feasible explanation for exercise-induced NT-proBNP elevations is the associated increase in myocardial wall stress.⁷ This would suggest a relation between NT-proBNP and exercise

duration that we could not confirm in the present study (5-42 km of running), but was found in the more intense exercise events (42-206 km of running and cycling).^{2,56}

Study limitations

Comparison among the considered running distances was limited as different runners were studied for each of the distances. Although the HAPPY population was not completely characterized, we believe that these results are instructive especially when compared to the trained 5 km runners. Furthermore, training was not given to all the participants and we had to deal with two different definitions for training.

Conclusion

When using the hs-cTnT assay, we found the distance of recreational, but competitive, running to be positively related to the asymptomatic increases in cTnT post-run concentrations, in contrast to NT-proBNP. Remarkably, a relatively short running distance in an untrained population resulted in significant hs-cTnT release. Greater effort in basic research to investigate the biological and pathological importance of exercise-induced cTn release and the effect of insufficient training on this release is needed.

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Cystatin C, a marker for renal function after exercise

Abstract: Renal impairment is common during and after severe exercise. In clinical practice, renal function is evaluated using serum creatinine, urine parameters, and equations to estimate the Glomerular Filtration Rate (GFR). However, creatinine levels may be biased by skeletal muscle damage and the GFR equations, requiring age, gender and body weight, are shown to be inadequate in normals. In the present study, we show that serum cystatin C and creatinine concentrations were elevated after marathon running in 26 and 46% of the 70 recreational male runners, respectively, possibly because of reduction in renal blood flow. The mean cystatin C increase was twice as low as compared to creatinine (21 and 41%, respectively), suggesting that cystatin C is indeed less biased by muscle damage. Future research has to reveal whether training diminishes the elevation in renal markers. Overall, cystatin C seems a more reliable method to establish renal function during and after extensive exercise.

Renal impairment is common during exercise.¹ Complications range from the asymptomatic increase of serum and urine parameters²⁻⁴ to the severe failure of renal function.^{5,6}

The first stage of Acute Kidney Injury (AKI) is defined as an abrupt reduction in kidney function (<48 h) with an absolute increase in serum creatinine $\geq 26.4 \mu\text{mol/L}$, a relative increase ≥ 1.5 -fold, or a reduction in urine output (AKIN criteria).⁷ Chronic kidney disease (CKD) is diagnosed in case of kidney damage or Glomerular Filtration Rate (GFR) $< 60 \text{ mL/min/1.73m}^2$ for ≥ 3 months, irrespective of its cause (KDOQI classification).⁸ However, accurate measurement of the GFR is complicated in sports medicine and has hardly been described. In clinical practice, serum creatinine, urine parameters, and estimates of GFR (eGFR) are used to study renal function. The guidelines⁸ recommend to estimate GFR using the 4-variable Modification of Diet in Renal Disease (MDRD) equation⁹ or, otherwise, to estimate creatinine clearance using the Cockcroft-Gault (C-G) equation.¹⁰

In sports medicine, however, serum creatinine concentrations might be influenced by skeletal muscle damage. Creatinine is a breakdown product from the muscles and depends on muscle mass¹¹ as has also been confirmed in top level athletes.¹² Banfi et al reported that for the interpretation of creatinine concentrations in athletes one should take into consideration the Body Mass Index of the athlete and the specific sport performed (training, aerobic/anaerobic metabolism, and competitive season).¹² Apart from the (biased) serum creatinine, the eGFR equations require data regarding age and gender (and for C-G also body weight).^{9,10,13} In addition, eGFRs are developed in CKD/diseased patients, are imprecise at higher values and, consequently, cause misclassification in normals or persons with extremes in body composition.^{14,15} Urine collection is quite demanding during sport activities and the accuracy of the 24-h urine is therefore questionable. There is thus need for a more reliable method to measure renal function after or even during exercise.

Cystatin C is a non-glycosylated 13 kD basic protein and member of the cysteine protease inhibitors. It is constantly produced by all nucleated cells and serum concentrations correlate with GFR.¹⁶ In contrast to creatinine, cystatin C levels are less affected by factors such as age, gender and muscle mass.^{11,17,18} This makes cystatin C a promising marker to estimate renal function during and after exercise. To the best of our knowledge, no studies have reported on cystatin C concentrations in sportsmen, whatever the sport discipline, either at rest or followed after exercise.

In this study, cystatin C concentrations were evaluated in comparison to creatinine and creatinine-based estimates of GFR in recreational male marathon runners. Serum concentrations were obtained prior to, immediately after, and the day after the marathon.

Materials and methods

The marathon study population consisted of 70 recreational male runners (Table 1), which is part of the population studied in chapters 3 and 4. The study was approved by the local ethical committee and all runners signed the informed consent. Serum was collected <2 h pre-run and <1 h post-run from the 70 runners and the day after the run serum was collected for logistic reasons from a selected group of 18 runners. The maximum temperature on the day of the marathon was 23.4°C with a south wind of <14 m/s. Aliquots were stored at -80°C. Serum creatinine (Jaffé reaction assay, reference range 60-115 µmol/L, as validated in our laboratory in 31,335 polyclinical male subjects using Bhattacharya analysis) and albumin concentrations (reference range 32.0-47.0 g/L) were measured on the Synchron LX 20 (Beckman Coulter). Cystatin C (reference range 0.53-0.95 mg/L, as given in the package insert) was measured on the BN ProSpec (Siemens). eGFR was estimated using the MDRD,⁹ the C-G¹⁰ adjusted for body surface area, and the Mayo Clinic Quadratic Equation (MCQE)¹³ equations, see equations 1 to 3, respectively. Data analysis was performed using SPSS, Version 13.0. Parameters were tested using the paired-samples *t*-test

Equation 1: eGFR according to MDRD (mL/min/1.73 m²)

$$= 186 \left(\frac{\text{creatinine}}{88.5} \right)^{-1.154} \times \text{age}^{-0.203} \times \text{gender}$$

if male: gender=1; if female: gender=0.742

Equation 2: eGFR according to C-G adjusted for body surface area (mL/min/1.73 m²)

$$= \frac{(140 - \text{age}) \times \text{weight}}{\text{gender} \times \text{creatinine}} \sqrt{\frac{\text{length} \times \text{weight}}{36}}$$

if male: gender=0.81; if female: gender=0.95; if BMI > 25 kg/m²: weight=25*(length)²

Equation 3: eGFR according to MCQE (mL/min/1.73 m²)

$$= \exp(1.911 + \frac{5.249}{\text{creatinine} / 88.5} - \frac{2.114}{(\text{creatinine} / 88.5)^2} - 0.00686 \times \text{age} + \text{gender})$$

if male: gender=0; if female: gender=-0.205

where creatinine is in µmol/L, age in years, weight in kilograms, and length in meters.

and the Wilcoxon Signed-Rank test. Parametric correlations were calculated with the Pearson's coefficient. Statistical significance was considered at $P < 0.05$.

Results

Pre-race cystatin C and creatinine concentrations were within the reference range. As shown in Table 1 (original measurements) and Figure 1 (after correction for the effect of dehydration using the ratio of albumin concentrations from the post versus pre-run serum samples), both markers were significantly increased after running the marathon ($P < 0.001$). After running, the mean cystatin C concentration of the runners was increased by 34%, while the mean creatinine concentration was increased by 53% (Table 1). Cystatin C was increased to above the upper reference limit in 46% of the 70 runners, while for creatinine this was true in 70% of the runners. After correction for the effect of dehydration, the mean cystatin C and creatinine post-run concentrations increased by 21 and 41%, respectively (Figure 1), and concentrations were increased to above the upper reference limit in 26 and 46% of the

Table 1: Baseline characteristics of the marathon study population (mean and 95% CI)

n	70 males					
age, years	47 (30-68)					
BMI, kg/m ²	23 (20-26)					
training, number of marathons completed ^a	8 (125)					
running time, h	3.71 (2.87-4.69)					
				pre vs 0 h	pre vs 1 d	
	pre	post 0 h	post 1 d ^b	P value	P value ^b	
cystatin C, mg/L ^c	0.71 (0.56-0.95)	0.95 (0.63-1.45)	0.73 (0.60-0.93)	<0.001	0.032	
creatinine, µmol/L ^c	86 (65-111)	132 (87-264) ^d	96 (68-168)	<0.001	0.078	
C-G, mL/min/1.73 m ²	90 (64-130)	61 (28-93)	76 (44-95)	<0.001	0.100	
MDRD, mL/min/1.73 m ²	90 (66-125)	58 (28-89)	80 (40-107)	<0.001	0.069	
MCQE, mL/min/1.73 m ²	114 (81-138)	71 (27-119)	88 (35-106)	<0.001	0.105	
albumin (g/L)	44.0 (36.0-48.2)	48.2 (39.4-53.0)	43.2 (35.9-47.3)	<0.001	0.495	

^a median and 97th percentile

^b for logistic reasons available for a selected group of 18 runners

^c pre- and post-run values followed a gaussian distribution according to their histogram and PP-plot

^d gaussian distribution was obtained after exclusion of one outlier (469 µmol/L)

Figure 1: Variation in renal functional parameters in recreational male marathon runners. Serum measurements were performed prior (pre), immediately after (post 0 h) and one day after the marathon (post 1 d). Serum concentrations were corrected for the effect of dehydration using the pre-run albumin level. Depicted are the mean (•) and 2× standard error of the mean (error bars).

runners, respectively. Figure 1 and Table 1 show that the renal marker concentrations returned to baseline concentrations within one day. Overall, cystatin C and creatinine concentrations were well correlated ($r = 0.702$).

Pre-race eGFR was $>60 \text{ mL/min/1.73 m}^2$ in all the 70 runners when using the C-G equation (99%), the MDRD and MCQE equation (100%). After running the marathon, eGFR was $< 60 \text{ mL/min/1.73 m}^2$ in 46, 55, and 33% of the runners when using the C-G, the MDRD and the MCQE equation, respectively. For albumin corrected values this was 29, 35, and 16%, respectively. All three eGFRs were highly correlated ($r > 0.950$, $P < 0.001$). For the higher eGFR concentrations ($>60 \text{ mL/min/1.73 m}^2$), the MCQE equation resulted in higher values (max deviation $+36 \text{ mL/min/1.73 m}^2$) as compared to the MDRD, whereas the C-G equation resulted in lower values (max deviation $-33 \text{ mL/min/1.73 m}^2$). The eGFRs were highly correlated to $1 / \text{creatinine}$ ($r = 0.879\text{--}0.899$, $P < 0.001$) and well correlated to $1 / \text{cystatin C}$ ($r = 0.614\text{--}0.638$, $P < 0.001$).

Discussion

Once serum creatinine concentrations rise above the upper reference limit, creatinine clearance has already been declined to half of normal ($<60 \text{ mL/min}$), as can be interpreted from the characteristic shape of the creatinine clearance curve.¹⁹ It is thus quite alarming

that after marathon running in half of our runners (46%) creatinine levels were elevated to above the upper reference limit (after correction for the effect of dehydration). This was accompanied by a mean increase in creatinine concentration of 41% (+35 $\mu\text{mol/L}$), indicating acute renal failure.⁷ Neumayr et al already suggested a temporary reduction of renal function after ultramarathon cycling, as creatinine concentrations increased by 20-33%.^{3,4} In all studies creatinine concentrations returned to baseline within one day, illustrating that exercise-induced renal impairment is temporary.^{2-4,20} In clinical practice estimates of GFR are used to study renal function. Recently, Lippi et al reported a decreased eGFR immediately after running a half marathon in 71% of the runners ($<60 \text{ mL/min/1.73 m}^2$, using the MDRD equation and after creatinine concentrations were corrected for the effect of dehydration).²⁰ In the present study, also after dehydration correction, we found a decreased eGFR in half this percentage of the marathon runners (35%), or when using the C-G and the MCQE equation in even fewer runners (16 and 29% of the runners, respectively). After marathon running, we calculated a 30% reduction in eGFR (Figure 1), while Neumayr et al measured a 25% reduction after ultramarathon cycling³ and Lippi et al measured a 16% reduction after running a half marathon.²⁰ However, although the equations for eGFR are recommended in the CKD guidelines,⁸ they systematically underestimate renal function at high values of GFR and overestimate it at lower values of GFR in normals¹⁵ and CKD patients with normal creatinine levels.²¹ The AKI guidelines do not recommend evaluation of GFR, either measured or estimated.⁷ As exercise-induced impairment of renal function seems an example of acute renal failure, we have to be careful in conclusions concerning eGFR.

Some studies suggest that the cystatin C level may be affected by factors other than GFR. Cystatin C may reflect inflammation²² and is increasingly excreted in subjects with a decreased creatinine clearance.²³ On the other hand, in contrast to creatinine, studies have shown that gender and muscle mass do not affect the cystatin C level.^{17,23} Furthermore, Bandaranayake et al recently showed that the within-subject variation (CV_I) and between-subjects variation (CV_G) of cystatin C was less than for creatinine (CV_I 4.5 and 6.1%; CV_G 13 and 17.4%, respectively).²⁴ Next to the PENIA assay for cystatin C measurements, similar results were recently obtained when using the PETIA assay (CV_I 4.5 and 5.8%, respectively).²⁵ Cystatin C and creatinine both had an index of individuality of 0.35 (<0.6),²⁴ illustrating that the population-based reference ranges are here of limited use.²⁶ The critical differences (including biological and analytical variation) were 12.6% (0.097 mg/L) and 16.7% (13.7

$\mu\text{mol/L}$) at the mean values of cystatin C (0.77 mg/L) and creatinine (82 $\mu\text{mol/L}$), respectively.²⁴ This means that the exercise-induced elevations of cystatin C and creatinine we measured in the present study are substantial (Table 1). Bandaranayake et al reported an analytical CV_A of 2.5% at 0.77 mg/L and 2.3% at 64 $\mu\text{mol/L}$ when using the BN II nephelometer (now Siemens) and the Synchron LX20 analyzer (as we used), respectively.²⁴ We measured a comparable CV_A for cystatin C on our BN ProSpec nephelometer (also Siemens), namely a CV_A of 2.6% and 2.3% at 0.52 mg/L and 0.95 mg/L, respectively (internal validation report). Thus, as shown in Figure 1, cystatin C elevation after marathon running (21%) was twice as low as compared to creatinine (41%), which might imply that the creatinine level after running was biased by skeletal muscle damage and cystatin C was not.

Previous studies on renal pathophysiology have shown that during exercise blood flow in the splanchnic and renal circulations decreases (up to 50%)^{27,28} to redistribute the blood to the skeletal muscles, heart and lungs.²⁹ Reduction of renal blood flow might explain the increase in renal markers observed immediately after marathon. The vasoconstriction though causes hypoxic damage to the nephron, causing an increased glomerular permeability with excretion of erythrocytes and protein into the urine.^{1,27} Training diminishes the adverse effect of exercise on renal function. Armstrong et al studied eighty rats after 15 min of treadmill running and reported a 52% reduction of renal blood flow in sedentary rats and a 14% reduction in trained rats.³⁰ This training effect might explain the discrepancies found in literature. No creatinine increase was found in 51 experienced healthy individuals after 100 km of running (maximal oxygen uptake 40 ± 7 mL/kg/min and 35 ± 6 mL/kg/min for males and females, respectively; no correction for the effect of dehydration reported).² A slight creatinine increase of 14% was reported in 17 trained males after running a half-marathon (≥ 5 years of training with maximal oxygen uptake 65 ± 5 mL/kg/min; after correction for dehydration) and a 33% increase was reported in ultra-marathon cyclists ($11,000 \pm 4,500$ trained km that year; no correction for dehydration reported).^{3,20} In the present study, we found a creatinine increase of 53% in 70 recreational trained males after marathon running (median of 8 marathons completed before; 46% after correction for the effect of dehydration). An even larger creatinine increase of 65% was found in 21 well-trained men after 70 km cross-country ski-race (no specification for the training status; no correction for dehydration reported).²⁷ As illustrated above, comparison of previous studies is complicated

because of differences in training status and sports disciplines. Future research has to reveal whether creatinine and cystatin C elevations are indeed dependent on training status.

In summary, renal investigation after extensive exercise is important to avoid injury in the long term. The present study shows preliminary data of cystatin C measurements in relation to prolonged strenuous exercise. After marathon running, the mean serum cystatin C concentration was twice as low as compared to creatinine, indicating that creatinine levels indeed might be biased by skeletal muscle damage. Furthermore, equations to estimate GFR are known to be inadequate in normals and trained subjects. Therefore, especially in sports medicine, cystatin C seems a more reliable method to establish renal function.

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Haemodialysis patients longitudinally assessed by high-sensitivity cardiac troponin T and commercial cardiac troponin T and cardiac troponin I assays

Background: Elevated cardiac troponin (cTn) concentrations predict an increased mortality in patients suffering from end-stage renal disease (ESRD). This study compares the performance of a pre-commercial high-sensitive cTnT assay (hs-cTnT) with two contemporary cTn assays in detecting cTn elevations in ESRD patients during a 6 month follow-up.

Methods: A total of 32 ESRD patients were followed for 6 months. cTn concentrations were assessed using the 4th generation cTnT and hs-cTnT assay (both Roche Diagnostics) and the Architect cTnI assay (Abbott Diagnostics).

Results: During follow-up, 26 (81%), 32 (100%) and 9 (28%) of the patients showed elevated cTn concentrations according to the current cTnT, the hs-cTnT, and the cTnI assays, respectively. The variation in concentrations measured within each patient had a median (IQR) magnitude of 0.03 µg/L (0.02-0.06), 0.017 µg/L (0.011-0.029) and 0.011 µg/L (<0.001-0.017), respectively. In addition, higher cTn concentrations were measured for patients who suffered from cardiac disease (P 0.004, 0.008, and <0.001, respectively).

Conclusion: According to hs-cTnT assay, all of the ESRD patients had elevated cTnT concentrations at least once during the follow-up. As elevated cTn concentrations are highly prognostic of adverse events, the use of serial measurements has thus identified additional patients at risk for such events. The fact that we find cTn concentrations to be higher in patients with a history of cardiac disease is in line with this. Additional studies in ESRD patients are needed to investigate the added diagnostic and prognostic value of the very low cTnT concentrations and variations only detected by the hs-cTnT assay.

Patients suffering from end-stage renal disease (ESRD) have a markedly reduced lifespan. Overall, the life expectancy of patients undergoing dialysis is about 4 times as low as for the general population.¹ The main cause of death in these patients is accounted for by cardiovascular events and over 55% of patients on dialysis suffer from congestive heart failure.^{1,2} Considering this high incidence of cardiovascular complications, there is a need for accurate and sensitive biomarkers which can be used for diagnosis and risk stratification in ESRD patients. Over the years, the cardiac troponins (cTn) have proven to be accurate and sensitive markers for assessing ischaemic cardiac damage and predicting cardiovascular death in ESRD patients.^{3,4} Nonetheless, cTn concentrations can be elevated in the absence of apparent cardiac damage or clinical symptoms^{5,6} and might be influenced by a decreased renal clearance in patients with severe renal disease.^{7,8} As the diagnosis of acute myocardial infarction (AMI) has been defined as: a rise and/or fall of cardiac markers – preferably cTnT or cTnI – detected with at least one value above the 99th percentile limit of the reference population and should be associated with clinical, electro-cardiographic or imaging findings,^{9,10} such cTn elevations can impede the diagnosis of an AMI (for instance when ESRD patients are presenting with clinical symptoms). Recent National Academy of Clinical Biochemistry (NACB) guidelines have addressed this issue and suggest that for patients with chronically elevated concentrations of cTn, changes in cTn (>20%) 6-9 hours after the onset of clinical symptoms are indicative of an AMI.¹¹ However, such changes in cTn concentrations might also occur in the absence of clinical symptoms.^{12,13} To date, little is known about the biological variation in cTn concentrations, although some studies have taken serial measurements to investigate the cTn serum concentrations over longer periods of time.¹²⁻¹⁶

The advent of new, more sensitive cTn assays could improve the usefulness of both single and serial cTn measurements. To date, most commercial cTn assays lack a sufficient analytical performance to accurately detect cTn concentrations in healthy subjects, either because the limit of detection (LOD) of the assay is higher than the reference concentrations or because the assay imprecision, expressed as the coefficient of variation (CV) is higher than 10% at the 99th percentile of the healthy subjects.¹⁷⁻¹⁹ Assays with sufficient sensitivity to determine the reference concentrations will improve the detection of “abnormal” cTn concentrations in ESRD and other patient populations. More precise assays will also be able to detect temporal changes in cTn concentrations which could not be detected to date. Such

increasingly confident measurements will not only identify patients with abnormal cTn concentrations and variations, but could also shed light on the discrepancy between cardiac troponin T (cTnT) and cardiac troponin I (cTnI). Until recently the number of ESRD patients showing increased cTnT concentrations was about three times as high as for cTnI (53% vs 17%).⁵ With the advent of new, more sensitive cTnI assays, the difference decreases, but the number of patients with cTn concentrations above the 10% CV limit of the assay is still larger for cTnT (59%) than for TnI (32%).²⁰

In this study, we investigate the performance of two recently developed cTn assays: the pre-commercial high-sensitive cTnT assay (hs-cTnT) (Roche Diagnostics, Mannheim, Germany) and the Architect i2000SR cTnI assay (cTnI) (Abbott Diagnostics, Wiesbaden, Germany) in measuring cTn concentrations in a population of haemodialysis patients. For comparison, cTnT was also measured using the current 4th generation cTnT immunoassay (Roche Diagnostics). In addition, we assessed the intra-individual variation in the cTn concentrations during a 6-month follow-up.

Methods

Patient population

A cohort of 44 chronic haemodialysis patients from the Department of Internal Medicine at the University Hospital Maastricht was selected to participate in this 6-month longitudinal study. The study protocol was approved by the medical ethical review committee of the University Hospital Maastricht / Maastricht University. All patients provided written informed consent. Blood samples were collected pre-dialysis at the start of the study and subsequently every two months for a period of 6 months. During the follow-up period 12 patients were hospitalized. The reasons for hospitalization were very diverse, briefly: pneumonia, stoma operation, removal of kidney transplant, kidney transplant, malaise after chemotherapy, rectal blood loss/diarrhea, nausea, vomiting, hip replacement, kidney transplant followed by ileus, hypoglycemia/collaps, dizziness/fall, subdural hematoma. Since one of our aims was to study the occurrence of cTn variations in a stable (e.g. with no acute worsening in the patients condition) population of ESRD patients, hospitalized patients were excluded from our analysis. Collected blood samples were allowed to clot and then centrifuged: obtained serum samples were stored at -80 °C until analysis. Four patients failed to have blood taken at one occasion. Clinical data were collected from the medical

records in our hospital. Patients could be divided into two groups on the basis of a history of cardiovascular disease (CVD). CVD was considered present when patients had a history of myocardial infarction, had required coronary intervention like percutaneous transluminal coronary angioplasty (PTCA), coronary artery bypass grafting (CABG) or suffered from congestive cardiac failure.

Laboratory methods

cTnT was measured on the Elecsys 2010 (Roche Diagnostics, Mannheim, Germany) using the 4th generation cTnT immunoassay with a limit of detection (LOD) of $<0.01 \mu\text{g/L}$ and a 10% CV of $0.03 \mu\text{g/L}$ (according to package insert). cTnT was additionally measured using the pre-commercial high-sensitive cTnT (hs-cTnT) assay on the Elecsys 2010. According to the manufacturers data (Jarausch, personal communication) the 10% CV was estimated at $0.012 \mu\text{g/L}$. cTnI was measured on the Architect i2000SR (Abbott Diagnostics, Wiesbaden, Germany). According to the manufacturer's data, the LOD of the assay was $0.009 \mu\text{g/L}$ and the 10% CV concentration was $0.032 \mu\text{g/L}$.

Precision profiles for the hs-cTnT and the cTnI assay were determined in our laboratory by repeated ($n = 20$) measurements in 7 pooled serum samples (cTnT concentrations ranging from 0.002 to $0.019 \mu\text{g/L}$ and cTnI concentrations ranging from 0.017 to $0.088 \mu\text{g/L}$) as described in chapter 3. The resulting 10% CV concentrations for the hs-cTnT and cTnI assays were found at 0.009 and $0.032 \mu\text{g/L}$, respectively. The LODs for the hs-cTnT and cTnI assays were calculated in our laboratory according to the Clinical and Laboratory Standards Institute EP17 guidelines. According to these guidelines the LOD is $(\text{mean}_{\text{blank}} + 1,645 \cdot \text{SD}_{\text{blank}}) + (1,645 \cdot \text{SD}_{\text{low concentration sample}})$. The LOB, determined in cTn negative serum (supplied to us by Abbott Diagnostics) by replicate ($n=10$) measurements revealed a LOB of $<0.001 \mu\text{g/L}$ for the hs-cTnT assay (mean and SD, $<0.001 \mu\text{g/L}$) and $0.006 \mu\text{g/L}$ for the cTnI assay (mean cTnI, $0.001 \mu\text{g/L}$; SD, $0.003 \mu\text{g/L}$). Replicate measurements ($n=20$) with the hs-cTnT assay in a serum sample containing a mean concentration of $0.002 \mu\text{g/L}$ showed a SD of $0.00072 \mu\text{g/L}$. For the cTnI assay, a mean concentration of $0.017 \mu\text{g/L}$ showed a SD of $0.003 \mu\text{g/L}$. The resulting LOD was thus established at $0.001 \mu\text{g/L}$ for the hs-cTnT assay and at $0.011 \mu\text{g/L}$ for the cTnI assay.

Statistical analysis

Data analyses were performed using SPSS, Version 15.0. Continuous variables are described as median and interquartile range (IQR) or as mean and standard deviation (SD). Categorical variables are described as absolute numbers and as percentages. Biovariability data was analyzed by calculating the intra-individual coefficient of variation (CV_I) and the range (min-max) of cTn concentrations measured during the follow-up. The CV_I was calculated by subtracting the analytical variation (CV_A) from the total variation (CV_{TOT}), such that CV_{TOT}^2 is $CV_I^2 + CV_A^2$. The analytical variation was estimated by extrapolating the CV_A from the precision profiles for the hs-cTnT and cTnI assays (chapter 3). For the 4th generation cTnT assay the CV_A was extrapolated from the precision profile in the package insert. The maximum percent change during the 6 month period for each of the patients was calculated as [(the maximum concentration measured during the 6 month period minus the minimum concentration) / minimum concentration]*100. Differences in cTn concentrations and variations between patients with and without cardiac disease were compared using the Mann-Whitney-U test. Concentrations measured below the analytical LOD were set equal to the LOD. The level of statistical significance was established at $P < 0.05$.

Results

Baseline measurements in the patient population

Table 1 shows the characteristics of the 32 ESRD patients enrolled in our study. Measurements at baseline revealed median cTn concentrations (IQR) as detected by the cTnT, hs-cTnT and cTnI assays of 0.02 µg/L (<0.01-0.05), 0.053 µg/L (0.032-0.076) and 0.014

Table 1: Baseline characteristics of the ESRD patient population.

Characteristic	n (%)
Men	21
Women	11
Age, y (min-max)	66 (35-91)
Time on dialysis, months (min-max)	33 (1 - 102)
Hypertension	28 (88%)
Diabetes	12 (38%)
History of ischaemic heart disease	10 (31%)
History of cardiac failure	9 (28%)
Patient with history of cardiac disease	17 (53%)

Table 2: Number of patients having above cutoff cTn concentrations at baseline and at least once during the 6-month follow-up.

Assay		LOD	10% CV	99 th percentile	AMI cutoff*
cTnT	Cutoff (µg/L)	< 0.01	0.03	<0.01	0.03
	Baseline	20 (63%)	12 (38%)	20 (63%)	12 (38%)
	Follow-up	31 (97%)	26 (81%)	31 (97%)	26 (81%)
hs-cTnT	Cutoff (µg/L)	0.001	0.009	0.016	0.016
	Baseline	32 (100%)	32 (100%)	30 (94%)	30 (94%)
	Follow-up	32 (100%)	32 (100%)	32 (100%)	32 (100%)
cTnI	Cutoff (µg/L)	0.011	0.032	0.013	0.032
	Baseline	17 (53%)	6 (19%)	16 (50%)	6 (19%)
	Follow-up	23 (72%)	9 (28%)	22 (69%)	9 (28%)

* According to recent NACB guidelines the 99th percentile from a healthy reference population is the recommended cutoff concentration in AMI. However, the assay imprecision (CV) should be ≤10% at the 99th percentile, otherwise the 10% CV concentration is used.

µg/L (0.011-0.027) respectively. Table 2 shows the number of patients having cTn concentrations above the different analytical cut-off values. At baseline 12 (38%), 30 (94%) and 6 (19%) patients showed cTn concentrations above the designated AMI cut-off values according to the cTnT, the hs-cTnT and the cTnI assays, respectively.

Serial measurements in the patient population

Figure 1 shows the range of cTn concentrations measured during six month follow-up in each patient. We found a median (IQR) cTn concentration of 0.037 µg/L (0.017-0.074), 0.055 µg/L (0.033-0.079) and 0.014 µg/L (<0.011-0.028) for the cTnT, hs-cTnT and cTnI assays respectively. As can be seen in Figure 1, the patients experienced large changes in cTn concentrations during the follow-up and the range of concentrations measured within each patient had a median (IQR) magnitude of 0.03 µg/L (0.02-0.06), 0.017 µg/L (0.011-0.029) and 0.011 µg/L (<0.001-0.017) according to the the cTnT, hs-cTnT and cTnI assays, respectively. This corresponded to a median within patient percent change of 150% (72-287), 44% (27-69) and 54% (0-114) for the cTnT, hs-cTnT and cTnI assays respectively. The total coefficient of variation (CV_{TOT}) was 40%, 16% and 20%, respectively. The CV_A belonging to the median concentrations measured during the follow-up (as estimated from the precision profiles) is 7% according to the cTnT assay (median, 0.037 µg/L), 5% according to the hs-cTnT assay (median, 0.055 µg/L) and 15% for the cTnI assay (median, 0.014 µg/L). The CV_I is thus 39, 15 and 13% for the cTnT, hs-cTnT and cTnI assays, respectively.

Figure 1: Range of cTn concentrations measured in 32 ESRD patients during six month follow-up. cTnT concentrations according to the **(A)** cTnT assay and **(B)** hs-cTnT assay and cTnI concentrations according to the **(C)** Architect cTnI assay.

Considering the magnitude of the variation it is not surprising that the serial measurements identified additional patients that had cTn concentrations above the previously mentioned cut-off concentrations (at least once) during the 6-month period. During the follow-up we found that 26 (81%), 32 (100%) and 9 (28%) patients had cTn concentrations above the AMI cut-off level according to the cTnT, hs- cTnT and cTnI assays, respectively (Table 2).

Patients with and without a history of cardiovascular disease

As can also be seen in Figure 1, large differences existed between the cTn concentrations and variations in patients with and without a history of CVD. Table 3 quantifies the differences in the cTn concentrations measured during the follow-up. The three assays showed significantly higher concentrations of cTn in the patients who suffered from CVD. In addition, as might be expected, the relative number of patients having elevated concentrations of cTn was higher in the group with CVD. This difference was particularly large for the cTnI assay where only 2 out of 15 patients without CVD had cTnI concentrations above the AMI cut-off concentration, compared to 7 out of 17 in the patients with CVD. Moreover, the variations in the cTn concentrations, as assessed by the ranges of concentrations measured during the follow up, were larger in the CVD patients. Note, however, that, as shown in Table 4, the variations in cTn concentrations were in part the result of analytical rather than biological variations.

When the results were assessed for the individual patients, we found a large degree of heterogeneity in the agreement between cTnT and cTnI elevations. In some cases, as with patients 23 and 24, one can identify severely elevated concentrations of both cTnT and cTnI, which both seem to increase/decrease at the same time. On the other hand, there were patients, such as 7, 15 and 18 which showed very strong elevations ($>0.1 \mu\text{g/L}$) in cTnT (according to both cTnT assays), yet virtually no elevations in cTnI concentrations. In general, it seemed that patients with elevated concentrations of cTnI also had elevated concentrations of cTnT, but patients with elevated concentrations of cTnT did not always have elevated concentrations of cTnI.

Table 3: cTn concentrations of patients with versus without a history of cardiovascular disease (CVD).

Assay	No history of CVD (n=15)			History of CVD (n=17)			P value	P value
	Concentration, median (IQR), µg/L	Within patient range, median (IQR), µg/L	Within patient change, median (IQR), %	Concentration, median (IQR), µg/L	Within patient range, median (IQR), µg/L	Within patient change, median (IQR), %		
cTnT	0.027 (0.01–0.051)	0.03 (0.01–0.04)	128 (42–292)	0.046 (0.024–0.093)	0.05 (0.02–0.09)	162 (80–303)	0.004	0.049
hs-cTnT	0.043 (0.025–0.066)	0.012 (0.007–0.025)	42 (28–54)	0.061 (0.044–0.091)	0.018 (0.011–0.058)	47 (24–101)	0.008	0.153
cTnI	0.011 (0.011–0.021)	0.001 (<0.001–0.015)	9 (0–65)	0.023 (0.011–0.039)	0.015 (0.007–0.022)	73 (47–145)	<0.001	0.013

Table 4: Intra-individual coefficients of variation in patients with and without a history of CVD.

Assay	No history of CVD (n=15)			History of CVD (n=17)		
	CV _{TOT}	CV _A	CV _I	CV _{TOT}	CV _A	CV _I
cTnT	35	10	34	47	6	47
hs-cTnT	14	5	13	17	5	16
cTnI	n.a.*	> 15%*	n.a.*	24	12	21

* The coefficients of variations for the patient without CVD could not be determined accurately for the cTnI assay.

Discussion

This study describes, for the first time, the performance of a pre-commercial hs-cTnT assay in measuring cardiac troponin elevations and variations in a population of ESRD patients during a 6-month follow-up. In addition, we compared its performance to the current 4th generation cTnT and the Architect cTnI assays.

In the past, several studies have shown the occurrence of elevated cTn concentrations in ESRD patients without apparent cardiac damage and clinical symptoms.²¹⁻²⁵ These elevated concentrations of cTn are of cardiac origin²⁶ and were shown to be highly predictive for a cardiovascular event.^{3,4,23} A meta- analysis combining results from 28 studies (3931 ESRD patients) showed that cTnT concentrations above 0.1 µg/L were associated with an increase in all cause mortality (relative risk: 2.64).³ Other studies have shown an increased mortality in ESRD patients with cTnT concentrations above 0.026 µg/L²³ and 0.01 µg/L.⁴ Due to the lack of standardization in the available assays, the prognostic value of cTnI is less conclusive and cutoff concentrations cannot be compared between assays. Nonetheless, elevated concentrations of cTnI have also been shown to be associated with an increased mortality in ESRD patients.^{4,21}

In agreement with previous studies,²¹⁻²⁵ we found that 12 (38%) of our patients had elevated concentrations of cTnT according to the current cTnT assay and 6 (19%) had elevated concentrations of cTnI according to the Architect cTnI assay. Using the recently developed pre-commercial hs-cTnT assay we found that the occurrence of elevated concentrations of cTnT is far greater than previously described and at baseline 30 (94%) of the ESRD patients showed elevated concentrations of cTnT.

By following the cTn concentrations at regular points in time, we have identified an additional number of patients with elevated cTn concentrations. During the follow-up, 26

(81%), 32 (100%) and 9 (28%) of the patients showed cTn concentrations above the AMI cut-off concentrations according to the cTnT, hs-cTnT and cTnI assays respectively. As elevated concentrations of cTn are highly prognostic of adverse events in ESRD patients, the use of serial measurements has thus identified additional patients at risk for such events. The fact that we find cTn concentrations to be higher in patients with a history of cardiovascular disease seems to be in line with this.

We should note however, that due to differences in the precision with which the 99th percentile can be determined, the AMI cut-offs for the assays are set at different concentrations. For the cTnT and cTnI assays, the 10% CV cut-off concentrations were used, whereas for the hs-cTnT assay, the 99th percentile cutoff was used. When we assessed the occurrence of cTn elevations above the 99th percentile cutoff, we found that the difference between the assays was much smaller. A total of 31 (97%) of the patients had cTnT elevations, 22 (69%) had cTnI elevations, as compared to 32 (100%) for the hs-cTnT assay. Considering the fact that the 4th generation cTnT and the Architect cTnI assays were not able to detect the 99th percentile with sufficient precision (CV <10%) these findings, however, should be interpreted with caution. Nonetheless, they suggest that more sensitive cTnI measurements could reveal that the occurrence of cTnI elevations in ESRD patients is more frequent than is presently thought. This idea is supported by recent findings with sensitive cTnI assays in ESRD²⁰ and chronic kidney disease patients,²⁷ which find more cTnI elevations than previously described.

In general, there seems to be a high degree of heterogeneity between the patients in the amount of variation in their cTn concentrations. For instance, according to the new hs-cTnT assay, 8 (25%) of the patients experienced changes in cTnT >0.03 µg/L, whereas another 8 (25%) of the patients had variations <0.01 µg/L during the 6-month follow-up. Thus, as can also seen in Figure 1, the patient population consisted of a group of patients with relatively stable cTn concentrations and a group with a high degree of variations in their cTn concentrations. Further research in a larger population is needed to identify if the patients with the higher degree of variation are at greater risk for adverse events.

According to recent NACB guidelines only patients with chronically cTn elevated concentrations together with changes in cTn (>20%) 6-9 hours after the onset of clinical symptoms are suffering from an AMI.¹¹ Therefore, none of our patients would have been classified as having an AMI. Nonetheless, as increases in cTn represent further (subclinical)

myocardial damage and an increased likelihood for cardiac events, they should not be ignored. It would therefore be wise to perform a detailed cardiac evaluation in patients showing large variations in cTn concentrations, even if these variations are not accompanied by clinical symptoms.

The increases in cTn, as seen in many of our patients, would, if not for the longitudinal study, have been not detected. Assessing cTn concentrations at regular points in time would therefore appear as a sensible tool to increase clinical vigilance for the presence of myocardial damage and as a means for possible intervention. This is in agreement with previous studies which provided evidence for the increased ability of serial versus single measurements to identify patients at risk for an event.¹²⁻¹⁶

In the setting of heart failure, previously undetectable concentrations of cTnT were shown to have important prognostic value.²⁸ The use of high-sensitivity cTn assays, like the hs-cTnT assay, will enable a better differentiation between cTn concentrations in healthy and diseased patients and could improve risk stratification. Regrettably, our study was limited by a small sample size and the absence of outcomes. Therefore, additional research is needed to show if this prognostic value exists in the setting of ESRD and how patients can further benefit from serial measurements with high-sensitivity cTn assays.

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The extent of coronary atherosclerosis is associated with increasing circulating levels of high-sensitivity cardiac troponin T

Objective: This study explored the relationship between coronary atherosclerotic plaque burden and quantifiable circulating levels of troponin measured with a recently introduced high sensitive cardiac troponin T (hs-cTnT) assay.

Methods and Results: Cardiac patients suspected of having coronary artery disease (CAD) but without acute coronary syndrome were studied. Cardiac troponin T levels were assessed using the fifth-generation hs-cTnT assay. All patients (n=615) underwent cardiac computed tomographic angiography (CCTA). On the basis of CCTA, patients were classified as having no CAD or mild (<50% lesion), moderate (50% to 70% lesion), severe (>70% lesion), or multivessel CAD (multiple >70% lesions). As a comparison, high-sensitivity C-reactive protein levels were measured. Progressively increasing hs-cTnT levels were found in patients with mild (median, 4.5 ng/L), moderate (median, 5.5 ng/L), severe (median, 5.7 ng/L), and multivessel (median, 8.6 ng/L) CAD compared with patients without CAD (median, 3.7 ng/L) (all $P < 0.01$). For high-sensitivity C-reactive protein and N-terminal pro-B-type natriuretic peptide, no such relationship was observed. In patients without CAD, 11% showed hs-cTnT levels in the highest quartile, compared with 62% in the multivessel disease group ($P < 0.05$). Multivariate analysis identified hs-cTnT as an independent risk factor for the presence of CAD.

Conclusion: In patients without acute coronary syndrome, even mild CAD is associated with quantifiable circulating levels of hs-cTnT.

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The release of cardiac troponins (cTn) into the circulation occurs with cardiomyocyte injury. cTn is a preferred marker for diagnosing acute myocardial infarction (AMI) and is used as a key diagnostic tool for decision making in patients presenting with chest pain. However, the current commercially available assays do not have analytic precision for reliable detection of minor myocardial damage.^{1,2} The variation between measurements at the upper reference limit of a reference population, for instance, exceeds 10%.² Furthermore, these immunoassays are unable to detect cTn in apparently healthy individuals harboring coronary artery disease (CAD). Recently, analytic improvements have been made in the lower part of the cTn measurement interval. Using the highly sensitive fifth-generation high sensitive cardiac troponin T (hs-cTnT) assay, we recently reported an almost gaussian distribution in a reference population (n = 479).³ For cTnI, a similar distribution was seen using the adapted AccuTnI assay from Beckman Coulter.⁴ Recent studies in patients suspected of having an acute coronary syndrome (ACS) found that ultrasensitive cTn assays substantially improve the early diagnosis of AMI.^{5,6}

Moreover, Sabatine et al showed that transient stress test-induced ischemia is associated with increase of cTnI as detected with an ultrasensitive cTnI assay.⁷ The level of cTnI increase was proportionally related to the extent of ischemia as assessed by nuclear perfusion imaging.

We hypothesized that mismatch between supply and demand in the heart, and the resulting episodes of cardiac ischemia, may result in quantifiable circulating levels of cardiac troponin T (cTnT) in patients with CAD, especially in patients with significant lesions. To investigate this question, we assessed the extent of coronary plaque burden on cardiac computed tomographic angiography (CCTA) and associated this with hs-cTnT levels in a group of patients suspected for having CAD but without ACS. As a comparison, we used 2 other widely used cardiac serum biomarkers, high-sensitivity C-reactive protein (hsCRP) and N-terminal pro-B-type natriuretic peptide (NT-proBNP).

Methods

Study population

A total of 646 patients were referred from the cardiology outpatient department for CCTA because of suspected CAD. Inclusion criteria were a recent history of cardiac typical or atypical chest pain, dyspnea, or collapse; at least 1 mL of serum for determination of

biomarkers; and a diagnostic CCTA-scan, defined as 7 or more interpretable coronary segments. The exclusion criterion was an hsCRP concentration >10 mg/L, indicating underlying inflammatory disease. The institutional review board and ethics committee at the Maastricht University Medical Center approved the study, and all patients gave informed consent.

Risk factor assessment

Cardiac risk factors were assessed by the referring cardiologists. Risk factors were gathered just before CCTA. Patients were classified as having diabetes if they were treated with a hypoglycemic agent or if they had a fasting plasma glucose ≥ 6.7 mmol/L. Patients were classified as smokers if they had smoked in the 12 weeks before CCTA. A family history of CAD was defined as having a first degree relative with a history of myocardial infarction or sudden cardiac death before the age of 60. We calculated the prospective cardiovascular Munster (PROCAM) risk score to estimate the 10-year risk of AMI and the Framingham risk score to estimate the 10-year risk of cardiovascular disease.^{8,9} The PROCAM risk score takes the following risk factors into account: age, gender, smoking status, diabetes mellitus, systolic blood pressure, myocardial infarction in first degree relatives before the age of 60, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides. The Framingham risk score takes into account: age, total cholesterol, HDL, systolic blood pressure, smoking, and diabetes mellitus.

CCTA acquisition

Patients received 5 to 20 mg of Metoprolol intravenously to lower the heart rate to <65 bpm, as well as sublingual nitroglycerin spray. Heart rate and ECG were monitored during CCTA. A native scan was performed to determine the calcium score using the Agatston method.¹⁰ Subsequently, CCTA was performed using 85 to 110 mL of contrast agent (Xenetix 350; Guerbet), which was injected in the antecubital vein at a rate of 6.0 mL/s, directly followed by 40 mL of intravenous saline (6.0 mL/s). In patients with a heart rate <65 bpm, a prospective-gated “step and shoot” protocol¹¹ was used. In patients with a heart rate >65 bpm, a retrospective-gated “helical” protocol with dose modulation¹² was used to obtain the best image quality at a minimal radiation dose. CCTA was performed using a 64-slice Multi Detector Computed Tomography scanner (Brilliance 64; Philips Healthcare) with a 64×0.625

mm slice collimation, a gantry rotation time of 420 ms, and a tube voltage of 80 to 120 kV depending on the patient's height and weight.

CCTA coronary plaque assessment

All CCTA-scans were independently analyzed by 2 cardiologists (M.H.M.W., E.M.L.), both blinded for patient details and both experienced in interpreting more than 1,000 CCTA scans. The interobserver agreement was excellent for the Agatston score (κ 0.96) and substantial for the CT plaque burden score (κ 0.79).

Figure 1: (A) Representative image of the coronary arteries when using CCTA **(B)** American Heart Association 16 segment coronary nomenclature diagram.^{13,14} Segments 1-3 are representing the proximal, mid, and distal right coronary artery (RCA), respectively. The posterior descending artery (RDP) and postero lateral (PL) branches are represented by 4 and 4a, respectively. The left main (LM), proximal, mid and distal left anterior descending (LAD) are represented by segment 5, 6, 7, and 8, respectively, while segment 9 and 10 represent diagonal D1 and D2. The proximal and mid circumflex (RCX) are represented by segment 11 and 13, while segment 12 and 14 represent the first and second obtuse marginal (OM) or PL branches, respectively. The 15th segment represents the RDP supplied by the RCX.

To indicate the location of coronary atherosclerosis, the 16-segment classification of the American Heart Association was used, as depicted in Figure 1.¹³ The coronary artery tree was assessed using the source images on the Cardiac Comprehensive Analysis software (Philips Healthcare) and Terra Recon AQNet Client reconstruction software. Coronary plaques were defined as visible structures within or adjacent to the coronary artery lumen, which could be clearly distinguished from the vessel lumen and the surrounding pericardial tissue. The degree of stenosis of atherosclerotic lesions was classified as none (no luminal stenosis), mild (1 or more lesions with diameter stenosis of 20% to 50%), moderate (1 or more lesions with diameter stenosis of 50% to 70%), or severe (1 or more lesions with diameter stenosis of >70%).¹⁴

CCTA burden scores

- CT plaque burden score: No CAD and mild, moderate, severe, and severe multivessel CAD were distinguished. The most diseased segment determined the final score.
- Segment-based score: Segments with mild diameter stenosis scored 1 point, segments with moderate diameter stenosis scored 2 points, and segments with severe diameter stenosis scored 3 points. The total score was the sum of all points divided by the number of assessable segments.
- Plaque involvement score: Segments with no CAD scored 0 points, and segments with CAD present scored 1 point. Total score ranged from 0 to 16.¹⁵

Agatston Score

The Agatston score, or coronary calcium score, was calculated using the calcium scoring software of Philips Healthcare, with a threshold of 130 Hounsfield units.¹⁰

Biomarker measurement

Blood samples were obtained just before CCTA, after an overnight fast. Samples were processed within 2 hours and stored at -80°C until analysis. Total cholesterol, HDL, triglycerides, glucose, and creatinine concentrations were measured using the Synchron LX20 (Beckman Coulter). LDL was calculated using the Friedewald equation, except for subjects with triglycerides >4.5 mmol/L and total cholesterol <1.3 mmol/L, in which case LDL was determined on the Cobas Mira Plus (Roche Diagnostics). hsCRP was measured on the BN ProSpec using the CardioPhase hsCRP assay (Siemens Diagnostics). As validated in our

laboratory (NCCLS EP5-A guidelines), the between-runs coefficients of variation (CV) at 0.25 and 45 mg/L were 3.7 and 1.0%, respectively. cTnT was measured on the Elecsys 2010 using the precommercial highly sensitive fifth generation cTnT assay (hs-cTnT) and the fourth-generation cTnT assay (Roche Diagnostics). The hs-cTnT assay was validated as reported in chapter 3 with the upper reference limit (99th percentile) at 0.016 µg/L, limit of detection at 0.001 µg/L, and 10% CV cutoff at 0.009 µg/L.³ Inter-assay CVs were 3.0 and 1.4% at 0.021 and 3.03 µg/L, respectively. For the cTnT assay, the upper reference limit was <0.01 µg/L, limit of detection was <0.01 µg/L, and 10% CV cutoff was 0.03 µg/L (given by manufacturer). NT-proBNP was also measured on the Elecsys 2010, with the limit of detection at 0.6 pmol/L and the inter-assay CV 6.8% at 8.78 pmol/L (given by manufacturer), and in our reference population, we measured the upper reference limit (97.5th percentile) at 28 pmol/L.

Statistical analysis

Data were analyzed using SPSS 15.0. To test for differences in patient characteristics, we used the Pearson χ^2 test for discrete variables and the 1-way analysis of variance test for continuous variables, including Bonferroni correction. The PROCAM, Framingham, cardiac marker concentrations, and plaque burden scores were normalized by natural logarithm transformation. For natural log transformation of the plaque burden scores we used the score plus 1 to also include patients with a score of 0. Gender differences were tested using the Mann-Whitney U test. Correlations were calculated using Spearman's correlation coefficient and tested with the 1-sample t test. Multinomial logistic regression analysis was performed using the stepwise method including variables with $P < 0.05$. Receiver-operating-characteristic curves were plotted for the likelihood ratio of having CAD considering PROCAM, Framingham, hs-cTnT, NT-proBNP, and hsCRP. The models studied included all possible combinations of 1 to 4 of these risk factors as described by Wald and Hackshaw.¹⁶ In short, the likelihood ratio for the presence of CAD was calculated for these parameters using MATLAB R2009a (see appendix attached to this chapter). The likelihood ratio for a combination of these parameters was obtained by the product of the individual likelihood ratios. The areas under the curves (AUC) were compared 1-sided as described by Hanley and McNeil.¹⁷ For statistical calculations, biomarker concentrations less than the limit of detection were set equal to the limit of detection. The threshold for statistical significance was $P < 0.05$.

Table 1: Patient characteristics overall and in relation to CT plaque burden score.

	All subjects (n=615)	no CAD (n=200)	CT plaque burden score			P value
			mild (n=242)	moderate (n=81)	severe (n=66)	
Age, y	57 ± 11	52 ± 12	59 ± 10	60 ± 10	59 ± 10	<0.001
Male sex, %	57.7	47.5	54.5	71.6	69.7	<0.001
BMI, kg/m ²	27 ± 4	26 ± 4	27 ± 4	27 ± 4	27 ± 4	0.028
Systolic BP, mmHg	142 ± 19	139 ± 18	141 ± 19	146 ± 20	148 ± 19	0.003
Diastolic BP, mmHg	80 ± 12	78 ± 12	79 ± 11	83 ± 12	82 ± 12	0.014
Smoking, %	27.5	24.6	23.1	32.4	42.1	0.023
Diabetes mellitus, %	9.7	6.2	11.8	7.6	13.1	0.162
Family history, %	40.3	38.5	39.4	45.3	42.9	0.858
Statin, %	42.4	27.7	42.6	53.8	61.5	<0.001
PROCAM risk, % *	5.1 (1.6-12.1)	2.2 (0.9-5.9)	5.9 (2.0-13.4)	7.5 (2.7-24.9)	9.5 (5.1-19.2)	<0.001
Framingham risk, % **	17.6 (10.2-29.9)	11.8 (7.1-18.2)	18.7 (10.8-29.9)	24.0 (15.6-35.3)	25.7 (16.1-38.3)	<0.001
Total cholesterol, mmol/L	5.3 ± 1.3	5.4 ± 1.2	5.2 ± 1.2	5.4 ± 1.3	5.2 ± 1.3	0.160
LDL, mmol/L	3.3 ± 1.1	3.3 ± 1.0	3.2 ± 1.1	3.4 ± 1.2	3.3 ± 1.2	0.418
HDL, mmol/L	1.3 ± 0.5	1.3 ± 0.5	1.3 ± 0.5	1.3 ± 0.5	1.1 ± 0.3	0.005
Triglycerides, mmol/L	1.7 ± 1.1	1.7 ± 1.0	1.7 ± 1.0	1.7 ± 1.1	1.9 ± 1.1	0.497
Glucose, mmol/L	5.9 ± 1.6	5.7 ± 1.6	6.0 ± 1.7	6.0 ± 1.5	5.9 ± 1.3	0.590
Creatinine, µmol/L	84.9 ± 16.6	82.5 ± 14.9	83.4 ± 15.0	87.6 ± 17.6	88.9 ± 17.1	<0.001
hsCRP, mg/L	1.36 (0.70-2.81)	1.24 (0.54-2.79)	1.32 (0.71-2.62)	1.25 (0.75-2.80)	1.92 (0.93-3.69)	0.040
hs-cTnT, ng/L	4.5 (3.0-7.0)	3.7 (3.0-5.4)	4.5 (3.0-7.2)	5.5 (3.5-8.3)	5.7 (3.7-8.4)	<0.001
cTnT, µg/L	<0.01	<0.01	<0.01	<0.01	<0.01	-
NT-proBNP, pmol/L	9.5 (4.3-19.8)	7.7 (3.7-17.9)	9.4 (4.8-17.3)	10.2 (3.9-22.0)	13.8 (5.3-26.5)	0.018
Agatston score	16 (0-180)	0 (0-0)	47 (7-184)	177 (28-415)	160 (28-592)	<0.001

Depicted are the mean (SD) for parameters with a gaussian distribution or the median (IQR) for parameters with a non-gaussian distribution.

* available for n=510; ** available for n=547; BMI, body mass index; BP, blood pressure.

Results

Baseline characteristics of the study population are presented in Table 1. There was a positive correlation between the extent of CAD and the levels of hs-cTnT. Figure 2A shows a progressive increase of hs-cTnT in patients with mild (median, 4.5 ng/L; interquartile range [IQR], <3.0 to 7.2 ng/L), moderate (median, 5.5 ng/L; IQR, 3.5 to 8.3 ng/L), severe (median, 5.7 ng/L; IQR, 3.7 to 8.4 ng/L), and multivessel (median, 8.6 ng/L; IQR, 5.3 to 14.3 ng/L) CAD compared with patients without CAD (median, 3.7 ng/L; IQR, <3.0 to 5.4 ng/L) as assessed with the CT plaque burden score ($P < 0.01$). For the other plaque assessment scores, similar data were observed. hs-cTnT concentrations revealed a correlation with CT plaque burden score and Agatston score ($r = 0.293$ and 0.353 , respectively, both $P < 0.001$). Similar correlations were found using the involvement score and the segment-based score. Figure 2B shows gender differences in hs-cTnT concentrations, as described previously.³ Moreover, 41% of patients without CAD had hs-cTnT values in the lowest quartile, compared with 12% in the multivessel group (Figure 3). By contrast, 11% of patients without CAD showed hs-cTnT levels in the highest quartile, compared with 62% in the multivessel group (χ^2 analysis, $P < 0.05$).

hsCRP did not show a significant correlation with any of the plaque burden scores ($r = 0.074$, $P = 0.06$ for CT plaque burden score; Figure 2C). NT-proBNP showed only modest correlation with plaque burden assessed by the different scores ($r = 0.131$, $P < 0.001$ for CT plaque burden score; Figure 2D).

Multivariate analysis (multinomial logistic regression, Table 2), using all significant risk factors from Table 1 ($P < 0.05$), identified gender, smoking, age, body mass index, HDL, and hs-cTnT as independent predictors for the presence of CAD. In the second and third models, in which clinical risk profiling according to the PROCAM and Framingham risk scores was used, respectively, hs-cTnT was again identified as an independent risk factor. As shown in Table 2, for a 1-unit increase in natural log-transformed hs-cTnT, the odds ratio of having a severe and severe multivessel lesion were 2.6 and 7.8, respectively ($P = 0.01$ and $P < 0.001$, respectively). Comparable odds ratios were obtained in the second and third models (Table 2). Overall χ^2 of the final regression model was 133.311 ($P < 0.001$). The criteria for classification accuracy were satisfied, as the proportional by chance accuracy rate was below the classification accuracy rate (36.8% and 46.9%, respectively).

Figure 2: (A) hs-cTnT concentrations show a progressive increase with enhanced coronary plaque burden, (B) with a distinction between males (stripes) and females (dots), (C) in contrast to hsCRP and (D) NT-proBNP concentrations. For all groups, $n=20$ to 242 , except for the females in the multivessel group, with $n=2$ (§). Indicated are the significant differences between groups after Bonferroni correction (*, as compared with the no-CAD group; o, as compared with the multivessel group, $P < 0.001$ in A except for the comparison between no-CAD and mild, $P < 0.05$ in C and D).

Figure 3: Distribution of hs-cTnT quartiles (Q1 to Q4) within the CT plaque burden score groups (χ^2 analysis, $P < 0.05$). In patients without CAD, 41% had hs-cTnT values in the lowest quartile, compared with 12% in the multivessel group. By contrast, 11% of patients without CAD showed hs-cTnT levels in the highest quartile, compared with 62% in the multivessel group.

Table 2: Odds ratios of significant risk factors for coronary plaque burden in relation to CT plaque burden score.

Variable	mild			moderate			severe			multivessel		
	OR	95% CI	P value	OR	95% CI	P value	OR	95% CI	P value	OR	95% CI	P value
Model 1												
gender (female=1)	0.74	0.44-1.26	0.268	0.26	0.12-0.57	0.001	0.55	0.24-1.25	0.155	0.06	0.01-0.56	0.013
smoking (yes=1)	1.21	0.67-2.17	0.525	2.51	1.19-5.30	0.016	2.83	1.28-6.26	0.010	2.82	0.83-9.56	0.095
age	1.07	1.04-1.09	<0.001	1.09	1.05-1.13	<0.001	1.08	1.03-1.12	<0.001	1.06	1.00-1.12	0.040
BMI	1.06	1.00-1.13	0.040	1.03	0.94-1.12	0.538	0.98	0.89-1.08	0.711	0.80	0.67-0.96	0.015
HDL	0.84	0.46-1.53	0.572	1.61	0.77-3.36	0.210	0.23	0.08-0.72	0.012	0.25	0.04-1.47	0.126
hs-cTnT	1.33	0.75-2.38	0.330	1.29	0.61-2.72	0.502	2.64	1.24-5.63	0.012	7.83	2.79-22.0	<0.001
Model 2												
statin (yes=1)	1.98	1.23-3.19	0.005	2.94	1.55-5.61	0.001	3.27	1.61-6.63	0.001	2.77	0.92-8.34	0.071
PROCAM	1.34	1.13-1.59	0.001	1.74	1.35-2.24	<0.001	1.94	1.46-2.59	<0.001	2.17	1.35-3.50	0.001
hs-cTnT	1.87	1.13-3.10	0.015	1.90	1.00-3.61	0.052	2.76	1.43-5.32	0.002	6.78	2.98-15.4	<0.001
Model 3												
statin (yes=1)	0.51	0.30-0.86	0.012	0.36	0.18-0.72	0.004	0.34	0.16-0.72	0.005	0.38	0.12-.24	0.109
Framingham	1.96	1.40-2.73	<0.001	3.16	1.92-5.22	<0.001	3.46	1.99-6.02	<0.001	3.12	1.29-7.53	0.011
hs-cTnT	1.57	0.88-2.80	0.126	1.81	0.89-3.69	0.102	2.21	1.06-4.62	0.035	4.49	1.75-11.48	0.002

The no CAD group is the reference group for all displayed CT score groups. Multinomial logistic regression analysis was performed using the Forward Stepwise method. Variables were included in case of a significance level of 0.05, and removed from the model in case of a significance level of 0.1. In the models, all variables from Table 1 ($P < 0.05$) were considered as possible predictors for plaque burden. For reasons of independency, in model 1 the risk scores PROCAM and Framingham were excluded, while model 2 and 3 did not include age, gender, BMI (only model 2), systolic and diastolic BP, smoking status and HDL. BMI indicates body mass index.

Figure 4A-C shows that hs-cTnT was superior to hsCRP and NT-proBNP to distinguish the subjects without CAD from those with any extent of CAD, ranging from mild to severe multivessel CAD. Furthermore, the AUC for the presence of CAD of hs-cTnT was 0.64 ($P < 0.001$), as plotted in Figure 5, whereas the AUCs of hsCRP and NT-proBNP did not significantly deviate from 0.5.

Figure 4: Probability distribution functions to distinguish the study populations without coronary plaque burden (no CAD, grey) and with any coronary plaque burden (mild to multivessel CAD, black dots) for the cardiac biomarkers **(A)** hs-cTnT, **(B)** hsCRP, **(C)** NT-proBNP and for clinical risk profiling when using the **(D)** PROCAM and **(E)** Framingham risk scores.

Also, clinical risk profiling using either the PROCAM or Framingham risk stratification model was not sufficient to differentiate the subjects without CAD from those with any extent of CAD, as shown in Figure 4D and E. The AUCs for the presence of CAD were 0.70 and 0.72, respectively, using receiver-operating characteristic analysis (Figure 5). Addition of hs-cTnT to PROCAM risk profiling added significantly (P 0.046) to the AUC for the presence of CAD (AUC 0.73), which was not the case for Framingham profiling (AUC 0.72, P 0.394). For hsCRP and NTproBNP, no significant addition to the AUC for the presence of CAD was observed for either the PROCAM method (P 0.464) or the Framingham method (P 0.298 and 0.192, respectively).

Figure 5: Receiver-operating-characteristic curve for the presence of coronary plaque burden. Addition of hs-cTnT to PROCAM risk profiling resulted in a significant increase of the AUC from 0.70 to 0.73 (P 0.046), in contrast to Framingham (AUC 0.72, P 0.394). Addition of hsCRP or NT-proBNP did not increase the AUC (P >0.1).

Discussion

In the present study, we assessed hs-cTnT levels and coronary plaque burden in 615 patients suspected of having CAD but without ACS. Our findings indicate that hs-cTnT correlates well with the CT plaque burden score and that even mild coronary atherosclerosis results in quantifiable circulating levels of hs-cTnT. In addition, hs-cTnT is an independent predictor for the presence of coronary atherosclerosis. For hsCRP, no such correlation was observed,

which is in line with studies concluding that hsCRP may only have limited value in clinical risk profiling.^{18,19} The fourth-generation cTnT level has shown tremendous clinical value in identification patients with acute cardiac ischemia. The detection of myocardial injury is an important step in making the clinical decision to admit patients with chest pain to the coronary care unit. Studies evaluating the clinical value of cTnT were performed in patients with unstable angina pectoris^{20,21} and patients who underwent coronary artery bypass grafting²² or percutaneous coronary intervention.²³ These studies unequivocally demonstrated that elevated levels of cTnT are associated with poorer prognosis and higher rates of major adverse cardiac events.

The fourth generation cTnT assay has a detection limit of 0.01 µg/L and is not sensitive enough to show variation in patients without cardiac ischemia. The novel, fifth-generation hs-cTnT assay has shown a gaussian variation in healthy subjects,³ and it was able to measure an increase of cTnT levels after transient ischemia.⁷ The ability to measure hs-cTnT variation in normal subjects prompted us to investigate the relationship between the extent of coronary plaque burden and circulating hs-cTnT levels.

A question remains as to what the meaning of circulating cTnT levels is, in patients with variable degrees of CAD who do not have ACS. Traditionally, it was thought that release of cTn is equivalent to myocardial necrosis. However, some animal studies have suggested that short episodes of ischemia may result in the release of cTnT, without demonstration of cell death.²⁴ Recently, Sabatine et al showed that a few minutes of exercise-induced ischemia in patients is sufficient to result in the release of quantifiable levels of cTn.⁷ It is unlikely that only minutes of ischemia would have resulted in myocardial cell death in the patients exhibiting significant ischemia. Recent work of our group demonstrated that 5-minute episodes of cardiac ischemia in a mouse model, followed by reperfusion, results in exposure of phosphatidylserine to the surface of cardiomyocytes and activation of caspase-3, both of which are indicative of apoptosis.^{25,26} However, no demonstration of cardiomyocyte cell death was made. In vitro studies have demonstrated that caspase-3 activation results in cleavage of cTn and subsequent release.²⁷ Therefore, elevated hs-cTnT levels observed in patients with CAD may be the result of activation of caspase-3 within cardiac myocytes and the resulting cleavage and release of cTnT, but they do not necessarily implicate myocardial cell death. However, even if hs-cTnT does not reflect cell death, activation of the apoptotic

program within cardiomyocytes would still be unwanted, and as such, increased release of hs-cTnT found in patients with variable degrees of CAD is undesirable.

The question is, what triggers sufficient ischemia to result in cell stress and caspase-3 activation or even cell death? In patients with severe lesions, transient cardiac ischemia could easily result from a mismatch in supply and demand during the day, triggered by physical exercise or emotional stress.⁷ However, it is puzzling how mild lesions would result in elevated levels of hs-cTnT. One could argue that even mild lesions may result in ischemia and cTnT release during periods when the metabolic demand of the heart exceeds the supply. An alternative mechanism is suggested by the data of Rittersma et al²⁸ who demonstrated that in 50% of the cases, organized older thrombi were visible at the site of the culprit lesion in AMI patients admitted for thrombectomy. This suggests that thrombus formation at the site of atherosclerotic lesions is not a rare event and is not necessarily linked to clinically manifest plaque rupture and vessel occlusion. Dislodgement of these thrombi in small coronary vessels could be a potential cause for microinjury. Virmani et al²⁹ reported that 25% to 40% of AMI cases were caused by plaque erosion, rather than rupture, and subsequent thrombus formation. Plaque erosion may therefore be an important cause of localized thrombus formation and subsequent dislodgement.

Whatever the mechanism of the cTnT release is, the fact remains that our data show that quantifiable circulating levels of hs-cTnT occur in patients with even mild CAD. Two important questions arise from this finding. The first question is whether measurement of this variation of hs-cTnT levels and its correlation with CAD may hold diagnostic importance or predictive value. Studies have shown that coronary atherosclerosis is responsible for at least two-thirds of ACS.³⁰ Therefore, the outcomes of our data suggest that hs-cTnT has the potential to become a serum biomarker that will improve identification of patients at risk for developing cardiac events. The second question is whether we should put mild CAD in a different perspective and treat it more aggressively, as these conditions are associated with significantly increased circulating cTnT levels. Because percutaneous intervention for mild lesions would be out of the question, pharmacological intervention aiming at regression of atherosclerotic lesions could be the way to go. Several trials have demonstrated that high-dose statin therapy results in measurable regression of atherosclerotic lesions.^{31,32} It remains to be seen whether atherosclerotic regression would be sufficient to reduce the cellular stress indicated by circulating hs-cTnT levels.

Study limitations

A study limitation may be the precision of assessment of coronary stenosis using CCTA. It is known that compared with conventional angiography, the accuracy of CCTA in the assessment of the extent of lesions is limited by spatial resolution constraints.³³ However, a large number of patients were studied, and we used different methods for the assessment of CAD, providing more or less equivalent correlations with hs-cTnT levels. Also, we demonstrated a good interobserver agreement, with a κ value of 0.79. Taken together, it is likely that the plaque burden, assessed with CCTA, is an adequate reflection of the actual plaque burden in the large patient cohort presented here. Another limitation is that participants were mainly Caucasian.

Conclusion

Coronary atherosclerosis in symptomatic patients without ACS is associated with quantifiable circulating levels of hs-cTnT, even in mild CAD.

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Appendix

```
%% LRhscTnT
```

```
% Method to calculate the likelihood ratio for hscTnT in patients with plaque burden (CT score > 1)
% versus the patients without (CT score = 1).
```

```
%% Import data from SPSS via Excel (manually)
```

```
% file 'all' contains all subjects (n=615)
```

```
% file 'plaque' contains subjects with CT score > 1
```

```
% file 'normal' contains subjects with CT score = 1
```

```
% with hsCRP, hs-cTnT, NTproBNP, PROCAM data, respectively, all after
```

```
% ln-transformation.
```

```
%% Method
```

```
% Columns are ID, ln hsCRP, ln hs-cTnT, ln NTproBNP, ln PROCAM, ln
```

```
% Framingham, respectively
```

```
hscTnT=all615(:,3);
```

```
hscTnT_normal=normal615(:,3);
```

```
hscTnT_plaque=plaque615(:,3);
```

```
% Gaussian distribution is described by
```

```
%  $1/(\text{standarddeviation} \cdot \sqrt{2\pi}) \cdot \exp(-(x-\text{mean})^2/(2 \cdot \text{standarddeviation}^2))$ 
```

```
% constant in Gaussian equation:
```

```
c_hscTnT_plaque=1/(std(hscTnT_plaque)*sqrt(2*pi));
```

```
c_hscTnT_normal=1/(std(hscTnT_normal)*sqrt(2*pi));
```

```
% constant in e-power of Gaussian equation:
```

```
ce_hscTnT_plaque=-1/(2*std(hscTnT_plaque)^2);
```

```
ce_hscTnT_normal=-1/(2*std(hscTnT_normal)^2);
```

```
for x=[1:615]
```

```
prob_hscTnT_plaque(x,1)=c_hscTnT_plaque*exp(ce_hscTnT_plaque*(hscTnT(x,1)-
mean(hscTnT_plaque))*(hscTnT(x,1)-mean(hscTnT_plaque)));
```

```
prob_hscTnT_normal(x,1)=c_hscTnT_normal*exp(ce_hscTnT_normal*(hscTnT(x,1)-
mean(hscTnT_normal))*(hscTnT(x,1)-mean(hscTnT_normal)));
```

```
LR_hscTnT(x,1)=prob_hscTnT_plaque(x,1)/prob_hscTnT_normal(x,1);
```

```
end
```


High-sensitivity cardiac troponin T: risk stratification tool in patients with stable chest pain

Background: Recent studies have demonstrated the association between increased concentrations of high-sensitivity cardiac troponin T (hs-cTnT) and the incidence of myocardial infarction, heart failure, and mortality. However, most prognostic studies to date focus on the value of hs-cTnT in the elderly or general population. The value of hs-cTnT in symptomatic patients visiting the outpatient department remains unclear. The aim of this study was to investigate the prognostic value of hs-cTnT as a biomarker in stable chest pain patients and to assess its additional value in combination with other risk stratification tools in predicting cardiac events.

Methods: We studied 1,088 patients (follow-up 2.2 ± 0.8 years) with stable chest pain who underwent coronary calcium scoring and coronary CT-angiography. Traditional cardiovascular risk factors and concentrations of hs-cTnT, N-terminal pro-brain-type natriuretic peptide (NT-proBNP) and high-sensitivity C-reactive protein (hsCRP) were assessed. Study endpoint was the occurrence of late revascularization, acute coronary syndrome, and cardiac mortality.

Results: Hs-cTnT was a significant predictor for the composite endpoint (highest quartile Q4 >6.7 ng/L, HR 3.55; 95%CI 1.88-6.70; $P < 0.001$). Survival analysis showed that hs-cTnT had significant predictive value on top of current risk stratification tools (Chi-square change $P < 0.01$). In patients with hs-cTnT in Q4 versus $<Q4$, a 2- to 3-fold increase in cardiovascular risk was noticed, either when corrected for high or low Framingham risk score, coronary calcium scoring, or CT-angiography assessment (HR 3.11; 2.73; 2.47; respectively; all $P < 0.01$). This was not the case for hsCRP and NT-proBNP.

Conclusions: Hs-cTnT is a useful prognostic biomarker in patients with stable chest pain. In addition, hs-cTnT was an independent predictor for cardiac events when corrected for cardiovascular risk profiling, calcium score and CT-angiography results.

The identification of patients at risk for acute cardiovascular events remains a challenge. One promising avenue to improve the identification of these patients is the use of serum biomarkers, which could provide a relatively easy and cost effective step in risk stratification. Several biomarkers have been evaluated with respect to their incremental diagnostic and prognostic value.^{1,2} However, none of these biomarkers has achieved widespread acceptance yet.

With the development of more accurate high-sensitivity cardiac troponin (hs-cTn) assays, new possibilities become available to improve risk stratification.³⁻⁵ Omland et al⁶ found a significant association between increased hs-cTnT concentrations and the incidence of cardiovascular death and heart failure in patients with stable coronary artery disease (CAD). Bonaca et al⁷ found an association between hs-cTnI concentrations and death as well as myocardial infarction in non ST-segment elevation acute coronary syndrome patients. However, most prognostic studies to date focus on the value of hs-cTnT in the elderly or the general population.⁸⁻¹¹ Less is known about the possible incremental value of hs-cTnT on top of existing risk stratification tools in patients with stable chest pain.

Chapter 7 describes we were the first to demonstrate the association between hs-cTnT and the extent of coronary plaque burden in stable chest pain patients, as determined by coronary computed tomographic angiography (CCTA).¹² We found that even mild CAD is associated with quantifiable circulating levels of hs-cTnT, which was confirmed by others.¹³ CCTA is a relatively new, non-invasive imaging technique and there is considerable interest in the role of CCTA and coronary calcium scoring (CCS) in risk prediction models.¹⁴

In this study, we investigated the incremental value of hs-cTnT on top of the Framingham risk score (FRS) and the extent of CAD as assessed with CCS and CCTA, in predicting cardiac events. As a comparison, we also studied high-sensitivity C-reactive protein (hsCRP) and N-terminal pro-B-type natriuretic peptide (NT-proBNP).

Methods

Study population

We studied 1,114 patients who were referred from the cardiology outpatient department for CCTA because of suspected CAD. Part of this population was studied in chapter 7. Included were patients with a recent history of (a)typical chest pain who gave at least one mL serum. Excluded were patients with missing data regarding their cardiac risk profile and

patients with a history of percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG). The study was approved by the institutional review board and ethics committee at the Maastricht University. All patients gave written informed consent.

Data assessment

Cardiovascular risk factors, coronary plaque assessment using CCTA and CCS, and biomarker measurements were assessed as described in chapter 7.

Follow-up

The composite study endpoint was the occurrence of revascularization (PCI/CABG) >90 days after CCTA, cardiac mortality and ACS, including myocardial infarction and unstable angina requiring hospitalization. ACS was defined as typical angina pectoris, cTnT elevation (>0.01 µg/L) and ST-segment elevation/depression of ≥1 mm, or at least two of these characteristics together with invasive angiographic confirmation of a culprit lesion.¹⁵ We censored follow-up when revascularization was performed within 90 days and after occurrence of the study endpoint. Patients were seen by their cardiologist on a regular basis, and all hospital visits, both outpatient department visits as well as emergency room visits, were recorded in the electronic patient records. Additionally, the national mortality records were checked. None of the attending clinicians had access to the results of the hs-cTnT, hsCRP and NT-proBNP measurements.

Statistical analysis

To test for differences in baseline patient characteristics, we used the Pearson χ^2 test for discrete variables and the *t*-test for continuous variables. FRS, hsCRP, hs-cTnT, and NT-proBNP concentrations, and CCS were normalized by natural logarithm transformation. For transformation of CCS we used the score plus 1 to also include patients with a score of 0. Logistic regression and survival analysis were used to study prediction of the composite endpoint of late revascularization procedures (PCI/CABG), ACS, and cardiac mortality. For Kaplan-Meier analysis, categories of independent variables were compared using the log-rank test. Cox proportional hazard regression was validated for proportionality using log-minus-log and for time dependency. It was used to evaluate the additive value of the cardiac biomarkers, based on the Chi-square change (-2 log likelihood ratio) and whether biomarkers remained significant predictors. Biomarker concentrations less than the limit of detection

were set equal to the limit of detection. The threshold for statistical significance was $P < 0.05$, two-sided unless stated otherwise. All data were analyzed using SPSS Statistics 18.0 (SPSS Inc., Chicago, IL, USA).

Table 1: Baseline patient characteristics.

Variable	All participants n=1,088	Event*		P value
		Non-cardiac n=1,046	Cardiac n=42	
Age, mean (SD), year	56 (11)	56 (11)	59 (11)	0.067
Male sex, %	53.8	53.6	57.1	0.655
Systolic BP, mean (SD), mmHg	142 (19)	141 (19)	149 (16)	0.010
Smoking, %	26.5	25.6	50.0	0.001
Diabetes mellitus, %	8.4	8.3	12.5	0.347
Positive family history, %	39.7	39.2	51.2	0.123
Framingham risk score (FRS), median (IQR), %	16.7 (9.3-27.2)	16.4 (9.2-26.4)	25.1 (14.6-48.1)	<0.001
Total cholesterol, mean (SD), mmol/L	5.3 (1.2)	5.3 (1.2)	5.6 (1.2)	0.179
LDL, mean (SD), mmol/L	3.3 (1.1)	3.3 (1.1)	3.5 (1.0)	0.303
HDL, mean (SD), mmol/L	1.3 (0.8)	1.3 (0.8)	1.2 (0.4)	0.581
Triglycerides, mean (SD), mmol/L	1.7 (1.2)	1.7 (1.2)	1.9 (1.1)	0.391
Glucose, mean (SD), mmol/L	5.8 (1.4)	5.8 (1.4)	6.0 (1.5)	0.471
Creatinine, mean (SD), $\mu\text{mol/L}$	97 (18)	97 (18)	106 (35)	0.296
hsCRP, median (IQR), mg/L	1.4 (0.7-3.1)	1.4 (0.7-3.1)	1.6 (1.0-4.8)	0.079
hs-cTnT, 5th generation assay, median (IQR), ng/L	4.1 (<3.0-6.7)	4.0 (<3.0-6.6)	6.8 (<3.0-10.3)	0.015
NT-proBNP, median (IQR), pmol/L	8.9 (4.1-18.1)	8.8 (4.0-18.4)	10.9 (6.5-16.2)	0.218
Coronary calcium score (CCS), median (IQR)	7 (0-122)	6 (0-110)	252 (8-644)	<0.001
CCTA luminal stenosis, %				<0.001
no CAD	36.8	37.9	9.5	
mild CAD (20-50%)	38.1	39.0	16.7	
moderate CAD (50-70%)	14.5	14.0	28.6	
severe CAD (>70%)	10.6	9.2	45.2	

* Cardiac events after follow-up of 2.2 ± 0.8 years: 55x PCI, 21x CABG, 6x AMI, 9x unstable angina, 1x cardiac mortality.

Results

Study population

From the 1,114 patients who underwent CCS and CCTA, 18 patients had a history of coronary revascularization and in 8 patients the cardiovascular history was missing. These patients were excluded. The remaining 1,088 patients were subject of this report. Baseline characteristics of the study population are presented in Table 1.

Follow-up information was available for all patients (mean follow-up time 2.2 ± 0.8 years). As a result of CCTA, 50 patients underwent (early) revascularization within 90 days (35x PCI, 15x CABG) and these were censored at the time of revascularization. Final survival analysis included a total of 42 patients who suffered a cardiac event: 26 patients underwent (late) revascularization >90 days after CCTA (20x PCI and 6x CABG), 15 patients suffered ACS (6x AMI and 9x unstable angina requiring hospitalization), and one patient died due to heart failure. The overall cardiac event rate was 4%.

Table 1 shows that patients who suffered a cardiac event consisted of significantly more smokers, had higher systolic blood pressure and FRS, higher hs-cTnT concentrations, higher CCS, and more severe lesions on CCTA.

Prognostic value of hs-cTnT

Logistic regression revealed that 1-unit increase in (ln-transformed) hs-cTnT concentration resulted in a significant increase in cardiac risk (HR 2.03, 95% CI 1.32-3.12, P 0.001), in contrast to NT-proBNP (HR 1.16, 95% CI 0.90-1.50, P 0.256) and hsCRP (HR 1.31, 95% CI 0.99-1.74, P 0.059). Comparable results were obtained when biomarker concentrations were corrected for age and gender.

Kaplan-Meier analysis shows that hs-cTnT and NT-proBNP concentrations were both significant predictors for the occurrence of cardiac events (P <0.001 and 0.009, respectively), in contrast to hsCRP (P 0.355). Moreover, Cox regression reveals that hs-cTnT was the only significant biomarker predicting for cardiac events, either when testing the biomarker concentrations as a continuous variable (Table 2: Model 1, 4, and 7, respectively) or when present in the highest quartile Q4 (Table 2: Model 2, 5, and 8, respectively).

Table 2: Survival analysis of cardiac biomarkers for the composite endpoint of cardiac events.

Model	Variable	χ^2	P value	HR	95% CI	P value
1	hs-cTnT (ln-transformed)	12.65	<0.001	2.07	1.38-3.10	<0.001
2	hs-cTnT in Q4 (>6.7 ng/L) *	17.30	<0.001	3.55	1.88-6.70	<0.001
3	hs-cTnT >URL (14 ng/L) **†	1.08	0.299	1.85	0.57-6.02	0.307
4	NT-proBNP (ln-transformed)	1.63	0.202	1.18	0.92-1.52	0.201
5	NT-proBNP in Q4 (>18 pmol/L) *	0.72	0.396	0.70	0.31-1.60	0.399
6	NT-proBNP >URL (36 pmol/L) **†	0.47	0.492	0.66	0.20-2.16	0.495
7	hsCRP (ln-transformed)	3.39	0.066	1.29	0.98-1.69	0.065
8	hsCRP in Q4 (>3.1 mg/L) *	0.86	0.355	1.38	0.70-2.73	0.357
9	hsCRP >URL (3 mg/L) **†	0.61	0.436	1.31	0.66-2.60	0.437

* Dichotomous variable (yes or no)

† URL = upper reference limit (used for diagnosis)

Additional value of hs-cTnT on top of FRS

Clinical risk profiling using FRS predicted significantly for the occurrence of cardiac events (Table 1, $P < 0.001$). This was confirmed using logistic and Cox regression for the ln-transformed FRS or when FRS was categorized as follows: <5% (low risk), 5-20% (intermediate risk), >20% (high risk) (all $P < 0.05$). Kaplan-Meier analysis confirmed this relation, as shown in Figure 2A (P 0.018). Noticeably, almost no cardiac events were observed in patients with FRS <5%.

When regarding time to event using Cox regression, hs-cTnT concentrations were significantly predictive on top of FRS (Table 3). This was true when hs-cTnT was added either to the individual parameters of the FRS (Model 1, Chi-square change 4.97, P 0.026) or to the complete FRS algorithm (Model 2, Chi-square change 4.42, P 0.035). To further illustrate, we noticed a 3-fold increase in cardiac risk in patients with hs-cTnT concentrations in Q4 as compared to <Q4, independent from high or low FRS (cutoff 20%) (Model 3, Chi-square change 10.56, P 0.001). In patients with FRS <20%, the cardiac event rate increased from 2.1% to 5.9% when hs-cTnT concentrations were in Q4 compared to <Q4. In patients with FRS >20%, the cardiac event rate increased from 3.6% to 10.6%, respectively. This is also

Figure 2 (next page): Kaplan-Meier analysis to classify survival based on risk profiling with **(A)** the Framingham risk score (FRS) as mentioned in the method section of chapter 7 and **(B)** FRS including hs-cTnT (cutoff 20% and Q4, respectively). Survival was also classified based on the extent of coronary plaque as assessed by **(C)** coronary calcium scoring (CCS) and **(E)** coronary computed tomography angiography (CCTA). The additive value of hs-cTnT on top of these assessments is shown in **(D)** and **(F)**, respectively.

Table 3: Framingham risk profiling for the composite endpoint of cardiac events.

	Without hs-cTnT			With hs-cTnT		
	HR	95% CI	P value	HR	95% CI	P value
Model 1						
Age	1.03	1.00-1.07	0.074	1.01	0.97-1.05	0.536
Male gender *	1.26	0.61-2.63	0.531	1.00	0.46-2.17	0.998
Total cholesterol	1.11	0.84-1.47	0.468	1.17	0.88-1.56	0.272
HDL cholesterol	0.92	0.45-1.88	0.812	0.87	0.37-2.03	0.743
Systolic blood pressure	1.02	1.00-1.03	0.071	1.02	1.00-1.04	0.048
Smoking *	3.73	1.83-7.60	<0.001	3.55	1.73-7.27	0.001
Diabetes mellitus *	1.32	0.44-3.94	0.618	1.10	0.36-3.40	0.863
hs-cTnT (ln-transformed)	-	-	-	1.93	1.12-3.35	0.019
Model 2						
Framingham (ln-transformed)	2.24	1.41-3.56	0.001	1.94	1.21-3.12	0.006
hs-cTnT (ln-transformed)	-	-	-	1.69	1.08-2.66	0.023
Model 3						
Framingham >20% *	2.33	1.19-4.55	0.013	1.79	0.89-3.57	0.101
hs-cTnT in Q4 *	-	-	-	3.11	1.58-6.11	0.001

* Dichotomous variable (yes or no)

illustrated using Kaplan-Meier analysis in Figure 1B (P 0.001). In contrast, no significant additional value was found for NT-proBNP and hsCRP.

Additional value of hs-cTnT on top of CCS and CCTA

Kaplan-Meier analysis shows an apparent gradient of adverse survival for more severe CAD (Figure 1C and 1E). Furthermore, hs-cTnT concentrations (median, IQR) were significantly higher in mild (4.2 ng/L, <3.0-7.3), moderate (4.7 ng/L, <3.0-7.3), and severe CAD (6.5 ng/L, 3.6-9.4) as compared to patients without CAD (3.3 ng/L, <3.0-5.3), all P <0.001. A similar trend was found for CCS (P <0.001). These data show that increasing concentrations of hs-cTnT were associated with the severity of CAD, which is in line with our previous results in part of this population.¹²

Cox regression in Table 4 shows the additional value of hs-cTnT on top of CAD assessment with CCS and CCTA. One unit increase in hs-cTnT (ln-transformed) resulted in a

minor increase in cardiac risk (Models 1, Chi-square change 3.29 and 2.511, P 0.070 and 0.113, respectively). Nevertheless, still a 2- to 3-fold increase in cardiac risk was noticed in patients with hs-cTnT concentrations in Q4, independent from high or low CCS (cutoff Agatston score 400) or luminal stenosis on CCTA (cutoff 70%) (Models 2, Chi-square change 7.20 and 7.24, respectively, both P 0.007). In patients with high CCS (Agatston score >400, n=85), the cardiac event rates increased from 4.3% to 24% when hs-cTnT concentrations were in Q4 as compared to <Q4. In patients with a CCTA lesion of >70% luminal stenosis (n=103), the cardiac event rates were 8.8% and 28% when hs-cTnT concentrations were in <Q4 and Q4, respectively. This is also illustrated using Kaplan-Meier analysis in Figure 1D and 1F for CCS and CCTA assessment, respectively (both P 0.001). Again, no significant additional value was found for NT-proBNP and hsCRP.

Table 4: Survival analysis of coronary plaque assessment for the composite endpoint of cardiac events.

	Without hs-cTnT			With hs-cTnT		
	HR	95% CI	P value	HR	95% CI	P value
CCS model 1						
CCS (ln-transformed + 1) ^a	1.40	1.20-1.64	<0.001	1.35	1.14-1.58	<0.001
hs-cTnT (ln-transformed)	-	-	-	1.62	1.00-2.64	0.052
CCS model 2						
CCS >400 ^b	5.68	2.72-11.86	<0.001	4.53	2.13-9.64	<0.001
hs-cTnT in Q4 ^b	-	-	-	2.73	1.32-5.62	0.007
CCTA model 1						
Luminal stenosis on CCTA:			<0.001			<0.001
No CAD = reference	1.00			1.00		
<50%	1.47	0.42-5.22	0.549	1.36	0.38-4.83	0.637
50-70%	7.25	2.27-23.11	0.001	6.55	2.04-21.02	0.002
>70%	23.98	8.10-70.96	<0.001	20.11	6.63-60.94	<0.001
hs-cTnT (ln-transformed)	-	-	-	1.46	0.94-2.26	0.090
CCTA model 2						
luminal stenosis on CCTA >70% ^b	11.33	5.98-21.47	<0.001	9.23	4.79-17.82	<0.001
hs-cTnT in Q4 ^b	-	-	-	2.47	1.29-4.77	0.007

^a To also include values equal to zero

^b Dichotomous variable (yes or no)

Discussion

Our study shows that in patients with stable chest pain suspected for CAD, hs-cTnT was a significant predictor for the composite endpoint of late revascularizations, ACS and cardiac mortality. Over three times as much cardiac events were found in patients with hs-cTnT concentrations in the fourth quartile (cutoff 6.7 ng/L, HR 3.55, $P < 0.001$) as compared to patients with hs-cTnT concentrations in the lowest three quartiles. Moreover, survival analysis showed that hs-cTnT significantly contributed to the identification of a subgroup of patients with higher risk for cardiac events. When using traditional risk factors, smoking (HR 3.55, $P 0.001$), hs-cTnT (HR 1.93, $P 0.019$), and systolic blood pressure (HR 1.02, $P 0.048$) remained the only significant predictors. Hs-cTnT remained significantly predictive independent from FRS (HR 1.7-3.1, dependent whether variables were continuous or categorized). In addition, hs-cTnT improved classification on top of the extent of CAD as assessed with CCS and CCTA. To illustrate, a 2- to 3-fold increase in cardiac risk was noticed in patients with hs-cTnT concentrations in the highest quartile, independent from high or low CCS (cutoff Agatston score 400) or luminal stenosis on CCTA (cutoff 70%) (HR 2.73 and 2.47, both $P 0.007$).

However, before widespread introduction of hs-cTn as a risk factor, it is of great importance to exclude false-positives. Reichlin et al showed that the positive predictive value of hs-cTnT in diagnosing acute myocardial infarction was only 19% (cut-off 2 ng/L, limit of detection) or 50% (cut-off 14 ng/L, 99th percentile), while the negative predictive value was nearly perfect (99-100%, dependent on cutoff).³ On the other hand, the present study as well as other studies has shown the adverse outcome of elevated hs-cTn on cardiovascular events.^{6,8-10,16} The log-normal reference change values (hours versus weeks) in healthy individuals for a delta increase of hs-cTnT were 85 and 315%, respectively.¹⁷ There are no results reported yet on optimal delta cutoffs for the long-term. Based on biological variation, deFilippi et al recently showed that the risk for heart failure or cardiovascular death were 1.7 and 1.8-fold, respectively, when hs-cTnT concentrations increased with >50% over two to three years.⁹

Question remains what the underlying pathophysiological mechanisms of elevated hs-cTnT concentrations in these patients are. Korosoglou et al¹³ concluded that the presence of non-calcified coronary plaques may result in continuous leakage of cTn, possibly due to

repetitive micro-embolization of atherosclerotic debris. In our study, we observed a stepwise increase in hs-cTnT concentrations with increasing atherosclerotic plaque burden which supports this explanation. Alternative explanations for cTn leakage which have been supposed are demand ischemia, myocardial ischemia (for example due to coronary vasospasm), direct myocardial damage, chronic renal insufficiency, or myocardial strain because of volume or pressure overload.¹⁸

Recently, two papers were published which feed the thought that the identification of patients at risk for a cardiovascular event may soon become easier and more accurate using hs-cTnT.^{8,9} DeFilippi et al⁹ performed serial measures of hs-cTnT in community-dwelling older adults. They found a significant association between baseline hs-cTnT concentrations, changes in hs-cTnT concentrations and the development of heart failure and cardiovascular death. De Lemos et al⁸ found an association between increased hs-cTnT and structural heart disease, especially left ventricular hypertrophy, and subsequent risk for all-cause mortality. However, the study by deFilippi was focusing on elderly with a mean age above 70 years, while in the study of de Lemos the vast majority of the population (77%) consisted of patients with FRS <10%. These characteristics are not typical for the patients presenting at the cardiology outpatient department. Therefore, it is not clear from those studies to what extent hs-cTnT would be of incremental value in stable chest pain patients presenting at the cardiology outpatient department. Moreover, the published studies focused on left ventricular hypertrophy and heart failure, respectively. It is not inconceivable that the main cause of the elevated hs-cTnT is the presence of atherosclerosis, because it is known that the majority of patients with heart failure have underlying coronary atherosclerotic disease.¹⁹ Moreover, hypertension is an important risk factor for atherosclerosis and also the major determinant of left ventricular hypertrophy.

In previous work, we demonstrated that even mild CAD is associated with increased concentrations of hs-cTnT and we suggested that hs-cTnT may become a potential serum biomarker to improve the identification of patients at risk for developing cardiovascular events.¹² From literature, it is known that the extent of CAD provides important prognostic information in both asymptomatic and symptomatic patients. Both high CCS and $\geq 50\%$ luminal stenosis on CCTA deprive prognosis significantly.²⁰ In this study, we show that measuring hs-cTnT provides additional value on these already strong prognostic parameters. In our opinion, these findings strengthen the hypothesis that hs-cTnT helps to identify the

patient at risk. Clinically, there is general consensus that a severe coronary stenosis (>70% on CCTA) may benefit from interventional procedures. However, less is known about management of patients with mild and moderate stenoses. There is increasing evidence that ACS may be predominantly caused by non-significant stenoses.²¹ A biomarker like hs-cTnT might improve risk stratification for cardiac events. However, due to the low event rate in our patient category, we could not prove this. Despite this, when using hs-cTnT, patients at risk for a future cardiovascular event could be better recognized in an early phase at relatively low cost. In patients with high concentrations of hs-cTnT, a more aggressive diagnostic or therapeutic approach may be beneficial to prevent cardiovascular events. These questions need to be addressed in large clinical trials.

This study has several limitations that merit comment. First, the follow-up period is relatively short and therefore we found relatively few events. Second, all patients were of European descent. It remains uncertain whether our results can be generalized to other populations. Third, we performed a single hs-cTnT measurement and it remains unclear in which manner hs-cTnT varies in time.

Conclusion

Hs-cTnT is a useful prognostic biomarker in patients with stable chest pain suspected for CAD. It appears that hs-cTnT is associated with the extent of CAD, assessed by CCS and CCTA, and that hs-cTnT is a significant predictor for a cardiac event (revascularization, ACS, and cardiac mortality). Even better performance was obtained when hs-cTnT concentrations were combined with Framingham risk profiling. Finally, though to a lesser extent, hs-cTnT also provided additive value to the assessment of CAD by computed tomography.

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Time- and temperature-dependent stability of NIST standard reference material 2921 in serum and plasma

Background: Cardiac troponin I (cTnI) immunoassays deviate more than 20-fold in results, limiting laboratory and study comparisons. Recently, well characterized cardiac troponin complex became available, being NIST standard reference material (SRM) 2921. NIST SRM 2921 was used for development of a higher-order reference assay and as model for serum based secondary reference material. However, we previously noticed instability of cTnI results for NIST SRM 2921 in plasma based pools and hypothesize this can be clarified by its susceptibility to fragmentation.

Methods: NIST SRM 2921 was spiked in cTn-negative serum and plasma pools, incubated at 4 and 37°C, and followed for 3 days. Stability of cTnI and cTnT results was evaluated using Abbott AxSYM and Roche immunoassays, respectively. Moreover, fragmentation was characterized applying the original testkit antibodies for immunoprecipitation and Western Blot detection.

Results: cTnI and cTnT assay results remained stable (>90%) when NIST SRM 2921 based pools were stored at 4°C. However, results reduced to 50-60% when samples were incubated for 3 days at 37°C, except for cTnT in plasma. For cTnI, Western Blot did not reveal fragmentation. For cTnT, Western Blot showed fragmentation in serum but not in plasma, and at 37°C a higher extent of fragmentation was confirmed.

Conclusions: We show that NIST SRM 2921 is susceptible to time- and temperature-dependent degradation that affects immunoassay results. For cTnT this appeared in serum but not in plasma, for cTnI another modification in immunogenicity seems to happen. Future research should thus continue to search for a representative and highly defined cTnI standard.

Standardization and harmonization purposes of cardiac troponin I (cTnI) immunoassays are still uncompleted as recently reviewed¹⁻⁴ and as discussed in the latest National Academy of Clinical Biochemistry guidelines.⁵ The more than 20 cTnI immunoassays that are commercially available deviate more than 20-fold in assay outcome, which is not an issue for the highly patented cTnT assay.⁶ Each cTnI assay uses different antibodies, calibrators, control materials, and detection techniques, limiting laboratory and multicenter study comparison.

The International Organization for Standardization prescribes the use of a reference measurement system that consists of the following components: (1) a primary standard reference material (SRM) to calibrate a higher-order reference measurement assay; (2) that is used to assign concentrations to secondary reference material, typically with a comparable matrix as the routine samples; (3) that are subsequently used by the manufacturers to assign values to the calibrators of their assay.^{1,3,7,8}

SRM 2921 provided by the National Institute of Standards and Technology (NIST) is cardiac troponin complex (cTnT, cTnI, and cTnC) purified from human heart tissue. It has been selected from 10 candidate SRMs^{9,10} and has extensively been characterized.¹¹ Recently, the development of the higher-order reference assay was initiated,^{1,12,13} as well as a Western Blot method to characterize the secondary reference samples.¹⁴ Here, NIST SRM 2921 spiked in serum was used as model for serum based reference samples. We however showed time-dependent reduction of cTnI levels in NIST SRM 2921 based pools using routine immunoassays.¹⁵ We expect that this instability of cTnI can be clarified by the susceptibility of cTnI to fragmentation,^{16,17} which will be relevant for future applications of NIST SRM 2921.

In the present study, we characterized the time- and temperature dependent stability of NIST SRM 2921 when spiked in cTn-negative serum and plasma pools during 3 days at 4 and 37°C. To do so, we performed Western Blot detection against cTnI and cTnT using original testkit antibodies. We applied the original AxSYM cTnI (Abbott Diagnostics) and cTnT (Roche Diagnostics) capture and detector antibodies, respectively, to optimize comparability between immunoassay and Western Blot results. The incubation at 37°C was in the meanwhile a model for the *in vivo* condition.

Methods

Mass spectrometric characterization of NIST SRM 2921

The ESI-MS system used was the 6410 Triple Quad LC/MS (Agilent). The Reversed Phase-HPLC column was the Zorbax 300SB-C18 column (Agilent, 100 x 2.1 mm, 300 Å, 3.5 µm). Solvents used were ultrapure H₂O with 0.1% HCOOH and HPLC grade acetonitrile with 0.1% HCOOH. Characteristic settings were a flow rate of 0.2 mL/min, a column temperature of 35°C, a mass range of m/z 100-1680 and 20 µL of NIST SRM 2921 solution was loaded (Hytest, certified by NIST, cTnT 36.9 mg/L, cTnI 31.2 mg/L). Further instrument settings were default. Gradient elution was performed from 2 to 60% acetonitrile in 30 min. Mass spectra of the cTn were deconvoluted using MagTran (Zhang, Version 1.03).

Incubation of spiked NIST SRM 2921 in cTn-negative serum and plasma pools

Serum (Venosafe) and heparin plasma (Lithium heparin, Venosafe) pools were collected from healthy controls (cTnI and cTnT <0.02 and <0.01 µg/L, respectively) and pre-incubated for 12 hours either at 4°C or 37°C. NIST SRM 2921 was spiked 1:1000 (v/v) in the pools in 3 steps (20x25x2) and immediately incubated. Aliquots were strictly collected at 0, 0.5, 2, 6, 24, 48, and 72 hours. cTnI and cTnT concentrations were immediately measured as specified below, while remaining aliquots were stored at -80°C until Western Blot analysis.

cTnI was measured in duplo on the AxSYM analyzer (Abbott Diagnostics) using the Troponin-I ADV assay (2nd generation) with a limit of detection at <0.02 µg/L, measuring range 0.02-22.78 µg/L, CV ≤10% at 0.16 µg/L, and diagnostic cut-off at 0.40 µg/L. Since cTnI concentrations were above measuring range, samples were automatically diluted 10x using Solution 4 Line Diluent (0.1 M phosphate buffer). cTnT was measured in duplo on the Cobas 6000 analyzer (Roche Diagnostics) using the 4th generation cTnT assay with a limit of detection at <0.01 µg/L, measuring range 0.01-25.00 µg/L, CV ≤10% at 0.03 µg/L, and 99th percentile cut-off at 0.01 µg/L. All assay characteristics were given by the manufacturer.

Immunoprecipitation using cTn testkit antibodies

Capture cTnI (epitope amino acid residue, a.a.r., 87-91 and 41-49) and cTnT (M11.7, epitope a.a.r. 136-147) antibodies were collected from the commercial AxSYM Troponin-I ADV (Abbott) and 4th generation cTnT (Roche) immunoassay, respectively, and treated as follows. For cTnI, capture antibodies were already conjugated to microparticles. For cTnT, 10 µg of

biotinylated M11.7 (1.5 mg/L) was added to 1 mL of magnetic beads coated with streptavidin (Invitrogen) and incubated for 90 min at RT. Additionally, beads were crosslinked with dimethyl pimelimidate (3x) as described previously.¹⁸ Immunoprecipitation was optimized to cTn binding >90% (data not shown). Subsequently, 200 µL of serum or heparin plasma was incubated with 50 µL of beads for 1 hour at 4°C. After being washed, the immunoprecipitate was eluted with 1 M glycine, pH 3 for 15 min at 56°C.

Western Blot using cTn testkit antibodies

Immunoprecipitates were mixed with 4x XT Sample Buffer (Bio-Rad) and 20x Reducing Agent (Bio-Rad), heated for 5 min at 95°C, and separated on a 12% Criterion XT SDS-PAGE gel (Bio-Rad) together with the Precision Plus Protein Standard (Bio-Rad). After transfer to the nitrocellulose membrane (Bio-Rad, 0.45 µm), blots were treated using the vacuum SNAP i.d. protein detection system (Millipore) according to manufacturer instructions. Block buffer was 0.5% non-fat dry milk Blocking-Grade Blocker (Bio-Rad) in PBS, 0.1% Tween-20 (PBST). Primary anti-cTnI solution was 1.5 µg/mL Troponin-I ADV (Abbott, epitope a.a.r. 24-40) diluted 1:1 with PBST, primary anti-cTnT solution was 10 µg/mL Roche M7 (as kindly provided by Roche Diagnostics, epitope a.a.r. 125-131), primary anti-cTnC solution was 2 µg/mL (Hytest), both in PBST. Secondary antibody solution for cTnI was streptavidin-HRP (R&D systems) 200-fold diluted, secondary antibody solution for cTnT and cTnC was 0.4 µg/mL goat anti-mouse peroxidase (Dako), all in PBST. Subsequently, membranes were incubated for 5 minutes in Super Signal West Femto Substrate (Thermo Scientific) and exposure was detected using the ChemiDoc XRS scanner (Bio-Rad). Bands were quantified using Quantity One software (Bio-Rad, Version 4.6.5.).

Results

Validation of NIST SRM 2921 by Western Blot using cTn testkit antibodies

Western Blot detection against cTnT in NIST SRM 2921 estimated intact and degraded cTnT at approximately 40 and 29 kDa, respectively (Figure 1). In comparison, mass spectrometry measured MWs of 34.5 and 26.8 kDa, respectively. The relative intensities were 81 and 19%, respectively, as based on the peak areas of the LC chromatogram.

Western Blot detection against cTnI identified 2 cTnI molecules in NIST SRM 2921 of approximately 28 and 25 kDa, with relative band intensities of 64 and 36%, respectively.

Deconvolution of cTnI mass spectra identified besides intact cTnI of 23.9 kDa also degraded cTnI of 23.7 and 23.6 kDa, all coeluting in one peak and thereby limiting estimation of relative abundance.

Figure 1: Validation of NIST SRM 2921 by Western Blot using cTn testkit antibodies. Reversed Phase-HPLC separation was followed by **(A)** mass spectrometry or **(B)** fractionation and subsequent Western Blot against cTnT (Roche detector antibody), cTnI (AxSYM detector antibody), and for comparison also against cTnC (Hytest). In the chromatogram are indicated the MWs (Da) as measured with LCMS. M, MW marker.

Incubation study

NIST SRM 2921 remained stable for at least 3 days when spiked in pooled cTn-negative serum or heparin plasma and stored at 4°C, as based on the assay results of the AxSYM cTnI and cTnT (4th gen) assays (Figure 2 and 3, top). In contrast, when stored for 3 days at 37°C, Figure 2 shows a gradual decrease for cTnI in serum (59%) and plasma (53%). Figure 3 shows a reduction of cTnT results in serum at 37°C (59%), but not that much in plasma (87%).

Western Blot analysis using the AxSYM antibodies shows no time-dependent degradation of cTnI in NIST SRM 2921, when spiked in serum or heparin plasma, and stored

Figure 3: Stability of cTnT in NIST SRM 2921 when spiked in pooled cTn negative serum and heparin plasma, respectively. **Top:** Relative concentrations (mean) compared to time zero as measured with the 4th generation cTnT assay (Roche) (n=2); **Bottom:** Samples also underwent immunoprecipitation and Western Blot using the Roche testkit antibodies. M, MW marker; P, loading control NIST spiked 1:400 in SDS buffer; N, cTn-negative serum incubated for 72 hours. MW_{estimated} of the bands was 40, 29, 19, and 16 kDa, respectively.

at 4 or 37°C (Figure 2). However, Western Blot detection reveals that cTnT in NIST SRM 2921 was susceptible to fragmentation when spiked in serum and stored at 4°C (Figure 3). Also, the 29 and 16 kD band showed after 3 days a 2.9- and 4.4-fold increase, respectively, compared to time zero. In heparin plasma no time-dependent degradation of cTnT was noticed, either at 4 or 37°C (Figure 3). When stored at 37°C cTnT degradation in serum was noticed to an ever higher extent. Here, only 15% was present of the 40 and 29 kD band after 3 days as compared to time zero, while the 19 and 16 kD band showed a 1.9 and 5.2-fold increase, respectively, as compared to time zero. Notice that the blots depicted in Figure 3 were overexposed, but this was required to visualize the appearance of cTnT fragments.

Discussion

In the present study, cTnI and cTnT in NIST SRM 2921 remained stable (relative concentration >90%) when spiked in cTn-negative pooled serum or heparin plasma at 4°C, as based on the AxSYM cTnI (Abbott) and 4th generation cTnT (Roche) immunoassay results. When incubated at 37°C, however, AxSYM immunoassay results showed a time-dependent decline. Since no time-dependent fragmentation was detected on Western Blot using the original testkit antibodies, this needs to be clarified by another modification in cTnI immunogenicity. For cTnT, Western Blot analysis revealed time-dependent fragmentation even at 4°C. Instability of cTnT in NIST SRM 2921 was even more obvious when incubated at 37°C, which now went along with a decline in immunoassay results.

For cTnI in NIST SRM 2921 yet an unknown modification other than fragmentation should clarify the instability of cTnI immunoassay results. It might be expected that our observation holds for most of the available cTnI assays, since most current cTnI manufacturers chose two or three antibodies that are directed against the stable mid region of amino acid 30-110.^{2,19} So, although cTnI proteolysis has been extensively described^{16,17} this should not affect the assay outcome of most of the cTnI immunoassays anymore. The capture (2x) and detector (1x) antibodies of the AxSYM cTnI assay are indeed almost similar to the other cTnI assays, although in different combinations of capture and detector antibodies, or with two similar antibodies possibly combined with a different third cTnI antibody.^{2,19}

It has to be noticed, however, that the stability of cTnI in the NIST SRM 2921 based pools stored at 4°C (with a 1000-fold dilution) was higher in the present Western blot study

as compared to the previous 6171-fold dilution (heparin plasma only).¹⁵ This discrepancy is thought to be related to the fact that we now pre-incubated the cTn-negative pools for 12 hours at 4°C before spiking, thereby limiting possible modification processes.

When spiked in serum, cTnT in NIST SRM 2921 appeared to be susceptible to time-dependent degradation whereas in heparin plasma it remained stable. The latter can likely be clarified by the protease inactivation in plasma. Harmonization of cTnT assays is of course not an issue because of patent reasons, but when using NIST SRM 2921 for EQA-surveys it seems preferable to use plasma.

Nevertheless, it remains questionable whether highly purified cTn, like NIST SRM 2921, is representative for native samples of acute coronary syndrome (ACS) patients. We previously showed that cTnT in serum was degraded after acute myocardial infarction,²⁰ while others showed this for cTnI.^{16,17} Since the extent of cTn degradation turned out to be dependent on sample time collection after the ischemic event, this indicates that cTn degradation happens intracellularly in myocytes. These studies were criticized, though, because they performed Western Blot detection with antibodies different from the ones used in the commercial testkits.^{21,22} Nowadays, both cTnI and cTnT assays use antibodies directed against stable mid regions. Still, the present study reveals for the first time that the original Roche antibodies detect intact cTnT as well as degraded cTnT molecules of approximately 29, 19, and 16 kDa. Moreover, we showed that (part of the) proteases targeting cTnT are still active in serum but not in heparin plasma. Apart from this, we previously reported better stability of cTnI and cTnT assay results in plasma of ACS patients than in plasma with spiked NIST SRM 2921.¹⁵ This all suggests that modification processes might already be completed for native cTn in patient blood, in contrast to the intact NIST SRM 2921 that is extracted from human heart tissue. In line with this hypothesis, harmonization of 7 cTnI assays also turned out to be independent whether serum samples were collected before or after 48 hours post onset of ACS.²³ Overall, it seems that the molecular structures of cTnT and cTnI in patients differ from the one in NIST SRM 2921 and this should be considered in the effort to harmonization and standardization of cTnI assays.

To date, standardization and harmonization of cTnI methods remains ongoing. First of all, standardization cannot be achieved as long as there is no clear definition for the cTnI measurand.^{24,25} NIST SRM 2921 was proposed as harmonizer,^{9,11} but was disappointing in calibrating cTnI immunoassays,¹⁰ and is nowadays proposed as primary reference material to

assign values to serum based secondary reference material.¹⁻⁴ To develop the whole reference measurement system, NIST SRM 2921 was also used as model for cTnI positive serum samples.^{13,14} In the present study, however, we show that it appears not to be stable in matrix based pools, in part because of susceptibility to fragmentation. Secondly, as discussed above, previous studies question its resemblance with native cTn in the blood circulation of patients.

For the time being, cTnI harmonization could be achieved by correcting results from a particular cTnI assay to the overall mean of cTnI assay results. This reduced the interlaboratory CV for cTnI results from 82-97% to 7-28%.¹⁰ However, the disadvantage is that the overall mean varies continuously, depending on the cTnI methods in use in the laboratories. Therefore, based on the harmonization potential and stability¹⁵ issues, it seems preferable to use patient material for cTnI harmonization purposes, despite the fact that collection of patient material is limited, as is the reproducibility of its processing.

In conclusion, we show that the highly characterized NIST SRM 2921 when spiked into human matrices is susceptible to time- and temperature-dependent degradation that also affects immunoassay outcomes. For cTnI not fragmentation, but another modification in immunogenicity seems to occur both in serum and plasma, while cTnT fragmentation appears to happen in serum but not in plasma. Nevertheless, future research should continue to search for a representative and highly defined cTnI standard, enabling manufacturers to correctly standardize their immunoassay.

Limitations

Stability studies were performed with cTnI and cTnT concentrations in the upper range of physiological concentrations (cTnI 119.5 µg/L; cTnT 19.3 µg/L) to ensure detection on Western Blot, especially regarding the Roche detector antibody (M7). Also, for simplicity reasons, only the AxSYM cTnI assay was considered.

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Circulating cardiac troponin T is degraded after acute myocardial infarction

Abstract: Despite cardiac troponins (cTn) are the preferred biomarkers to diagnose an acute myocardial infarction (AMI), their structures in the circulation are not correctly understood yet. We and others previously showed cTnT and cTnI degradation in patient sera but this was disputed because of antibody differences with the commercial immunoassays. Recently, it was reported by using gel filtration chromatography that cTnT in serum is predominantly intact and free and cTnI is present in complex with cTnC. However, non-denaturing separation data should always be interpreted with great care. In the present study, after extensive calibration of the above condition complemented with Western Blot detection using the commercial antibodies, we evidently proved that cTnT in AMI serum as measured with the 4th generation cTnT assay is completely degraded into smaller fragments. Furthermore, cTnI was only partly degraded and we confirmed that cTnI is mainly in complex with cTnC. The clinical impact of the present study is dependent on the validation study in a larger patient population, but might have immense consequences for cTnT assay calibration and the interpretation of clinical results, especially when disease specific modifications patterns will be identified.

Despite the fact that cardiac troponins (cTn) are the preferred biomarkers to diagnose and monitor an acute myocardial infarction (AMI),¹⁻³ their structural aspects in the blood circulation are not correctly understood yet. We previously showed that circulating cTnT is completely degraded in hemodialysis patients⁴ and in patients after AMI.⁵ However, these and other cTnT and cTnI degradation results⁵⁻⁹ were disputed because the antibodies of the commercial immunoassays were different from the ones used for Western Blot detection.¹⁰ In contrast, Fahie-Wilson et al reported that the predominant form of cTnT is intact free, unbound cTnT in patients with kidney failure¹¹ and similar results were very recently found in serum of patients after AMI.¹⁰ They did not find any evidence for the presence of smaller molecular forms of cTn in the circulation. Like described previously,¹² they used gel filtration chromatography (GFC) to study the non-covalent complexation of cTn in serum. However, since GFC separation takes place under non-denaturing conditions, molecular weight assignment of cTn elution profiles should not be based solely on the molecular weights of globular protein calibrators. This is especially important for elongated structures like cTnT as we discussed before.^{13,14}

Correct understanding of the structural aspects of cTn might possibly assist in unraveling the pathophysiology of cTn release from damaged myocytes. In the past it has been hypothesized that during ischaemia cytosolic cTn are released relatively fast from myocytes in the intact form (<1 day after onset of symptoms), while the slower necrotic and proteolytic degradation is required to liberate cTn bound to the contractile apparatus.¹⁵ The cytosolic pool of both cTnT and cTnI is estimated to be 5-10% of the total cTn content as based on solubility studies of myocardial tissue,^{15,16} though has been criticized.^{17,18} In comparison, under milder ischemic conditions like after prolonged exercise it has been proposed that cTn elevations could lead to the release of only intact cytosolic cTn, the so called reversible damage.¹⁹

Moreover, it would be of great clinical importance to know which molecular cTn structure(s) exactly react with the commercial cTnT and cTnI immunoassays. Degradation and other immunogenic modifications might affect the harmonization and standardization purposes of the different cTnI immunoassays.^{2,20} Furthermore, in case modification patterns are disease specific, it might even affect the interpretation of elevated cTn concentrations in routine work-up in the clinics.

In the present pilotstudy, we investigated the molecular structures of cTn circulating in patient blood after AMI. Non-covalent complexation of cTn proteins in serum was studied using GFC as illustrated in Figure 1. The subsequent fractions were analyzed by cTnT (Roche Diagnostics) and cTnI (AxSYM, Abbott Diagnostics) immunoassays and Western Blot detection with antibodies similar to the ones used in the commercial assays.

Figure 1: Schematic presentation of study design.

Methods

Samples analyzed by gel filtration chromatography

Purified human cTn complex (Hytest) and purified free cTnT, cTnI, and cTnC (all Advanced Immunochemical) were dissolved as prescribed by the manufacturer and characterized with LCMS as described in chapter 9. Subsequently, samples were diluted in running buffer to 17.5 mg/L (theoretically). When being spiked in cTn negative pooled serum (cTnT and cTnI <0.01 µg/L), cTn complex was diluted to cTnT 22.1 µg/L and cTnI 168.6 µg/L, free cTnT was diluted to cTnT 19.9 µg/L, and free cTnI was diluted to cTnI 97.2 µg/L (all measured).

A series of serum samples was collected in the routine lab of an AMI patient (female, 84 years). The diagnosis at presentation at the emergency department was ST-elevated myocardial infarction with inferior and right ventricular infarctions. The patient was treated in the right coronary artery with PCI and 2 stents. Biomarkers measured during clinical work-up are depicted in Table 1 indicating a typical release curve for AMI.

Table 1: Release curves CK and cTnT during clinical work-up of the selected AMI patient.

Time, days*	0.12	0.34	0.58	0.90	1.69	1.91	2.91	3.93	4.80	7.57	8.00
CK, U/L	705	2595	4042	3697	1667	1250	289	120	122	47	60
cTnT, µg/L	1.53	9.26	n.d.	n.d.	23.12	21.07	n.d.	n.d.	n.d.	n.d.	5.15

* Time after onset of symptoms

n.d. not determined

Fractionation by gel filtration chromatography (GFC)

Gel filtration chromatography (GFC) was performed on a HP 1100 system (Agilent) equipped with a Sephacryl-S100 column (GE Healthcare, 1.6x60 cm) and a diode array detector. The column was equilibrated with 0.26 mol/L NaCl, 2.5 mmol/L $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 0.02 mol/L Tris, 6 mmol/L NaN_3 , and 1 g/L bovine serum albumin buffer, pH 7.4¹⁰ and operated at 0.5 mL/min. The void volume (V_0) determination and calibration was performed using the Gel Filtration Calibration kit (GE Healthcare) by applying Dextran Blue (2000 kDa) and conalbumin (75.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), and ribonuclease (13.7 kDa) supplemented with myoglobin (Sigma, 16.9 kDa). Dextran Blue (1 mg/mL) and protein standards (3-4 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 in duplo (mean for batch in Figure 3 and 4 was 39.0 and 37.3 mL, respectively). For the serum samples, 1.0 mL was loaded on the column. For each sample loaded, fractions of 1.25 mL were collected and kept on ice until immunoassay measurements or storage at -80°C.

Characterisation of GFC fractions by immunoassays

cTnT was measured on the Elecsys 2010 (Roche Diagnostics) using the 4th generation cTnT assay with the limit of detection <0.01 µg/L and coefficient of variation (CV) 10% cutoff at 0.03 µg/L. cTnI was measured on the AxSYM (Abbott) using the Troponin-I ADV assay with CV <10% from 0.27-4.00 µg/L. NT-proBNP was also measured on the Elecsys 2010 with limit of

detection 0.6 pmol/L and inter-assay CV 6.8% at 8.78 pmol/L. Albumin was measured on the Synchron LX20 (Beckman Coulter) using the microalbumin assay with measuring range 2.0-970 mg/L. All assay characteristics were given by the manufacturer.

Immunoprecipitation and Western Blot detection of the GFC fractions of the AMI patient

Catcher cTnI (epitope amino acid residue, a.a.r., 87-91 and 41-49) and cTnT (M11.7, epitope a.a.r. 136-147) antibodies were collected from the commercial AxSYM Troponin-I ADV (Abbott) and 4th generation cTnT (Roche) immunoassay, respectively. The subsequent preparation of the cTnT and cTnI antibody conjugated beads was performed as described in chapter 9. Next, 200 to 1000 μ L of the GFC fractions (dependent on the cTn concentration) were precipitated with 50 μ L of anti-cTnT or anti-cTnI coated beads for 1 hour at 4°C. After being washed, the immunoprecipitate was eluted with 1 M glycine, pH 3 for 15 min at 56°C for cTnT or for 5 min at 95°C for cTnI.

Western Blot detection against cTnT, cTnI, and cTnC were performed as described in chapter 9. In short, the primary antibody solutions were 1.5 μ g/mL Troponin-I ADV reagent 3 (Abbott, epitope a.a.r. 24-40) diluted 1:1 with 0.1% PBS-Tween 20 (PBST), 10 μ g/mL Roche M7 (as kindly provided by Roche Diagnostics, epitope a.a.r. 125-131) in PBST, and 2 μ g/mL anti-cTnC (Hytest, 7B9) in PBST, respectively.

Results

Figure 2 illustrates the high purity of the commercially purified human cTn complex (A) and the free cTnT (B), cTnI (C), and TnC (D). Most abundant masses were assigned to intact cTnT (MW_{measured} 34.5 kDa), cTnI (MW_{measured} 23.9 kDa), and cTnC (MW_{measured} 18.4 kDa), as based on the expected masses (cTnT, UniProtKB ID P45379, 35.9 kDa; cTnI, UniProtKB ID P19429, 24.0 kDa; cTnC, UniProtKB ID P63316, 18.4 kDa). Similar masses were measured for the free unbound cTn, except for cTnI with MW_{measured} 23.6 kDa. Furthermore, 13 and 11% of cTnT in the cTn complex and free cTnT, respectively, were degraded to 26.8 kDa as based on quantification of the peak area in the LCMS chromatogram (shoulder cTnT peak in Figure 2A and B).

When purified cTn complex was separated by gel filtration chromatography (GFC), as depicted in Figure 3A, the elution profiles of cTnT and cTnI overlapped (peak at 2-3 mL). On the contrary, when the free purified cTns were loaded (one after the other) cTnT and cTnI

Figure 2: LCMS chromatogram of purified human cTn complex **(A)**, free cTnT **(B)**, cTnI **(C)**, and cTnC **(D)** using Reversed Phase-HPLC gradient separation from 2% to 60% acetonitrile in 15 min. The inserts show the measured masses after deconvolution of the m/z spectra belonging to the main peaks, with labeled the most abundant measured mass.

Figure 3: Human purified cTn complex (**A, B**) and free cTnT, cTnI, and cTnC (**C, D**) spiked in buffer and separated using GFC. Fractions were characterized by the 4th generation cTnT and AxSYM cTnI immunoassays (**A, C**), and Western Blot analysis (**B, D**) using the commercial testkit cTnT and cTnI antibodies (and cTnC) as further specified in the methods section. Indicated on the blots are the positive control P, cTn complex spiked in running buffer; the MW marker; and the negative control N, running buffer.

eluted around 3 and 18 mL, respectively (Figure 3C). These results were confirmed by Western Blot analysis using the detector antibodies of the commercial cTnT and AxSYM-cTnI immunoassays and cTnC, as shown in Figure 3B and 3D.

Figure 4 shows that elution characteristics remained comparable when cTn was spiked in cTn negative human serum. Results were confirmed by immunoprecipitation and Western Blot detection (data not shown). Furthermore, cTnT - either in complex (peak at 3 mL) or free (peak at 6 mL) - eluted just before serum albumin (peak at 11 mL). Free cTnI spiked in serum eluted around V_0 and between albumin and NT-proBNP (peak at 15 mL), possibly being the dimer and monomer, respectively.

Figure 4: Human purified cTn complex (A) and free cTnT (B) and cTnI (C) spiked in cTn neg serum (cTnT/cTnI <0.01 $\mu\text{g/L}$) and AML patient serum samples from 3, 14, 22, 46 hours after admission to the emergency department (D, next page) that were separated using gel filtration chromatography. Fractions were characterized by cTnT (-●-, 4th generation) and cTnI (·○·, AxSYM) immunoassays. Internal serum markers were albumin (-■-, 68 kDa) and NT-proBNP (·□·, approx 13 kDa).

The cTnT and cTnI elution profiles of the AMI patient changed with time, dependent on the time of admission to the emergency department, as shown in Figure 4D. Moreover, as depicted in Figure 5, immunoprecipitation and Western Blot detection revealed that the first cTnT peak (5 mL) belonged to almost intact cTnT of $MW_{estimated}$ 27 kDa and the second cTnT peak (22 mL) belonged to smaller cTnT degradation products of $MW_{estimated}$ 15-17 kDa. For cTnI, as depicted in Figure 6, both intact and degraded cTnI were identified in the peak at 15 mL of $MW_{estimated}$ 28, 25, 18, and 15 kDa.

In conclusion, Figure 7 illustrates that the elution characteristics of cTn either in complex or in the free, unbound state differed from to the calibration proteins. These globular protein standards eluted in a linear relation as expected. However, all the cTn proteins, either in complex or free, deviated clearly from the calibration line, which was especially the case for free cTnT.

Figure 5: Western Blot analysis against cTnT of AMI patient serum samples fractionated in Figure 4D using the commercial Roche antibodies as further specified in the methods section. Considered are the samples from 3 (A), 14 (B), and 22 (C) hours after admission to the emergency department. Indicated on the blots are the positive control P, cTn complex spiked in running buffer (cTnT 1 µg/L); the marker M with MW labeled (kDa); and the negative control N, running buffer. P and N also underwent immunoprecipitation before loaded on the gel.

Discussion

After AMI, the circulating cTnT forms are completely degraded, as evidently shown in the present study after extensive validation but in contrast to findings of others.^{10,12} Concerning cTnI, we confirm the predominant form in AMI serum is most probably the binary cTnI-cTnC complex in the presence of both intact and degraded cTnI.

Investigation of the cTnT and cTnI forms in serum was performed by separation with non-denaturing technique of GFC. This enables detailed characterization of non-covalent

Figure 6: Western Blot analysis against cTnI of AMI patient serum samples fractionated in Figure 4D using the commercial Abbott AxSYM antibodies as further specified in the methods section. Considered are the samples 3 (A), 14 (B), and 22 (C) hours after admission to the emergency department. Indicated on the blots are the positive control P, cTn complex spiked in running buffer (cTnT 1 µg/L); the marker M with MW labeled (kDa); and the negative control N, running buffer. P and N also underwent immunoprecipitation before loaded on the gel.

Figure 7: Calibration of the gel filtration column according to globular protein standards (●) as further specified in the method section. Indicated are the elution characteristics of human cTn complex, (free) cTnT, and cTnI (all ▲) as interpreted from Figure 4A-C. Calibration curve of the standards follows equation $y = -0.028x + 2.1175$ (R^2 0.9788).

complexes present in serum, but also requires extensive and careful validation. We reported cTnT and cTnI elution profiles of our AMI patient similar to the ones reported by Bates et al.¹⁰ We used the same type of gel filtration column, running buffer, and calibration proteins, except that our column was longer. However, we disagree in the assignment of the main cTnT peak that elutes after serum albumin. Bates et al assigned this cTnT peak to free, intact cTnT as based on elution profiles of the globular calibration proteins. We show that their separation conditions were unable to differentiate between cTnT in complex and in the unbound state. With Western Blot detection using the Roche antibodies, we reveal that the cTnT peak eluting after albumin should be assigned to completely degraded cTnT forms of approximately 15-17 kDa. Apart from this, both studies show the main cTnI peak coelutes with serum albumin and assign this to the binary cTn IC complex which predominance was already shown by Katrukha et al.²¹ In conclusion, especially for cTnT with its elongated structure,²² non-denaturing separation data should be interpreted with great care¹⁴ and our extensive validation evidently proofs cTnT in serum is completely degraded.

The cTnT and cTnI degradation patterns have been studied before,⁵⁻⁷ but interpretation of results has always been limited since the used antibodies differed from the ones in the commercial immunoassays.¹⁰ This could explain our discrepancy with Labugger et al, who

reported the predominance of approximately 26 kDa degraded cTnT in AMI serum, though they too reported the presence of mainly intact cTnI.⁶ To put an end to these discussions, we put a lot of effort to apply the original cTnT (Roche Diagnostics) and AxSYM cTnI (Abbott Diagnostics) antibodies in our immunoprecipitation and Western Blot analyses, as kindly provided by Roche or as collected from the commercial kits.

The present study shows that the commercial cTnT assay predominantly detects cTnT fragments in AMI serum instead of intact cTnT. Since only one cTnT manufacturer is on the market, these new insights will not affect the cTnT harmonization and standardization purposes. Nevertheless, for calibration reasons it might be worthwhile to precisely know which cTnT form is predominantly present in patient serum.²⁰ By contrast, for the more than 20 available cTnI immunoassays, research in degradation patterns has prompted the manufacturers to choose anti-cTnI antibodies directed against the stable cTnI domain at amino acid residues 30-110.^{23,24} To date, results of the cTnI immunoassays still deviate up to around 3-fold and cTnI assays might thus still differ in their reactivity to degraded cTnI forms.

The circulating cTnT and cTnI forms could be specific for the time-phase after onset of AMI symptoms, as suggested from the elution profiles in the present study and Langendorf-reperfusion studies in rat hearts.⁷ In previous research, we already showed time-dependent degradation of cTnT in 20 AMI patients with no intact cTnT present anymore 12 hours after onset of symptoms,⁵ although at that time we did not use the Roche antibodies yet. In addition, Hessel et al found intact and degraded cTnT and cTnI forms in necrotic rat myocytes,²⁵ while only intact cTnI was released in the absence of necrosis via a stretch-related mechanism mediated via integrins.²⁶

The release of intact cTnT and cTnI forms might thus be a sign of early or mild ischemia. Intact cTn forms would originate from the cytosolic pool of the myocytes.¹⁹ However, Jeong et al have reported cytotoxicity of nonmyofilament-associated cTnT,¹⁸ indicating that under healthy conditions cTnT is not present in the cytosol. Also, in comparison to intact cTnT, they found larger toxicity for the cTnT mid- and C-terminal region¹⁸ which are known for their interactions to the sarcomeres and are highly conserved among muscle types.²⁷ It has also been shown that under stress conditions the N-terminus is cleaved off due to increased calpain-1 and caspase-3 activities^{28,29} and could be an adaptation to sustain cardiac function.^{30,31} This all indicates that a higher level of cell death is reached from the moment

the N-terminus is removed from cTnT. In relation to the present study, the first fragment that was detected after onset of AMI symptoms is thus probably N-terminal degraded cTnT (MW 25-27 kDa, a.a.r. 79/98-298). The smaller fragments detected later on after onset of symptoms could derive from the midregion, closer to the location of the epitopes of the two Roche antibodies (a.a.r. 125-147).

It would also be intriguing to find disease specific cTnT or cTnI forms. In End-Stage Renal Disease patients, we previously found degraded cTnT forms only, ranging in size from 8 to 25 kDa though without using the Roche antibodies.⁴ These cTnT fragments could be due to impaired renal clearance of renewed myocytes^{32,33} or because of myocardial ischemia. Furthermore, as shown by the group of Van der Velden, failing hearts are characterized by a lower extent of cTnI phosphorylation³⁴ and an impaired diastolic function when cTnI is truncated at the C-terminus.³⁵

Future research should validate whether cTnT fragmentation differs among AMI patients to investigate clinical consequences. The present study was limited to one AMI patient only. However, the obtained GFC elution profiles were similar to results obtained by Bates et al¹⁰ and, therefore, first effort was put in the extensive validation by Western Blot analysis. In addition, it remains to be elucidated whether fragmentation patterns differ in cTnT-positive patients not suffering from AMI, like with renal insufficiency or after extensive exercise.

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General discussion

Specifications of a perfect cardiac biomarker to diagnose acute myocardial infarction (AMI) are at least heart specificity, early detection after first symptoms, and a long half-life.¹ The current preferred biomarkers to diagnose AMI are cardiac troponins (cTn), either cardiac troponin T (cTnT) or I (cTnI).^{2,3} The main advantage of cTn is its cardiac specificity.⁴ This is in contrast to previous markers, like lactate dehydrogenase (LDH), aspartate aminotransferase, creatine kinase, or creatine kinase muscle-brain type. Or the ones that did not even reach routine clinical practice, like myoglobin or heart-type fatty acid-binding protein, which are also present in skeletal muscles. Second advantage of cTn is the timeframe of release. After onset of symptoms cTn concentrations increase within several hours and remain elevated for approximately a week. Nevertheless, faster and better diagnosis of AMI and also prognostic applications were expected by lowering the detection limit of current cTn immunoassays.

New insights due to high-sensitivity troponin assays

– Measurable troponin concentrations in healthy individuals

Improvements have lately been achieved in lowering the detection limit of cTn immunoassays. Previously, cTn concentrations elevated after myocardial infarction to detectable levels, while cTn concentrations in a healthy control group were not detectable. When validating the new high-sensitivity cTnT (hs-cTnT) assay, as described in *chapter 3*, it became clear that cTnT concentrations in a reference population follow a typical gaussian like distribution in the lower measuring range. We were the first who validated the hs-cTnT assay when it was only pre-commercially available. Only Latini et al had reported on the hs-

cTnT assay before, but they focused on the prognostic value in stable chronic heart failure patients.⁵ The bell-shaped cTnT distribution was later confirmed by other researchers.^{6,7} In *chapter 3* we also validated the second generation Architect cTnI assay, but this turned out to be a less sensitive assay. Though, a bell-shaped cTnI distribution in seemingly healthy individuals was obtained when using research high-sensitivity cTnI (hs-cTnI) assays.^{8,9}

As summarized in *chapter 2*, the hs-cTnT assay is nowadays the only commercially available cTn assay that measures the diagnostic cutoff concentration (99th percentile of seemingly healthy individuals) with sufficient precision (CV <10%) as recommended by the guidelines.^{2,3} The predicate of “guideline acceptance” has also been reported for research hs-cTnI assays,¹⁰ like the Singulex Erenna,¹¹ Nanosphere VeriSens,¹² and the Beckman Coulter Access assay.⁸ However, it remains to be elucidated whether “guideline acceptance” is required for diagnostic purposes. The clinical performance of the Architect cTnI assay to diagnosis AMI was namely not inferior to the hs-cTnT and Access hs-cTnI assay.¹³ On the other hand, when applying the hs-cTn assays for prognostic and screening purposes,¹⁴ it might become more important to measure with sufficient precision in the lowest concentration range. With the hs-TnT assay we were able to measure 5%, 98%, and 14% of the cTnT concentrations with CV <10% in our reference population (*chapter 3*), post-run concentrations of the marathon runners (*chapter 3 and 4*), and the stable chest-pain patients (*chapter 7*), respectively. The research Singulex Erenna assay is nowadays the only assay available – for research only – that achieves CV <10% across most of the reference concentrations.¹⁰ Although this requirement in assay precision was to some extent arbitrarily chosen,² further assay improvements seem essential when applying hs-cTn concentrations for prognostic purposes.

The pathophysiology of increased cTn concentrations in the blood circulation of healthy individuals is not understood yet. Previously, the heart was considered to be a post-mitotic organ in which the number of myocytes is established at birth and is lost with age or disease. Recent research, however, has shown the existence of renewal of myocytes, which will be very slow with around half of the myocytes exchanged at the age of 70,¹⁵ or rather faster since another study claims replacement of the entire heart several times during life-time.¹⁶ On the other hand, survival analysis by us (*chapter 8*, stable chest-pain population, 2-year follow-up) and others (general and elderly population, 6 and 12-year follow-up, respectively)^{17,18} revealed adverse survival in subjects with cTn concentrations even within

the reference range. It might thus still be true that measurable cTn concentrations in any circumstance should be avoided.

Consequently, proper selection criteria for a reference control population remain unclear. As reported in *chapter 3* and reviewed in *chapter 2*, we found a relatively high 99th percentile cutoff concentration of 16 ng/L for the hs-cTnT assay. We used cardiac biomarkers to simply screen the healthiness of the reference population. Half of the validation studies (47%) did not report their inclusion criteria. As expected, lower cTnT⁷ and cTnI¹⁹ upper reference limits were reported for the studies that extensively screened the subjects with a complete scale of questionnaires, ECG, (stress) echo, and biomarkers. Nevertheless, we were the first who reported a significant sex effect, and this was confirmed by others,^{6,7,20} while an age effect was not that obvious. Optimal diagnosis of AMI was though reported to be age dependent (cutoff, 70 years) when using the hs-cTnT or Architect cTnI assay.²¹ Overall, so far, the clinical impact to use cardio-healthy, sex- and age-matched control groups is not clear for diagnostic purposes of hs-cTn concentrations, but could especially be relevant for their prognostic use.

– Improved diagnosis of AMI

First clinical studies validated whether the improved hs-cTnT and hs-cTnI assays indeed accelerate the process to diagnose AMI.^{13,21,22} They reported perfect negative predictive values of around 100% but, this went along with low positive predictive values.

It was suggested to reduce the number of false positive diagnoses by serial sampling, as discussed in *chapter 2*. The guidelines proposed a change of 20%, as based on 3 times the standard deviation.² Optimal diagnosis in evolving non-ST-elevated myocardial infarction patients was however achieved by the 99th percentile cutoff at presentation in the emergency department with in addition a change of >117% within 3 hours or >243% within 6 hours.²³ Secondly, first results on biological variation of cTnI²⁴ and cTnT²⁵ concentrations suggest that cTnT is more suitable for serial sampling than cTnI. To detect an increase in cTnT concentrations, log-normal concentrations should increase on the short- and long-term (4-hour and 8-weeks period, respectively) with at least 85% and 315%, respectively.²⁵

Finally, it should be reconsidered whether the 99th percentile cutoff concentration of a healthy reference control group is the best diagnostic cutoff to diagnose AMI. One might question whether it would be better to use the 97.5th percentile, which is less sensitive to

outliers and is common for other biomarkers. On the other hand, the best way to validate diagnostic performance is by ROC curve analysis for each cTn immunoassay separately.²¹

– **Exercise-induced troponin elevations: how innocent are they?**

Exercise-induced cTn concentrations have occasionally been noticed after prolonged strenuous exercise but in the absence of clinical symptoms. It has been hypothesized whether these cTn elevations are caused by early or minor myocardial necrosis or by so called reversibly damaged myocytes, in which cytosolic cTn is released for instance via integrin-mediated²⁶ or blebbing mechanisms.²⁷

As we previously reviewed for cTnT²⁸ and as summarized in *chapter 4* for cTnI, cTn elevations were noticed with the contemporary cTn immunoassays in none to around half of the athletes immediately after prolonged strenuous exercise. This was dependent on the type of exercise, the assay, and/or the cutoff used. Echocardiographic research showed temporarily cardiac dysfunction after marathon running,^{29,30} but no myocardial necrosis could be confirmed using magnetic resonance imaging.³¹ We were the first who studied exercise-induced cTnT elevations using the hs-cTnT assay. As described in *chapter 3 and 4*, post-run hs-cTnT concentrations elevated above the 99th percentile cutoff in almost all (86%) recreational marathon runners with a 10-fold (median) increase. In addition, pre-run concentrations were within the reference range and post-run concentrations returned to baseline 1 day after the marathon event. The exercise-induced hs-cTnT concentrations could neither be clarified by renal dysfunction because creatinine and cystatin C concentrations increased with only 30% (*chapter 5*), nor by dehydration as comparable results were obtained after correction for dehydration (marathon runners, *chapter 3 versus 4*). This would suggest that the exercise-induced cTnT elevations are either minute or asymptomatic for myocardial injury.

We furthermore showed that post-run cTnT concentrations turned out to be dependent on running distance (*chapter 4*). This would suggest an association of cTnT elevations with exercise intensity or duration.^{31,32} We could not confirm this for runners running the same distance. However, in the marathon runners we noticed a negative association of experience on post-run cTnT concentrations (*chapter 3*). A training effect has been suggested by a few others^{29,33} and has also been proven in animal models.³⁴ As is generally known, this might emphasize that sufficient training is protective for the heart. A so called athlete's or

endurance-trained heart sustains long intervals of volume overloads of the left ventricular. Predominant cardiac adaptations are the increasing left and right ventricular internal diameter and the addition of new sarcomeres in-series to existing sarcomeres, which is known as eccentric hypertrophy.^{29,35,36} Moreover, cardiac remodelling due to training, in contrast to pathological hypertrophy, results in an increase in capillary density and a normal mitochondria-to-myofibril ratio.³⁷ This indicates that sufficient training protects the heart from ischemia but that too extensive or prolonged exercise might result in real cardiac damage and fibrosis.³⁸

– Elevated troponin concentrations in End-Stage Renal Disease

In End-Stage Renal Disease (ESRD) patients cTn concentrations are known to be elevated without further apparent cardiac damage or ischemia.³⁹ It is unclear whether such chronic cTn elevations are caused by cardiac disease or by the decrease or complete absence of renal clearance.⁴⁰

As described in *chapter 6*, in almost all ESRD patients (94%) cTnT concentrations were elevated when using the hs-cTnT assay, compared to around half of the patients when using contemporary cTn assays. Additional patients with elevated cTn concentrations were identified by serial measurements during 6 months follow-up. Interestingly, significantly higher cTn concentrations were found in patients with a history of cardiovascular disease, with larger intra-individual variability, compared to patients without this history. These results suggest that the chronic cTn elevations are caused by a combination of coronary and renal disease.

– Prognostic value of troponin in stable chest-pain subjects

The identification of patients at coronary risk remains an ongoing challenge. Complicated are especially patients who visit the cardiology outpatient department with complaints of chest-pain but with a normal ECG and a negative exercise stress test. Traditional risk factors and prediction algorithms like Framingham risk profiling turned out to be of limited use.^{41,42} With the development of more accurate high-sensitivity cTn assays, new possibilities seem available to improve risk stratification in patients with a possible cardiovascular risk.

Chapter 7 describes that hs-cTnT concentrations in stable chest-pain patients (n=615) were associated with the extent of coronary plaque as detected with coronary computed

tomographic angiography (CCTA). Significantly higher hs-cTnT concentrations were noticed in subjects with already mild coronary plaque lesion (diameter stenosis <50%) ($P < 0.05$). Such a trend was confirmed by Korosoglou et al, who in addition made the distinction between calcified, non-calcified, and remodelling plaques ($P < 0.001$).⁴³ Remarkably, 97% of the hs-cTnT concentrations were within the reference range (<16 ng/L). Secondly, we showed that hs-cTnT was a significant predictor for coronary plaque, in contrast to other (cardiac) biomarkers like hsCRP and NT-proBNP. It remains puzzling, however, how cTnT is related to coronary atherosclerosis, whether cTnT is locally released due to myocardial necrosis or by mild ischemia.

The association of hs-cTnT with left ventricular hypertrophy, chronic heart failure, and cardiovascular death has been reported in elderly¹⁸ or in the general population,^{17,44} but these are not the typical patients visiting the cardiology outpatient department. Kaplan-Meier analysis in *chapter 8* confirmed an adverse cardiovascular survival in stable chest pain patients with relatively higher hs-cTnT concentrations ($n=1,088$ with follow-up 2.2 years, $P < 0.001$) for the composite endpoint of late revascularization (after 90 days), ACS, and cardiac death. Comparable results were obtained when all-cause mortality was included (data not shown). To a smaller extent such a trend was also observed for NT-proBNP ($P 0.009$) but not for hsCRP ($P 0.355$). Moreover, over three times as much cardiovascular events were found in patients with hs-cTnT concentrations in the fourth quartile (HR 3.55, $P < 0.001$), as compared to patients with hs-cTnT concentrations in the lowest three quartiles. Hs-cTnT remained significantly predictive when corrected for traditional risk factors or when risk factors were combined in the Framingham risk score (HR 1.7-3.1). Omland et al showed an even stronger additive value of hs-cTnT to traditional risk factors in patients with stable coronary artery disease ($n=3679$).⁴⁵ The incremental value of hs-cTnT might thus be useful for prognostic use at the cardiology outpatient department, especially since hs-cTnT is a relatively inexpensive test¹⁴ that seems more powerful than previous cardiac biomarkers.⁴⁶ Further research with longer follow-up data and double blinded randomization studies are now needed to show whether and, if so, how cardiologists should adapt treatment of their outpatients based on cTn concentrations in the lower range.

Harmonisation and standardization of troponin assays

More than 20 cTnI immunoassays are nowadays commercially available,⁴⁷ but assay results deviate up to 20-fold.⁴⁸ This is not an issue for the highly patented cTnT immunoassay (Roche Diagnostics). Panteghini emphasized the need for an internationally recognized reference measuring system.^{49,50} In collaboration with the National Institute of Standards and Technology (NIST), standard reference material (SRM) 2921 was selected from 10 candidates.⁵¹ NIST SRM 2921 is a highly characterized cTn TIC complex that is purified from human heart tissue.^{52,53} Unfortunately, first attempts of cTnI assay harmonization were unsuccessful when using NIST SRM 2921 based pools⁵³ and we previously observed a time-dependent decline in cTnI assay results.⁵⁵

In *chapter 9*, we showed that cTnI and cTnT assay results remained stable (recovery >90%) when NIST SRM 2921 was spiked in human serum and plasma pools and kept at 4°C. However, assay results reduced to 50-60% when the pools were incubated at 37°C, except for cTnT results in plasma (87%). Subsequently, we applied the commercial antibodies of the 4th generation cTnT and AxSYM cTnI assay, respectively, for Western Blot analysis. For cTnI, we did not detect time-dependent cTnI fragmentation, yet another cTnI modification in immunogenicity appears to happen. For cTnT, we detected time-dependent degradation in serum but not in plasma. For the incubation at 4°C, cTnT degradation did not affect assay results, in contrast to the higher extent of degradation found when incubated at 37°C.

In conclusion, these limitations of NIST SRM 2921 do not support to use it for cTnI harmonization purposes. Patient material seems instead preferable, despite the fact that it is less specified and characterized.⁵⁵ The susceptibility of cTnT degradation in serum but not in plasma is probably clarified by the protease inactivation in plasma. Though serum and (heparin) plasma samples showed a good correlation for cTnT results ($r=0.995$),⁵⁶ its impact on calibration and clinical samples might remain of importance. Nevertheless, future research should continue to search for a highly defined cTnI standard that eventually will enable manufacturers to standardize the cTnI immunoassays.

Fully degraded cTnT in the blood circulation after AMI

Despite cTn are the preferred biomarkers to diagnose and monitor AMI,^{2,3} their structural aspects in the blood circulation are not yet correctly understood. We previously showed that

cTnT is completely degraded in serum of hemodialysis patients³⁹ and in patients after AMI especially after 12 hours of onset of symptoms.⁵⁷ These and other cTn degradation results⁵⁷⁻⁶¹ were disputed because the antibodies of the commercial immunoassay were different from the Western Blots.⁶² By contrast, Fahie-Wilson and colleagues reported the predominant forms of cTnT being free, unbound cTnT both in patients after AMI⁶² and in patients with kidney failure.⁶³ They used gel filtration chromatography (GFC) to study the non-covalent complexation of cTn in serum, as described before.⁶⁴ However, since GFC separation takes place under non-denaturing conditions, molecular weight assignment of cTn elution profiles should not be based solely on globular protein standards. This is especially important for elongated structures like cTnT as we discussed before.^{65,66}

In *chapter 10* we extensively calibrated the Sephacryl S-100 GFC column, which was also used by Fahie-Wilson and colleagues, using human purified cTn TIC complex and free cTnT, cTnI, and cTnC. These elution profiles proof that this column is unable to separate cTnT in TIC complex from free, unbound cTnT, as measured by using the 4th generation cTnT and Axsym cTnI assay and confirmed with Western Blot analysis using the same commercial antibodies. Next, we evidently showed that cTnT in the blood circulation after AMI is completely degraded into mainly 15-17 kDa cTnT fragments. We furthermore confirm that cTnI is predominantly present in the cTn I-C complex^{62,67} and, when using Axsym antibodies, only partly degraded.

The clinical impact of these new insights depends on future research. First, cTnT degradation should be validated in a larger patient population. Secondly, characterization of cTnT in plasma samples will show whether degradation happens intra- or extracellularly. Thirdly, disease specific degradation or post-translational modifications might exist.^{68,69} Finally, although only one manufacturer is on the market for cTnT, assay calibration and quality controls for inter- and intra-laboratory variability might improve using patient serum instead of purified cTnT spiked in serum or buffer.

Directions for future research

As can be concluded from this thesis, lowering the detection limit of cTn assays has led to the improved identification of patients at cardiovascular risk. However, the hs-cTn assays appeared to be especially useful for the “rule out” of acute coronary syndrome. It is now of great clinical relevance to minimize false-positively assigned subjects and thus to improve

the distinction of a healthy cardiac condition from one with preliminary signs of cardiovascular disease.

In-depth future research should therefore focus on the molecular mechanism when and in what form cTn is released from damaged myocytes. The release of cTn at the cellular level was started by Hessel et al^{26,70} in primary rat cells and in our group in HL1 cells (Jacobs, PhD thesis, Maastricht University, 2012). Exposure of these cells to anoxia or electrical stimuli (tachypacing) revealed a simultaneous release of cTn and cytosolic LDH. This indicates that myocardial cell membranes either after ischemia or prolonged exercise are ruptured irreversibly via the necrotic cell death pathway. It has to be noticed, however, that already a significant amount of cTn was lost intracellularly before it was detected in the medium. Since this was not the case for LDH, this might suggest that intracellular breakdown of cTn was started before the onset of necrosis. More extensive research should validate the different phases of cell death (apoptosis, necroptosis, and necrosis) in the ischemia and exercise models⁷¹⁻⁷³ to demonstrate in detail whether cTn release differs for the different triggers. There should also be focused on the reversibility of cell viability, to see whether cTn release takes place in the cell death pathway before or after the point-of-no-return. Ideally, it would be preferable to visualize leakage of cTn in individual myocytes using molecular imaging.⁷⁴ Piper et al showed that reversible injury happens via blebbing,^{27,75} in which the cytosolic cell content is released via the formation of vesicles. Blebbing has indeed been shown in apoptotic cells and not in necrotic cells,^{71,75} but has not been related to cTn release yet.

Furthermore, certain cTn forms or fragments might be associated with particular conditions. As hypothesized in the introduction, exercise-induced cTn elevations are thought to be characterized by intact, cytosolic cTn only. We put much effort in lowering the detection limit of our Western Blot method to characterize the cTn structures released after marathon running. Unfortunately, until now, we reached a detection limit for cTnT in serum around 0.2 µg/L. Also the identification of cTn fragments using mass spectrometry remains limited due to the complexity of blood.⁷⁶ Current mass spectrometric methods that measure low-abundant proteins in blood use sophisticated nano-flow instrumentation and only focus on small parts of the protein (1-4 tryptic peptides).^{77,78} Finally, apart from these technical challenges, other modifications of interest might be the phosphorylation status of cTnI,⁶⁸ cTnT,⁷⁹ and other sarcomeric phosphoproteins⁸⁰ in relation to calcium sensitivity and thereby cardiac contraction in general.

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Samenvatting

Hart- en vaatziekten zijn de belangrijkste oorzaak van overlijden wereldwijd. Het grootste aandeel wordt veroorzaakt door ischemische ziekten, zoals een acuut myocard infarct (AMI). Eigenschappen van de ideale biomerker om AMI te diagnostiseren zijn specificiteit voor hartschade, een vroege detectie na het ontstaan van de eerste symptomen en een lange halfwaardetijd. Op dit moment voldoen de cardiale troponines (cTn), cardiale troponine T (cTnT) of I (cTnI), het meest aan deze criteria. De troponines zijn eiwitten die een regulerende functie hebben in het contractiemechanisme van het hart. Volgens de meest recente internationale richtlijnen kan de diagnose AMI gesteld worden wanneer sprake is van een stijging of daling van cTn concentratie met tenminste één waarde boven de 99^{ste} percentiel van een referentie/controle groep, samen met andere klinische bevindingen en beeldvorming.

De huidige cTn immunoassays zijn echter niet (of met onvoldoende precisie) in staat om cTn concentraties te detecteren in de bloedcirculatie van gezonde personen en de 99^{ste} percentiel concentratie als afkapwaarde kan daarom niet behoorlijk bepaald worden. Bovendien zijn er sterke verschillen in cTnI resultaten tussen verschillende laboratoria en klinische studies. Er zijn namelijk meer dan 20 cTnI assays commercieel verkrijgbaar die tot wel 20 maal kunnen variëren in uitslagen, dit in tegenstelling tot de cTnT assay waar nog altijd patent op zit. Tenslotte zijn er tegenstrijdige resultaten beschreven betreffende de structuur van cTn in de bloedcirculatie. In dit proefschrift trachten we de analytische beperkingen van de huidige cTn metingen te overwinnen.

– Meetbare troponine concentraties bij gezonde personen

Zeer recentelijk zijn er verbeteringen bereikt in het lage meetbereik van de cTn assays. Dit heeft geleid tot de zogenaamde hoog-sensitieve (hs) cTn immunoassays zoals bediscussieerd in *hoofdstuk 2*. Wij hebben als eerste gepubliceerd over toepassing van de hs-cTnT assay in gezonde personen. In *hoofdstuk 3* hebben we laten zien dat cTnT concentraties in de bloedcirculatie van deze gezonde referentiepopulatie een typisch gaussische verdeling heeft met een 99^e percentiel afkapwaarde van 16 ng/L. We ontdekten een verschil in concentratie tussen mannen en vrouwen, terwijl een relatie met leeftijd minder duidelijk was. Waarom cTn bij gezonde personen in bloed kan worden aangetoond is nog onduidelijk. Voorheen

werd verondersteld dat myocyten vanaf geboorte bestaan en dat veroudering en ziekten leiden tot verlies van myocyten. Recent onderzoek heeft echter aangetoond dat aanmaak van nieuwe myocyten wel mogelijk is.

Desalniettemin, de hs-cTnT assay was de enige commercieel verkrijgbare cTn assay die de diagnostische afkapwaarde kon meten met de aanbevolen precisie (variatie coëfficiënt <10%), zoals besproken in *hoofdstuk 2 en 3*. Het blijft vooralsnog onduidelijk of deze richtlijn in precisie noodzakelijk is voor diagnostische doeleinden. Voor prognostische doeleinden zou het echter veel belangrijker kunnen zijn om het hele referentiegebied te meten met optimale precisie en om geslachts- en leeftijdsafhankelijke afkapwaarden toe te passen.

– Nieuwe inzichten in het lage meetbereik van troponine

Met de huidige cTn assays werden af en toe cTn concentraties gemeten bij personen zonder duidelijke klinische aanwijzingen voor een AML. Dit zou mogelijk kunnen duiden op het feit dat cTn kan vrijkomen uit beschadigde myocyten zonder dat necrose van de hartspiercel is opgetreden. Doordat wij als een van de eersten beschikten over de hs-cTnT assay waren wij in de gelegenheid om verschillende populaties met een cardiaal risico te bestuderen, met cTnT concentraties in het lage meetbereik.

Inspannings-geïnduceerde verhoging van cTn concentraties zijn nu en dan gemeten na langdurige inspanning maar in de afwezigheid van klinische symptomen. Zoals bediscussieerd in *hoofdstuk 4* was dit ook afhankelijk van het type inspanning, de gebruikte assay, en de daarbij gekozen afkapwaarde. In *hoofdstuk 3 en 4* laten we zien dat cTnT concentraties na het lopen van een marathon waren gestegen in nagenoeg alle lopers (86%) en één dag na de loop weer zakten. Deze inspannings-geïnduceerde verhoging van cTn konden we niet verklaren door uitdroging (*hoofdstuk 3 versus 4*) of door een verminderde renale klaring (*hoofdstuk 5*). We vonden een positieve associatie met de gelopen afstand (*hoofdstuk 4*) en een negatieve associatie met training (*hoofdstuk 3*). Dit alles suggereert dat cTn verhoging door inspanning symptomatisch is voor cardiale schade en dat met voldoende training het hart hiertegen wordt beschermd.

Daarnaast blijft het onduidelijk waarom patiënten met terminale nierinsufficiëntie een chronisch verhoogde cTn concentratie vertonen, of dit komt door cardiale ziekte of door verminderde of zelfs afwezige renale klaring. *Hoofdstuk 6* beschrijft dat cTnT concentraties waren verhoogd tot boven de 99^{ste} percentiel afkapwaarde in nagenoeg al deze patiënten

(94%). Opvallend was dat de patiënten met een historie van cardiovasculaire ziekte significant hogere cTnT concentraties vertoonden met een hogere intra-individuele variabiliteit in vergelijking tot de patiënten zonder deze historie. Dit doet vermoeden dat de chronische cTn verhoging wordt veroorzaakt door een combinatie van zowel coronaire als renaal lijden.

De identificatie van patiënten met coronaire lijden blijft een grote uitdaging. Traditionele risicofactoren, predictiemodellen, en biomerkers worden tot nu toe beperkt toegepast. In *hoofdstuk 7* hebben we patiënten bestudeerd met stabiele klachten van pijn-op-de-borst. We vonden een significante associatie tussen cTnT concentraties in de circulatie en de mate van atherosclerose in de coronaire arteriën zoals gedetecteerd met computed tomografie. Een dergelijke associatie vonden we niet voor de biomerkers NT-proBNP en hsCRP. Onderzoek naar de overlevingsduur in *hoofdstuk 8* bevestigde inderdaad dat cTnT was geassocieerd met het krijgen van een cardiaal event (revascularisatie, acuut coronaire syndroom, of cardiaal overlijden). We vonden meer dan drie maal zoveel cardiale events in patiënten met een hs-cTnT concentratie in het vierde kwartiel (>6.7 ng/L), ook wanneer gecorrigeerd voor traditionele risicofactoren. In mindere mate vonden we zelfs een verbeterde classificatie bovenop de detectie van coronaire plaque met computed tomografie.

Alles te samen ondersteunen de resultaten in dit proefschrift de hypothese dat elke meetbare cTn concentratie moet worden geassocieerd met cardiaal lijden, dat te allen tijde vermeden dient te worden.

– Harmonisatie en standaardisatie van troponine assays

Harmonisatie- en standaardisatiepogingen van de verschillende cTnI assays laten nog zeer te wensen over, ook met gebruik van het gezuiverde en goed gekarakteriseerde standaard referentiemateriaal NIST SRM 2921. In *hoofdstuk 9* laten we haar instabiliteit zien na standaard additie in serum en heparineplasma, wat beperkingen geeft in de harmonisatie van cTnI assays. Op dit moment lijkt cTn-positief patiëntenmateriaal de voorkeur te hebben als harmonisator, ondanks dat dit moeilijk te verkrijgen is en minder goed is gespecificeerd en gekarakteriseerd.

– **Volledig gedegradeerde troponine T in de bloedcirculatie**

Tegenstrijdige resultaten zijn beschreven over de structuur van cTn in de bloedcirculatie. Wij laten in *hoofdstuk 10* duidelijk zien dat cTnT na een AMI volledig is gedegradéerd in serum. Daarnaast bevestigen we dat cTnI met name voorkomt in het cTn I-C complex en slechts gedeeltelijk is gedegradéerd. Klinische consequenties zijn afhankelijk van een validatie studie in een grotere AMI populatie en of er eventueel ziekte afhankelijke fragmenten of fragmentpatronen geïdentificeerd kunnen worden.

– **Richtingen voor toekomstig onderzoek**

Het is van groot klinisch belang om fout-positieve toekenning van patiënten met cardiaal lijden te minimaliseren. Toekomstig onderzoek moet zich daarom richten op het nog beter onderscheid kunnen maken tussen een gezonde myocyte en een myocyte met de eerste tekenen van cardiovasculair lijden. Dit kan bereikt worden door bijvoorbeeld het moleculaire mechanisme, wanneer en in welke vorm cTn vrijkomt uit beschadigde myocyten, verder uit te diepen of door een nog betere of complementaire cardiale biomarker te ontdekken.

Summary

Cardiac and/or vascular disease is the leading cause of death worldwide. The highest incidence accounts for ischemic diseases, like an acute myocardial infarction (AMI). Specifications of a perfect biomarker to diagnose AMI are heart specificity, early detection after first symptoms, and a long half-life. The current preferred biomarkers are cardiac troponins (cTn), either cardiac troponin T (cTnT) or I (cTnI), being regulatory proteins of the cardiac contractile apparatus. As recently redefined by the international guidelines, AMI is diagnosed with the detection of a rise and/or fall of cTn with at least one value exceeding the 99th percentile of a reference control group, together with clinical and imaging findings.

Up to now, however, the contemporary cTn immunoassays were unable to detect cTn concentrations in the blood circulation of healthy individuals (or with insufficient precision) and the 99th percentile cutoff concentration could thus not be properly determined. In addition, comparison of cTnI results among different laboratories and clinical studies remained limited since more than 20 cTnI assays are commercially available, in contrast to the highly patented cTnT assay, that deviate up to 20-fold in assay result. Finally, conflicting results have been reported on the structural aspects of cTn in the blood circulation. In this thesis, we attempt to overcome these analytical limitations in the cTn measurements.

– Measurable troponin concentrations in healthy individuals

Improvements in the lower cTn measuring range have lately been achieved, resulting in the so called high-sensitivity (hs) cTn immunoassays as reviewed in *chapter 2*. By using the hs-cTnT assay, as described in *chapter 3*, we were the first who showed that cTnT concentrations in the blood circulation of a healthy reference population follow a typical gaussian distribution and we reported the 99th percentile cutoff at 16 ng/L. We found a significant difference between sexes, while an age effect was not that obvious. The reason for the presence of cTn concentrations in healthy individuals remains unclear. It was previously considered that the number of myocytes is established at birth and is lost with age or disease, though recent research has shown the existence of (slow) renewal of myocytes. Nevertheless, the hs-cTnT assay was the only commercially available cTn assay that measured the diagnostic cutoff concentration with sufficient precision according to the guidelines, as can be concluded from *chapter 2 and 3*. It remains to be elucidated, however,

whether the predicate “guideline acceptance” is required for diagnostic purposes. For prognostic and screening purposes, it might become more important to measure with optimal precision across the complete reference range and to apply sex- and age-specific cutoff concentrations.

– **New insights in the lower troponin measuring range**

With the contemporary assays, measurable cTn concentrations have occasionally been noticed without the clear evidence of AMI. This raises the possibility that cTn can be released from damaged myocytes but without typical myocardial necrosis. The availability of the hs-cTnT assay enabled us to investigate several populations at (low) coronary risk in more detail.

Exercise-induced cTn concentrations have occasionally been noticed after prolonged strenuous exercise but in the absence of clinical symptoms. This turned out to be dependent on the type of exercise, the assay, or the cutoff used. As described in *chapter 3 and 4*, after marathon running, post-run cTnT concentrations elevated to above the 99th percentile cutoff in almost all runners (86%) and returned to baseline one day after the run. The exercise-induced cTnT concentrations could neither be clarified by a diminished renal function (*chapter 5*), nor by dehydration (*chapter 3 versus 4*). We furthermore found a positive association to running distance (*chapter 4*) and a negative association to experience (*chapter 3*). Overall, this indicates that exercise-induced cTnT elevations are symptomatic for minor myocardial injury and that sufficient training protects the heart from real cardiac damage.

In addition, it remains unclear whether chronic cTn elevations in patients with End-Stage Renal Disease are caused by cardiac disease or by the decrease or absence of renal clearance. *Chapter 6* describes that cTnT concentrations were above the 99th percentile cutoff in almost all patients (94%). Interestingly, in the patients with a history of cardiovascular disease, significantly higher cTnT concentrations were found with higher intra-individual variability compared to patients without this history. This might suggest that the chronic cTn elevations are caused by a combination of coronary and renal disease.

The identification of patients at coronary risk remains an ongoing challenge. Traditional risk factors, prediction models, and biomarkers turned out to be of limited use so far. In *chapter 7*, we investigated patients with stable chest-pain. We found a significant association between circulating cTnT concentrations and the extent of coronary atherosclerotic plaque

as assessed with coronary computed tomography. Such an association we did not find for NT-proBNP and hsCRP. Survival analysis in *chapter 8* indeed confirmed that cTnT was associated with the occurrence of a cardiac event (revascularization, acute coronary syndrome, or cardiac death). We found more than three as much cardiac events in patients with hs-cTnT concentrations in the fourth quartile (>6.7 ng/L), also when hs-cTnT was corrected for traditional risk factors. To a lesser extent, improved risk classification was even obtained on top of coronary plaque assessment.

Overall, the results shown in this thesis support the hypothesis that any measurable cTn concentration is associated with cardiac injury that should be avoided at any time.

– Harmonization and standardization of troponin assays

Harmonization and standardization efforts for cTnI assays have been of limited success when using the purified and highly characterized standard reference material NIST SRM 2921. In *chapter 9*, we showed its instability when spiked in serum and heparin plasma, and its limited ability in harmonization of cTnI results. For now, cTn-positive patient material seems preferable, despite the fact that it is difficult to obtain and less specified and characterized.

– Fully degraded troponin T in the blood circulation

Conflicting results have been reported on the structural aspects of cTnT in the blood circulation. We evidently show in *chapter 10* that cTnT after AMI is completely degraded in serum. Furthermore, we confirm that cTnI is predominantly present in the cTn I-C complex and only partly degraded. Clinical consequences are dependent on the validation in a larger AMI population and whether disease specific modification or modification-patterns exist.

– Directions for future research

It is of great clinical relevance to minimize false-positively assigned subjects at coronary risk. Future in-depth research should therefore focus on improving the distinction between a healthy myocyte and preliminary signs of cardiovascular disease. This could possibly be achieved by unraveling the molecular mechanism when and in what form cTn is released from myocytes, or by identifying an even better or complementary cardiac biomarker.

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Lieve Tim, jouw brede schouders geven mij letterlijk en figuurlijk de steun die ik nodig heb en ik hoop dat dat voor eeuwig zo mag blijven.

Curriculum vitae

Alma Mingels was born on November 14th, 1981 in Veldhoven, the Netherlands. She finished her secondary education (atheneum) in 2000 at the S.G. Sophianum in Gulpen. In the same year, she started the study of Biomedical Engineering at the Eindhoven University of Technology. Her graduation research project was performed at the Laboratory of Macromolecular and Organic Chemistry under supervision of prof. dr. Bert (E.W.) Meijer, dr. Maarten Merkx, and ing. Joost van Dongen. She investigated (modified) protein structures using liquid chromatography and mass spectrometry. During her Master, she also did an internship at the University of Delaware, United States of America, in the group of prof. Kristi Klück. Subsequently, in 2007 she started as a PhD student at the department of Clinical Chemistry of the Maastricht University Medical Center under supervision of prof. dr. Marja van Dieijen-Visser and dr. Will Wodzig. This was in close collaboration with the Cardiovascular Research Institute Maastricht (CARIM) at the Maastricht University, where she completed several additional courses in statistics and cardiovascular diseases. The most important results are described in this thesis and have been presented in part at (inter)national congresses like the IFCC WordLab 2011 in Berlin. Chapter 7 was awarded with an abstract prize in 2010, handed out by the Dutch Society of Clinical Chemistry (NVKC). She will continue her work at the department of Clinical Chemistry in Maastricht, where as of December 2011 she started her residency in Clinical Chemistry.

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