Cardiac troponins

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Cardiac troponins: State of the (He)Art

towards optimization of interpretation

Cardiac troponins: State of the (He)Art - towards optimization of interpretation
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Cardiac troponins: State of the (He)Art

towards optimization of interpretation

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht, op gezag
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Contents

Chapter 1	General introduction	7
Chapter 2	Strong diurnal rhythm of troponin T, but not troponin I, in a patient with renal dysfunction	21
Chapter 3	Diurnal rhythm of cardiac troponin: consequences for the diagnosis of acute myocardial infarction	27
Chapter 4	Twenty-four-hour biological variation profiles of cardiac troponin I in subjects with or without chronic kidney disease	63
Chapter 5	Origin of cardiac troponin T in chronic kidney disease	77
Chapter 6	Mass spectrometric identification of cardiac troponin T in urine of patients suffering from acute myocardial infarction	97
Chapter 7	Large variation in measured cardiac troponin T concentrations after standard addition in serum or plasma of different individuals	115
Chapter 8	Combining cardiac troponin I and cardiac troponin T for the early diagnosis of acute myocardial infarction	121
Chapter 9	Effect of a six-month resistance-type exercise training program on the course of cardiac troponin T levels in (pre)frail elderly	151
Chapter 10	Effect of exercise training on the course of cardiac troponin T and I levels: three independent training studies	157
Chapter 11	Prognostic value of basal cardiac troponin levels on mortality in the general population	173
Chapter 12	General discussion	197
Supplemental	Summary, Samenvatting	207
	Valorization	217
	Dankwoord, Curriculum Vitae, List of publications	221

General introduction

Acute myocardial infarction: a major burden of morbidity and mortality

Despite the decreasing prevalence of cardiovascular disease in the Western World¹, acute myocardial infarction (also known as heart attack) remains an important burden of morbidity and mortality worldwide². In the Netherlands, with approximately 17 million inhabitants, each year 70 000 people are diagnosed with acute myocardial infarction^{3,4}. This etiology substantially contributes to mortality –acute myocardial infarction is considered as the cause of death in approximately 5 000 people yearly–, morbidity and related public health expenses^{3,5-7}.

Chest pain, an important indicator of acute myocardial infarction, is reported by approximately 10% of all subjects visiting the emergency department⁸, a population of which 15-20% does actually have an acute myocardial infarction⁹. Correct and rapid distinction of subjects with and without myocardial infarction assists the allocation of treatment to those who need it and prevents unnecessary resource use. Therefore, accurate diagnostics may, besides prevention and improved treatment, contribute to lower the burden of acute myocardial infarction⁹⁻¹⁶.

The perfect biomarker for the diagnosis of acute myocardial infarction

The diagnosis of acute myocardial infarction is based on the presence of characteristic clinical symptoms, like chest pain, accompanied by electrocardiogram (ECG) changes or by elevated levels of cardiac proteins (nowadays cardiac troponins)^{17,18}. Whereas deviations on the 12-lead ECG are only clearly present in 30-40% of patients with a myocardial infarction¹⁹⁻²², cardiac biomarkers in the blood play an important role in the diagnosis of acute myocardial infarction. During the last decades, an evolution in biomarkers used for the diagnosis has taken place. Subsequently, aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatine kinase (CK), creatine kinase MB isoenzyme (CK-MB), and cardiac troponins were the biomarkers used in a clinical setting to detect acute myocardial infarction²³. Time and again, a biomarker was replaced by another marker with more 'desirable characteristics'²⁴⁻²⁶. Table 1 shows an overview of biomarker characteristics that are considered to be important^{26,27}.

Figure 1. Sensitivity and specificity depend on the cut-off concentration. If there is an overlap in biomarker concentrations of people with and without a certain disease, it is not possible to have a test with a 100% sensitivity and a 100% specificity. In this case a higher sensitivity will be accompanied by a lower specificity, and vice versa. A cut-off concentration with a high sensitivity is suitable for the safe rule-out, and one with a high-specificity for the rule-in of subjects.

In general, the preferred characteristics of cardiac biomarkers strongly depend on their intended use²⁸. In case of the diagnosis of acute myocardial infarction, its accurate recognition – the allocation of subjects in a group with and without this specific etiology – is the main goal. Therefore, an important aspect for the evaluation of a biomarker for the diagnosis of acute myocardial infarction is the accuracy: The amount of agreement between the results of the biomarker test under evaluation (index test) and the gold standard diagnosis^{29,30}. The accuracy is often represented by the sensitivity (detection of true-positives: a positive result when the disease is truly present) and specificity (detection of true negatives: a negative result when the disease is truly absent) at a selected cut-off point²⁷, or by the area under a receiver operating characteristic (ROC) curve³⁰. Hypothetically, the ideal test makes the perfect distinction between subjects with and without myocardial infarction, and should, therefore, present both a maximum, 100%, sensitivity and specificity. However, in reality, there is often an overlapping range in biomarker concentrations between subjects with and without disease. Hence, the definition of a cut-off point is required. This causes a high sensitivity (almost all subjects that truly have an acute myocardial infarction have a positive test result) at the expense of lower specificity (a high number of false positive test results), or the other way around (Figure 1).

Table 1. Desirable characteristics of biomarkers for the diagnosis of acute myocardial infarction^{26,27}

General features	Acceptable to the patient
	Add to clinical assessment
	Ability to influence therapy
	Ability to improve patient outcome
	Cost-effective
	Applicable to (almost) all patients, always
	High myocardial specificity
	Release proportional to injury size or ischemic burden
	Suitable for early diagnosis: immediate release
	Permit long time window for diagnosis
	Permit diagnosis of recurrent ischemia
Test features	Ability to detect even small infarctions
	No special sample preparation needed
	Rapid test / Short turnaround time
	Test readily available
	Easy interpretable
	Single measure representative
Assay / measurement features	Internationally standardized
	High accuracy
	High precision
	Compatible with existing logistics

Cardiac troponins as biomarkers for acute myocardial infarction

According to the universal definition, acute myocardial infarction is cardiomyocyte death due to prolonged myocardial ischemia, an imbalance between oxygen demand and supply¹⁷. As a result of this process of ischemic injury and subsequent cell necrosis, cardiomyocyte content is released into the peripheral blood stream. After a finite period of time, cardiac ischemia will be reflected by increasing concentrations of several biomarkers, like cardiac troponins, in the peripheral blood^{31,32}.

Cardiac troponins form part of the intracellular content of cardiomyocytes. The cardiac troponin complex, consisting of a troponin T, I and C protein, fulfills a key role in cardiomyocyte contraction by regulation of the binding between the actin and the myosin filaments. In general, troponins are structurally bound to the actin filaments of the contractile apparatus of the cardiomyocytes³³. In addition, it has been suggested that a smaller portion (3-8 %) may be present as unbound protein in the cytosol³⁴⁻³⁶. Two proteins of the cardiac troponin complex, cardiac troponin T (cTnT) and cardiac troponin I (cTnI), are

exclusively expressed in the heart, and are therefore considered as cardiac specific biomarkers^{33,37-39}.

Figure 2. Release of cardiac troponins following acute myocardial infarction. In the healthy heart, cardiac troponins are limited to the cardiomyocytes where they play an essential role in muscle contraction coupling³³. In cardiac ischemia, altered cell membrane integrity causes release of troponin into the circulation as the contractile apparatus breaks down^{32,35}.

The detection of cardiac troponins: from conventional to high-sensitivity assays

The discovery of cardiac specific troponins triggered the interest in cardiac troponins as cardiac specific biomarkers. The first immunoassay for the detection of cTnI in serum was developed in 1987⁴⁰, followed by the first cTnT assay in 1989⁴¹. These initial assays were characterized by several ‘undesirable properties’ such as an extended turn-around time, low analytical sensitivity and cross-reactivity with skeletal muscle troponins²³. In the subsequent years, the continuous evolution in assays led to improvement of cardiac specificity, analytical sensitivity, and assay precision at the low measurement range^{23,42-45}. In 2010, the first high-sensitivity assay was implemented and in the following years multiple high-sensitivity assays became commercially available and are now widely used in most Western European countries^{46,47}. A troponin assay can be considered as a high-sensitivity assay if at least 50% of the general population exhibits measurable levels below the 99th percentile⁴⁸⁻⁵².

As shown in table 2, there are multiple conventional and high-sensitivity cTnI assays in the marketplace and, due to intellectual property rights, there is only one manufacturer that produces cTnT assays. Because cTnT and cTnI are different proteins with distinct

characteristics, their measured concentrations will differ in individuals^{33,53,54}. Nevertheless, it is important to consider that this is also the case for cTnI measured with different assays since the assays are not standardized and have various combinations of antibodies, incubation conditions, and detection technology⁴⁹. As a result, the guidelines on the diagnosis of acute myocardial infarction use different, assay-specific cut-off values^{18,55}.

Table 2. Analytical characteristics of the troponin assays used throughout this thesis^{49,50,52}

Assay	Manufacturer	LoB ^a , ng/L	LoD ^b ,ng/L	99 th percentile, ng/L	CV at 99 th percentile, %
<i>Conventional assays</i>					
cTnI	Abbott	NA	9	28	14
cTnI	Beckman Coulter	<10	10	40	10
cTnT	Roche	10	NA	<10	NA
<i>High-sensitivity assays</i>					
hs-cTnI	Abbott	0.7 – 1.3	1.1 – 1.9	26.2	4.0 – 6.0
hs-cTnI	Siemens	NA	0.5	9	<10
hs-cTnI	Singulex	NA	0.09	10.1	9.0
hs-cTnT	Roche	3	5	14	8.0

^aThe highest apparent analyte concentration expected to be found when replicates of a sample containing no analyte are tested. ^bLowest analyte concentration likely to be reliably distinguished from the LoB and at which detection is feasible⁵⁶.

The interpretation of cardiac troponins in the era of high-sensitivity assays

In the year 2000, before the introduction of the high-sensitivity assays, cardiac troponins were designated as the preferred biomarkers for the diagnosis of acute myocardial infarction^{17,57-60}. According to the first consensus document, a maximal concentration of cTnT or cTnI exceeding the decision limit (99th percentile upper reference limit of a healthy reference population) on at least one occasion during the first 24 hours after the index clinical event was considered as a biochemical indicator for myocardial injury⁵⁷. To be more specific in the detection of acute coronary events, the interpretation of cardiac troponins was further specified as a dynamic elevation, rise and/or fall, of cardiac troponin with at least one value above the 99th percentile (if compatible with the clinical presentation)^{50,58}. This definition of acute myocardial infarction remained the same after the introduction of the high-sensitivity assays, with the proviso that the increased sensitivity of the new assays

allowed a shorter time interval between two consecutive cardiac troponin assessments (three instead of six hours), and hence allows a faster diagnosis^{9,61-64}.

Figure 3. 0 h/1 h rule-in and rule-out algorithm. Based on assays-specific cut-off values acute myocardial infarction can be rapidly ruled-in and ruled-out at presentation or within one hour. Patients who do not qualify for ‘rule-out’ or ‘rule-in’ require a further high-sensitivity cardiac troponin assessment (e.g. at 3 h)¹⁸. *Only applicable if chest pain onset >3h

In 2015, the European Society of Cardiology presented its new guidelines with two alternative algorithms for the diagnosis of acute myocardial infarction based on high-sensitivity cardiac troponins; a 0 h/3 h algorithm and a 0 h/1 h algorithm. Whereas the interpretation of cardiac troponin levels in the 0 h/3 h algorithm is not substantially different from that of the previous 0 h/6 h algorithm (one value above the 99th percentile and a rise/fall defined by a delta-change-value), the 0 h/1 h algorithm is based on a different approach (Figure 3)^{18,65-70}.

The expense and opportunities of high-sensitivity cardiac troponin assays

The ability to measure previously undetectable concentrations of cardiac troponins expedited the clinical use of lower cut-off levels and significantly increased the sensitivity to detect (small) acute myocardial infarctions^{9,12}. However, the increase in sensitivity due to lower cut-off values came at the expense of a decreased specificity¹²: Elevations of cardiac troponin levels turned out not to be specific for acute myocardial infarction, but were also associated with several other acute and chronic conditions like myocarditis, heart failure, pulmonary embolism, stroke, stable coronary artery disease, type 2 diabetes mellitus, decreased renal function and as the result of prolonged exercise^{32,71-80}. Since the cut-off values of the current diagnostic algorithms are specifically aimed at not missing subjects with acute myocardial infarction (thus the sensitivity is rather high), this may lead to an overdiagnosis or even an overtreatment of patients with persistently elevated cardiac troponin concentrations^{81,82}. Therefore, the further optimization of interpretation of cardiac troponin concentrations in diagnosis seems to be indicated and prompts questions like: How may these conditions affect the diagnosis of acute myocardial infarction? And, how can diagnostic algorithms, or even assays, be adjusted for these effects?

Notwithstanding, the introduction of the high-sensitivity assays also facilitated the exploration of basal cardiac troponin concentrations and their characteristics in larger cohort studies. Several of these studies showed that (slightly, even below the 99th percentile) elevated cardiac troponin concentrations reflect future or (sub)clinical disease, and are associated with an unfavorable prognosis, an increased risk for cardiovascular disease and mortality^{77,78,83-87}. These findings indicate a potential role of cardiac troponins in secondary prevention. Though, there is no current consensus on the interpretation, treatment or follow-up of subjects with (slightly) elevated cardiac troponins without acute clinical symptoms. Future research in this field is required.

Lastly, the capability to measure cardiac troponin concentrations in a majority of subjects also facilitated the large-scale comparison of cTnT and cTnI concentrations. Although the current guidelines equally recommend both cardiac troponins for the diagnosis of acute myocardial infarction¹⁸, it became clear that not only the biochemical, but also the biological

characteristics (for example the presence of a circadian rhythm and the variation in a healthy reference population) differ^{33,88-91}. This leads to additional questions regarding dissimilarities between both cardiac troponins in diagnosis and prognosis.

Outline of this thesis

This thesis focuses on research that is related to the optimization of interpretation of cardiac troponin concentration for both diagnosis and prognosis.

Chapter 2 presents a case which illustrates four important challenges we face in the interpretation of cardiac troponins (discussed in the following chapters): First, the variation in cardiac troponin concentrations over the day (*chapters 3 and 4*). Second, the effect of impaired renal function on cardiac troponin levels (*chapters 5 and 6*). Third, the differences between cTnT and cTnI (*chapters 7 and 8*). Fourth, persistently elevated basal cardiac troponin concentrations and their clinical significance (*chapters 9, 10 and 11*).

Chapter 3 shows a diurnal rhythm that is present for cTnT, but not for cTnI, and examines its effect on the diagnosis of acute myocardial infarction.

Chapter 4 quantifies the hour-to-hour biological variation in cTnI and its time-dependency over 24 hours in subjects with or without chronic kidney disease.

Chapter 5 employs the circadian rhythm in cTnT concentrations, described in *chapter 3*, to examine if the kidneys, by impaired renal clearance, or the heart, by increased release, can be considered as the main driver behind elevated cTnT concentrations in chronic kidney disease patients.

Chapter 6 uses mass-spectrometry to study if cTnT can be identified in the urine of patients with acute myocardial injury.

Chapter 7 presents a series of experiments which examine the variation in measured concentrations after the addition of a standardized amount of cardiac troponins to the serum and plasma of different individuals.

Chapter 8 explores whether combining cTnT and cTnI might overcome limitations of individual cardiac troponins, and increase the number of rule-outs after a single blood draw.

Chapters 9 and 10 describe the prevalence of basal elevated cardiac troponin concentrations in (frail) elderly, and assess the effects of prolonged exercise training on these levels.

Chapter 11 contains a meta-analysis to the prognostic value of cTnT and cTnI on cardiovascular and all-cause mortality in the general population.

The final chapter, *chapter 12*, contains a general discussion of the work presented in this thesis and provides directions for future research.

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Strong diurnal rhythm of troponin T, but not troponin I, in a patient with renal dysfunction

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Introduction

Cardiac troponin T (cTnT) and I (cTnI), measured with high-sensitive assays, are the preferred biomarkers for the diagnosis of acute myocardial infarction^{1,2}. Serial cardiac troponin measurements, and the interpretation of a 'troponin rise-or-fall' in patients with chest pain discriminate between acute and chronic troponin elevations¹. This diagnostic concept assumes that troponin levels in patients without acute cardiac injury fluctuate randomly around an individual's homeostatic setpoint. However, it has recently been demonstrated that cTnT exhibits a diurnal rhythm^{3,4}. The amplitude of the diurnal cTnT rhythm and its diagnostic implication in patients with chronically elevated cTnT levels are unexplored. Also, whether cTnT and cTnI are equally affected by rhythmic diurnal oscillation is unknown.

Methods

A 63-year-old woman with decreased renal function (estimated Glomerular Filtration Rate (eGFR) 14 ml/min/1.73m²) was included in our observational study to the effect of renal function on the diurnal rhythm of cTnT and cTnI (ClinicalTrials.gov: NCT02210897). This study was carried out according to the principles of the Declaration of Helsinki and approved by the Institutional Review Board and Ethics Committee of Maastricht University Medical Center. The participant provided written informed consent. Her medical history included nephroangiosclerosis, dyslipidemia, hypertension, pacemaker implantation for chronotropic incompetence, hyperparathyroidism caused by parathyroid adenoma, aortic insufficiency, and gout. Chronically elevated cardiac troponin levels in this patient were first observed in 2011, when she was evaluated at the emergency department after a collapse. At the time, laboratory analysis with a conventional, non-high-sensitive, assay showed an increased cTnT level (0.84 µg/L; 99th percentile <0.01 µg/L), which remained elevated during hospitalization (0.77 µg/L and 0.79 µg/L, 6 and 16 hours after presentation respectively). Coronary angiography and pulmonary CT angiography found no substrate, and the elevated cardiac troponin levels were therefore considered unrelated to an acute coronary event or pulmonary embolism. In the end, she was discharged with a diagnosis of vasovagal collapse. As part of the present study, she was restricted to the laboratory environment over a period

of 25 hours and serial venous blood samples were drawn every hour. She took her regular medication (allopurinol, amlodipine, doxazosine, ferrofumarate, furosemide, irbesartan, sodium bicarbonate, nebivolol, pantoprazole, prednisolone and sevelamer) and was completely asymptomatic during these 25 hours. High-sensitivity cardiac troponin T (hs-cTnT, Roche Cobas) and I (hs-cTnI, Abbott Architect) were measured in duplicate in all samples. Analyses were performed using IBM SPSS Statistics for Windows 23.0 (IBM Corp., Armonk, New York, USA) and GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, California, USA).

Results

In line with laboratory results from 2011, baseline hs-cTnT concentration at the start of the study, day 1 8.30 AM, was highly elevated and strongly exceeded the 99th percentile threshold (147.7 ng/L, 99th percentile = 14 ng/L). Interestingly, hs-cTnI at baseline was within the reference range (10.3 ng/L, 99th percentile = 26.2 ng/L). hs-cTnT concentrations showed a diurnal rhythm and fitted a cosine model⁵ ($R^2 = 0.90$), with a maximal concentration change over 24 hours of 50.9 ng/L. In contrast, hs-cTnI levels remained virtually unchanged over time with a maximal difference of only 2.6 ng/L over 24 hours and no visual cosine fit. The concentrations of hs-cTnT and hs-cTnI over time are shown in figure 1. hs-cTnT levels exceeded the diagnostic criteria, both baseline level and 1-hour delta-change values, of the current European Society of Cardiology (ESC) guidelines to rule-in subjects for AMI¹.

Conclusion

This observation warrants further evaluation of the diurnal troponin T rhythm in subjects with chronically elevated hs-cTnT levels, and examination of its effect on the diagnostic accuracy in patients with chronically elevated troponin levels.

Figure 1. The course of hs-cTnT and hs-cTnI in a 63-year old woman with decreased renal function. The dotted line indicates the cut-off level for the immediate ruling-in of suspected non-ST-elevation myocardial infarction without additional serial testing using high-sensitive cardiac troponin assays at presentation as recently published in the 0h/1h rule-in and rule-out algorithms of the ESC guidelines . According to this algorithm patients are ruled in the acute coronary syndrome treatment protocol if they present with a concentration of hs-cTnT (Roche) or hs-cTnI (Abbott) above 52 ng/L (immediate rule-in without additional serial testing) or when hs-cTnT (Roche) or hs-cTnI (Abbott) concentration increases in the first hour with 5 or 6 ng/l, respectively^{1,6,7}. The 1h delta values for hs-cTnT in this patient vary between 0.1 and 20.0 ng/L, and exceed the cut-off value of 5 ng/L eight times, especially around noon and in the early morning. The 3h delta values for hs-cTnT vary between 0.1 ng/L and 32.1 ng/L. The subject slept in supine position between 11.30 PM and 7.00 AM (shaded area).

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Diurnal rhythm of cardiac troponin: consequences for the diagnosis of acute myocardial infarction

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Abstract

Background

Interpretation of serial high-sensitivity troponin (hs-cTn) measurements for the diagnosis of acute myocardial infarction (AMI) assumes random fluctuation of hs-cTn around an individual's homeostatic set-point. The aim of this study was to challenge this diagnostic concept.

Methods

Study-1 examined the presence of a diurnal hs-cTn rhythm by hourly blood sampling, day-and-night, in 24 individuals without a recent history of AMI. Study-2 assessed morning versus evening diagnostic accuracy of hs-cTnT and hs-cTnI in a prospective multicenter diagnostic study of 2782 unselected patients, presenting to the emergency department with acute chest pain.

Results

In study-1, hs-cTnT, but not hs-cTnI, exhibited a diurnal rhythm, characterized by gradually decreasing concentrations throughout daytime, rising concentrations during nighttime, to peak concentrations in the morning (mean 16.2ng/L at 8:30A.M and 12.1ng/L at 7:30P.M.). In study-2, the hs-cTnT rhythm was confirmed by higher hs-cTnT levels in early-morning presenters compared to evening presenters with an adjudicated diagnosis of non-cardiac disease. The diagnostic accuracy (area under the receiver-operator-characteristic curve (AUC)) of hs-cTnT at presentation, 1-hour, and for the combination of absolute changes with presenting concentration, were very high and comparable among patients presenting early-morning as compared to evening (all AUC>0.93). Hs-cTnI exhibited no diurnal rhythm with no differences in AUC among early-morning and evening presenters.

Conclusions

Rhythmic diurnal variation of hs-cTnT is a general phenomenon that is not seen with hs-cTnI. While the diurnal hs-cTnT rhythm does not seem to affect the diagnostic accuracy of hs-cTnT for AMI, it should be considered when using hs-cTnT for screening purposes.

Introduction

The early diagnosis of acute myocardial infarction (AMI) is mandatory for the timely initiation of evidence-based therapy and based on three key diagnostic tools: clinical assessment, the ECG, and cardiac troponin (cTn), a quantitative marker of cardiomyocyte injury¹. Troponin assays target one of two proteins – cardiac troponin T (cTnT) or cardiac troponin I (cTnI) – with distinct biochemical properties, but comparable diagnostic performance². The advent of high-sensitivity cTn assays for both cTnT (hs-cTnT) and cTnI (hs-cTnI) has expedited the early diagnosis of AMI³⁻⁵, but also resulted in the detection of previously unnoticed cardiomyocyte injury in multiple other conditions such as diabetes, chronic kidney disease, frailty, or even apparently healthy elderly individuals^{6,7}. Key to accurately discriminate between acute and chronic causes of cardiomyocyte injury is serial testing and the assessment of kinetic hs-cTn changes as recommended in the current guidelines⁸⁻¹¹. A critical assumption when interpreting changes between serial measurements is that hs-cTn blood concentrations fluctuate randomly around a homeostatic set-point. This dogma of random fluctuation was recently challenged by the intriguing observation that hs-cTnT exhibited a diurnal rhythm in a small group of male subjects with type 2 diabetes¹². The diurnal hs-cTnT rhythm was characterized by peak-levels during morning hours, a gradual decrease during the day, and a rising pattern at nighttime¹².

The generalizability of the diurnal rhythm of hs-cTnT beyond male type 2 diabetes patients and whether hs-cTnT and hs-cTnI are equally affected is unknown. It is also unknown, whether -if confirmed- diurnal rhythmic oscillation in hs-cTn blood concentration affect the diagnostic accuracy of hs-cTn for AMI. This possibility is substantiated by the observation that the magnitude of physiological change over the day, exceeded clinically applied delta-change criteria for AMI in some individuals^{1,12}.

The present study was conducted to quantify the rhythmic diurnal oscillation of hs-cTnT and hs-cTnI in currently asymptomatic women and men. Next, we analyzed data from a multicenter diagnostic study, enrolling patients presenting to the emergency department with suspected AMI, to determine whether rhythmic diurnal oscillation and thereby the time of presentation affects the diagnostic accuracy of hs-cTnT and hs-cTnI.

Materials and methods

Two studies were conducted: Study 1 was a standardized biological variation study with hourly blood sampling day and night, to ascertain and quantify circadian rhythms of hs-cTnT and hs-cTnI (January 2013 to May 2014); Study 2 was a prospective diagnostic study (APACE; Advantageous Predictors of Acute Coronary syndrome Evaluation). APACE is an ongoing prospective international multicenter study aiming to advance the early diagnosis of AMI^{2,4,13,14}. Both studies were carried out according to the principles of the Declaration of Helsinki and approved by the local Institutional Review Board and Ethics Committee at each institution. Written informed consent was obtained from all participants.

Study 1: Diurnal rhythm of cardiac troponin under standardized conditions

The study population consisted of 24 individuals; 19 males and 5 females (mean age 72 ± 7 years, Supplementary Table 1). Exclusion criteria were: a history of AMI or stroke less than 12 months prior to inclusion. The diurnal hs-cTnT variation of 7 male subjects has been described before as part of a biological variation study of hs-cTnT in patients with type 2 diabetes (Supplementary Figure 1A and 2, participants 4, 5, 7, 11, 16, 20, 24)¹². To extend these pilot findings to both sexes and individuals with normal glucose homeostasis, 17 additional subjects (12 men, 5 women) were included in the present study. Participants were asked to refrain from exhaustive physical activities and exercise training 2 days before the test period. After an overnight fast, subjects arrived at the laboratory by car or public transportation. From 8.30 AM till 9.30 AM the next day, subjects were restricted to the laboratory environment and sampled hourly (8 mL) via an antecubital venous catheter. At night (11.30 PM till 7.00 AM), extension lines were used to minimize disturbance of participants' sleep. Meals were consumed at 8.30 AM, 12.30 PM and 5.30 PM (breakfast, lunch and dinner, respectively). Blood samples were collected in both serum (sensitive cTnI [s-cTnI] Beckman Coulter) and EDTA tubes (hs-cTnT Roche and hs-cTnI Abbott) and measured in duplicate.

Study 2: Diurnal rhythm of cardiac troponin and diagnostic accuracy

To investigate the clinical effects of rhythmic diurnal hs-cTn oscillations, we evaluated whether the time of presentation to the emergency department affected the diagnostic accuracy of hs-cTn for AMI in the APACE study. The study population consisted of 2782 consecutive patients older than 18 years presenting to the emergency department between April 2006 and June 2013 with symptoms suggestive of AMI with onset or peak of symptoms within the last 12 hours (Supplementary Table 2). Patients with renal failure requiring dialysis were excluded from the study.

For this analysis patients were excluded if the adjudicated diagnosis was ST-elevation MI (n=108) (as biomarkers in general do not have a role in the rapid diagnosis of ST-elevation AMI), if the final diagnosis remained unclear after adjudication and at least one cTn level was elevated (possibly indicating presence of AMI) (n=92), or if the exact presentation time was not recorded (n=48).

Adjudication of the final diagnosis and follow-up of the patients is extensively described in the Supplementary Methods. Briefly, two independent cardiologists adjudicated the final diagnosis by reviewing all available medical records - patient history, physical examination, results of laboratory testing (including cTn levels), radiologic testing, ECG, echocardiography, cardiac exercise test, lesion severity and morphology in coronary angiography - pertaining to the patient from the time of ED presentation to 90-day follow up. In situations of disagreement about the diagnosis, cases were reviewed and adjudicated in conjunction with a third cardiologist.

Cosinor rhythmometry results from study 1 were used to divide the day in two timeframes: a 'hs-cTn high level phase' (early-morning) and a 'hs-cTn low level phase' (evening). Diagnostic accuracy of hs-cTn at presentation, at 1h, and the combination of absolute changes with baseline concentration were the primary diagnostic end points, as they reflect the most important diagnostic variables in clinical practice^{1,9,10}.

Laboratory analysis

Details on the four cTn assays (hs-cTnT Roche Diagnostics, hs-cTnI Abbott Diagnostics, hs-cTnI Siemens, s-cTnI Beckman Coulter) used in this manuscript are described in the Supplementary Methods.

Statistical analysis

Diurnal troponin changes were analyzed by fitting the data to a cosine curve by using the method of cosinor-rhythmometry^{12,15}. Evidence of a diurnal rhythm was indicated by a significant cosinor model fit. Receiver-operating characteristics (ROC) curves were constructed to assess the sensitivity and specificity of hs-cTn for the diagnosis of AMI in early-morning presenters and evening presenters. The comparison of independent areas under the ROC curves (AUC) was performed as recommend by Hanley and McNeil¹⁶. Similarly, AUCs were compared in early-morning presenters and evening presenters for the prognostic accuracy of hs-cTn at presentation to predict all-cause mortality during follow-up.

All hypothesis testing was two-tailed and a p-value of less than 0.05 was considered statistically significant. Analyses were performed using IBM SPSS Statistics for Windows 22.0 (SPSS Inc, Chicago, IL) and MedCalc 10.3.4 (MedCalc Software, Mariakerke, Belgium).

Results

High-sensitivity cardiac troponin T exhibits a diurnal rhythm

To investigate the diurnal rhythm of hs-cTn, blood samples were collected hourly over a time span of 25h (8.30 AM to 9.30 AM the next day – Supplementary Figure 1). In all participants, we found rhythmic circadian hs-cTnT variation, characterized by gradually decreasing concentrations throughout daytime (mean 16.2 ng/L at 8.30 AM and mean 12.1 ng/L at 7.30 PM) and rising concentrations during nighttime, to peak concentrations in the morning (mean 16.2 ng/L at 8:30A.M. the next day - Figure 1A, Supplementary Figure 2-4). Subjects with the highest hs-cTnT concentrations displayed the largest amplitude of the diurnal oscillation, illustrated by the highly significant correlation between hs-cTnT level at 8.30 AM and the range of individual changes during the 25h period (Pearson's R^2 0.70; $p < 0.001$ –

Supplementary Figure 5). Based on the rhythmic variation, we tested the hs-cTnT data for a periodic signal using single cosinor analysis under the assumption of a 24h period. In all but one individual (participant 1), the observed pattern correlated significantly to the fitted cosine model, as described by the mesor, amplitude and acrophase (range R^2 0.44-0.91; all $p \leq 0.001$ - Table 1). Correction for possible posture-induced changes in plasma volume did not abrogate the diurnal rhythm (Supplementary Figure 6)¹⁷. In addition, we could not relate the diurnal rhythm to the 25h variation in cortisol, thyroid stimulating hormone or testosterone in terms of the time of peak and the maximal individual changes during the day (Supplementary Methods, Supplementary Figure 7).

Figure 1. Diurnal variation of hs-cTnT, but not hs-cTnI, is a general phenomenon. A) Mean $\pm$ SEM 25h hs-cTnT profile and fitted cosine curve. Subjects slept in a supine position between 11.30 PM and 7.00 AM (shaded area). The curve is described as $Z(t) = 13.70 + 1.7 \cdot \cos(15t - 337.19)$, with a R^2 of 0.92 ($p < 0.001$). The time of peak was calculated at 6.58 AM. B) Mean $\pm$ SEM 25h cTnI profile.

High-sensitivity troponin I levels fluctuate at random

Distinct from hs-cTnT, hs-cTnI (Abbott) and s-cTnI (Beckman Coulter) levels fluctuated randomly around the subject's homeostatic setpoint. Mean hs-cTnI and s-cTnI concentrations were 6.5 ng/L and 5.0 ng/L at 8.30 AM and 6.1 ng/L and 5.3 ng/L at 7.30 PM, respectively (Figure 1B, Supplementary Figure 8 and 9). Accordingly, in all but one participant, the 25h hs-cTnI and s-cTnI profile did not fit significantly to a cosine curve. Even

participant 24, with hs-cTnI and s-cTnI values above the 99th percentile upper reference limit, and with the most prominent diurnal oscillations for hs-cTnT, showed relatively stable hs-cTnI and s-cTnI levels during the 25h test period (Supplementary Figure 8 and 9). One individual had a hs-cTnI day-curve that could be fitted to a cosinus model (Supplementary Figure 8, participant 7). This curve, however, was characterized by an acrophase that was almost 10h out of phase compared to the mean hs-cTnT acrophase (10.30 PM vs. mean hs-cTnT acrophase of 6.58 AM).

Diurnal rhythm of high-sensitivity cardiac troponin in chest pain patients

To investigate whether rhythmic diurnal hs-cTn oscillation is also evident in a clinical setting, we compared hs-cTnT (Roche) and hs-cTnI (Abbott and Siemens) concentrations from 2782 patients included in the APACE trial. Baseline hs-cTn values were available for 2700, 2281 and 1812 patients (Roche, Abbott and Siemens, respectively). The adjudicated final diagnosis was AMI in 17.5%, unstable angina in 9.8%, cardiac non-coronary disease in 14.3%, non-cardiac chest pain in 53.3%, and unknown (but not AMI) in 5.1%. In a multivariate analysis, admission time was a marginally significant predictor of baseline hs-cTnT values (Supplementary Table 3), but not hs-cTnI Abbott and hs-cTnI Siemens (data not shown).

APACE patients were classified in one of two timeframes that were derived from the observed diurnal rhythm of hs-cTnT under standardized conditions (Figure 1A): a hs-cTnT high-level phase in the early-morning (11.00 PM till 2.00 PM) and a hs-cTnT low-level phase in the evening (2.00 PM till 11.00 PM). Hs-cTnT also showed a diurnal rhythm in patients with adjudicated diagnoses of non-cardiac disease and other cardiac disorders with significantly higher hs-cTnT levels in early-morning presenters compared to evening presenters. These differences were statistically significant, but small in absolute terms, and were not seen in patients with AMI or unstable angina (Table 2, Supplementary Table 4).

The diurnal rhythm of hs-cTnT overall did not affect diagnostic accuracy for AMI. Diagnostic accuracy of hs-cTnT as quantified by the area under the receiver-operating characteristics curve (AUC) at presentation, at 1h and with the combination of absolute changes with baseline concentration were very high and comparable among patients presenting in the

Table 1. The individual diurnal variation of hs-cTnT is significantly described by a cosinor model

Participant ^a	Mesor (ng/L)	Amplitude (ng/L)	Acrophase (h)	R ²	P-value
1	5.6	0.2	10.45 AM	0.23	0.05
2	7.3	1.2	5.39 AM	0.82	<0.001*
3	9.4	2.4	5.47 AM	0.89	<0.001*
4	8.4	1.7	7.16 AM	0.66	<0.001*
5	8.6	0.9	6.29 AM	0.65	<0.001*
6	8.9	0.9	5.01 AM	0.70	<0.001*
7	10.0	1.0	7.35 AM	0.55	<0.001*
8	10.6	0.9	5.57 AM	0.54	<0.001*
9	10.5	0.9	5.12 AM	0.68	<0.001*
10	12.7	0.7	2.56 AM	0.44	0.001*
11	12.9	2.4	6.03 AM	0.81	<0.001*
12	12.8	1.3	5.18 AM	0.64	<0.001*
13	15.6	3.8	6.03 AM	0.81	<0.001*
14	13.8	2.2	8.17 AM	0.86	<0.001*
15	15.3	2.7	8.53 AM	0.77	<0.001*
16	17.5	4.0	6.37 AM	0.91	<0.001*
17	16.3	1.9	10.26 AM	0.57	<0.001*
18	16.1	1.4	8.26 AM	0.79	<0.001*
19	19.7	3.7	5.29 AM	0.90	<0.001*
20	19.5	4.1	9.08 AM	0.80	<0.001*
21	19.4	2.2	4.35 AM	0.86	<0.001*
22	17.8	1.9	5.29 AM	0.79	<0.001*
23	18.3	2.4	8.19 AM	0.82	<0.001*
24	21.7	2.3	9.18 AM	0.74	<0.001*

The cosinor model is described as: $Z(t) = M + A \cdot \cos(\omega t + \phi) + e(t)$, where $Z(t)$ represents the measured hs-cTnT concentration at a given time (t), M the mesor (value about which oscillation occurs), A the amplitude (half the difference between the peak and the nadir value), ω the angular frequency (degrees per unit time, with 360° representing a complete cycle), ϕ the acrophase (timing of maximal value in degrees) and $e(t)$ the error between the cosinor model and the measurement. ^aSubjects were ranked on the basis of their visual range of hs-cTnT concentration over the day: generally, high subject numbers correspond to a wider hs-cTnT dispersion. The numbering used in Table 1 is extended to Supplementary Figure 1, 2, 8, 9 allowing direct comparisons throughout the paper.

*Significant after Bonferroni correction for multiple testing, $p=0.0021$ (0.05/24)

early-morning and patients presenting in the evening (all AUC >0.90; Table 3; Figure 2). In contrast, AUC of absolute changes for hs-cTnT within the first hour were significantly lower in the early-morning (AUC 0.90, 95%CI 0.89-0.92) as compared to the evening (AUC 0.95, 95%CI 0.93-0.97, $p = 0.01$). No significant difference was seen in absolute changes within 2h, 3h and 6h between the two timeframes (Supplementary Table 5a). An additional analysis comparing diagnostic accuracy of absolute changes of day presenters (8 AM to 8 PM) and night presenters (8 PM to 8 AM) showed no systematic difference (Supplementary Table 5b). In patients presenting very early (≤ 3 hours) after symptom onset ($n=1490$, 16.3% with AMI), differences in AUC between early-morning presenters and patients presenting in the evening seemed to be more pronounced but without systematic significance (Table 4).

The prognostic accuracy of hs-cTnT at presentation for all-cause mortality within one year, as quantified by the AUC was moderate-to-high and comparable among all adjudicated diagnoses between early morning presenters and evening presenters (Supplementary Table 6).

Hs-cTnI (Abbott and Siemens) did not exhibit a diurnal rhythm, levels were similar among all adjudicated diagnoses between early-morning presenters and evening presenters (Table 2, Supplementary Table 4). In addition, diagnostic accuracy of hs-cTnI was very high and comparable among patients presenting in the early-morning and patients presenting in the evening (all AUC > 0.92, Table 3, Table 4, Supplementary Table 5).

Table 2. Early-morning and evening levels of hs-cTnT and hs-cTnI according to final diagnosis

		Early-morning 11.00 PM – 2.00 PM	Evening 2.00 PM – 11.00 PM	
Timepoint	Adjudicated diagnosis	Median (IQR)	Median (IQR)	p-value ^a
<i>hs-cTnT Roche, ng/L</i>				
0h	AMI	60 (28-153)	60 (25-119)	0.94
	Unstable angina	11 (7-16)	12 (6-16)	0.73
	Cardiac cause not CAD ^b	14 (7-30)	10 (6-26)	0.04
	Non-cardiac cause	6 (4-10)	5 (3-8)	<0.001
	Unknown	7 (4-10)	5 (3-10)	0.20
1h	AMI	68 (32-193)	88 (34-164)	0.49
	Unstable angina	12 (7-17)	13 (7-16)	0.52
	Cardiac cause not CAD ^b	15 (7-30)	10 (6-27)	0.07
	Non-cardiac cause	6 (4-10)	5 (4-8)	0.004
	Unknown	7 (4-10)	6 (4-10)	0.34
<i>hs-cTnI Siemens, ng/L</i>				
0h	AMI	199 (27-1270)	158 (22-559)	0.35
	Unstable angina	6 (3-14)	7 (4-17)	0.55
	Cardiac cause not CAD ^b	9 (3-50)	6 (2-24)	0.45
	Non-cardiac cause	2 (1-5)	2 (1-5)	0.09
	Unknown	3 (2-7)	2 (1-4)	0.17
1h	AMI	275 (46-1357)	203 (55-974)	0.58
	Unstable angina	6 (4-15)	8 (4-17)	0.66
	Cardiac cause not CAD ^b	10 (3-70)	6 (3-23)	0.21
	Non-cardiac cause	3 (1-5)	2 (1-6)	0.36
	Unknown	4 (2-7)	3 (1-4)	0.22
<i>hs-cTnI Abbott, ng/L</i>				
0h	AMI	114 (22-628)	107 (21-424)	0.82
	Unstable angina	6 (3-12)	8 (4-13)	0.49
	Cardiac cause not CAD ^b	9 (4-32)	7 (3-18)	0.46
	Non-cardiac cause	3 (2-5)	3 (2-5)	0.39
	Unknown	3 (3-5)	3 (2-4)	0.44
1h	AMI	151 (28-871)	217 (38-613)	0.52
	Unstable angina	6 (4-12)	9 (4-14)	0.46
	Cardiac cause not CAD ^b	10 (3-50)	7 (4-15)	0.40
	Non-cardiac cause	3 (2-5)	3 (2-6)	0.58
	Unknown	3 (2-5)	3 (2-3)	0.85

^aCompared with the Mann Whitney U test. ^bCAD; coronary artery disease

Figure 2. Diagnostic performance of hs-cTnT and hs-cTnI for AMI in early-morning and evening presenters. ROC curves show the diagnostic accuracy of hs-cTnT (A) and hs-cTnI (B (Abbott) and C (Siemens)) for AMI in patients presenting in the early morning (11.00 PM–2.00 PM) and in the evening (2.00 PM–11.00 PM).

Table 3. Diagnostic accuracy of cTnT and cTnI in early-morning and evening presenters

	Early-morning 11.00 PM – 2.00 PM	Evening 2.00 PM – 11.00 PM	
	AUC (95% CI)	AUC (95% CI)	p-value
<i>hs-cTnT Roche, ng/L</i>			
0h	0.93 (0.92-0.94)	0.94 (0.92-0.96)	0.58
1h	0.94 (0.93-0.95)	0.97 (0.95-0.98)	0.10
Δ1h	0.90 (0.89-0.92)	0.95 (0.93-0.97)	0.010
0h+Δ1h	0.96 (0.95-0.97)	0.98 (0.96-0.99)	0.20
<i>hs-cTnI Siemens, ng/L</i>			
0h	0.92 (0.90-0.93)	0.94 (0.92-0.96)	0.32
1h	0.93 (0.92-0.95)	0.96 (0.94-0.98)	0.24
Δ1h	0.93 (0.91-0.94)	0.95 (0.92-0.97)	0.39
0h+Δ1h	0.94 (0.92-0.95)	0.96 (0.94-0.98)	0.26
<i>hs-cTnI Abbott, ng/L</i>			
0h	0.93 (0.91-0.94)	0.94 (0.92-0.96)	0.50
1h	0.94 (0.91-0.94)	0.96 (0.94-0.98)	0.09
Δ1h	0.92 (0.91-0.94)	0.94 (0.92-0.96)	0.35
0h+Δ1h	0.94 (0.92-0.95)	0.97 (0.95-0.98)	0.11

Table 4. Diagnostic accuracy of cTnT and cTnI in early-morning and evening presenters (≤3h since symptom onset)

	Early-morning 11.00 PM – 2.00 PM	Evening 2.00 PM – 11.00 PM	
	AUC (95% CI)	AUC (95% CI)	p-value
<i>hs-cTnT Roche, ng/L</i>			
0h	0.90 (0.89-0.92)	0.93 (0.91-0.96)	0.26
1h	0.94 (0.92-0.95)	0.98 (0.96-0.99)	0.050
Δ1h	0.90 (0.88-0.92)	0.95 (0.93-0.97)	0.01
0h+Δ1h	0.95 (0.93-0.96)	0.98 (0.96-0.99)	0.06
<i>hs-cTnI Siemens, ng/L</i>			
0h	0.91 (0.88-0.93)	0.94 (0.90-0.96)	0.36
1h	0.94 (0.92-0.96)	0.96 (0.93-0.98)	0.63
Δ1h	0.93 (0.91-0.94)	0.95 (0.92-0.97)	0.41
0h+Δ1h	0.95 (0.93-0.97)	0.97 (0.94-0.98)	0.61
<i>hs-cTnI Abbott, ng/L</i>			
0h	0.92 (0.90-0.94)	0.94 (0.91-0.97)	0.42
1h	0.94 (0.92-0.96)	0.97 (0.94-0.99)	0.25
Δ1h	0.91 (0.90-0.94)	0.94 (0.92-0.96)	0.20
0h+Δ1h	0.94 (0.92-0.96)	0.97 (0.94-0.99)	0.19

Effect on sensitivity, specificity, positive predictive value and negative predictive value

Using the ESC2015 NSTEMI cutoff values¹, specificity of hs-cTnT, but not hs-cTnI, is slightly lower in the early morning compared to the evening. (Supplementary Table 7) However, inclusion of the 1h delta-change value, which is the most important determinant to increase specificity in clinical practice, neutralizes the specificity difference between morning and evening presenters, hence limiting the clinical relevance of the baseline difference. Negative and positive predictive value of hs-cTnT are also similar in early-morning and evening presenters (Supplementary Table 7).

Discussion

The aim of the present study was to validate the diurnal troponin rhythm as a general phenomenon and to evaluate whether diurnal oscillation affect the diagnostic accuracy for AMI. We report seven major findings:

First, we identified rhythmic diurnal hs-cTnT variation as a general phenomenon, characterized by gradually declining levels during daytime followed by rising concentrations during nighttime to peak concentrations at again (early) morning hours. The rhythm is irrespective of sex and glucose metabolism status, with the largest amplitude of the oscillation seen in stable subjects with the highest hs-cTnT concentrations. Second, the diurnal rhythm is restricted to hs-cTnT, with no evidence for rhythmic variation detected in hs-cTnI and sensitive cTnI as quantified by three different cTnI assays. cTnT (36 kDa) and cTnI (24 kDa) are components of the thin filament of cardiomyocytes with possibly a small proportion existing as unbound protein in the cytosol^{18,19}. Release of cTn in the circulation is strongly associated with myocardial injury, although it is unclear whether detectable levels in healthy individuals reflect protein turnover or cardiomyocyte apoptosis⁶. As measured in serum from AMI patients, circulating cTn consists of intact and degradation forms that are ultimately cleared by the kidneys²⁰. We can only speculate which physiological differences between cTnT and cTnI underlie the observed differences in the diurnal rhythmicity. Renal function may play a role, as various observations suggests that deteriorating renal function is stronger related to elevated cTnT levels than cTnI levels²¹⁻²⁵. However, it is unknown

whether diurnal variations in renal function would be sufficient to cause rather pronounced relative changes in hs-cTnT in the normal range. Accordingly, differences in cTn metabolism including repair mechanisms may also play a role. Third, these results could be reproduced in the large APACE study: significantly higher hs-cTnT levels were evident in early-morning presenters compared to evening presenters with an adjudicated final diagnosis of non-cardiac disease. Fourth, diagnostic accuracy of hs-cTnT for AMI overall was very high in patients presenting in the early-morning as well as patients presenting in the evening and not significantly different between the two. Fifth, the physiological rise and fall of hs-cTnT during the day significantly affected the diagnostic accuracy of absolute changes within the first hour after presentation with lower AUC in the early-morning (when non-cardiac disease and healthy persons have higher levels) as compared to the evening (when non-cardiac disease and healthy persons have lower levels). This result could not be reproduced in changes within 2, 3 and 6h. As in current clinical practice absolute changes are not used as a primary diagnostic variable, but only secondary to the absolute level at presentation and at 1h, or in conjunction with the absolute level at presentation/1h, the clinical impact – if any – of the circadian rhythm in hs-cTnT seems to be very small overall. Sixth, in patients presenting very early (<3h) after chest pain onset, the difference in AUC seemed to be more pronounced. This observation can be well explained that in early presenters the true circadian baseline level has still a stronger impact on hs-cTnT blood concentrations in AMI, as the added hs-cTnT component resulting exclusively from the AMI event is much smaller very early versus later after chest pain onset. As the magnitude of the difference in AUC was small, the clinical relevance of this difference seems to be very small at most also in early presenters. The circadian rhythm in hs-cTnT may well however affect the diagnostic accuracy of hs-cTnT when used as a screening tool for cardiovascular disease, where much smaller differences exist between health and disease. Accordingly, screening with its need for serial testing over years should be scheduled on the same time of the day in the individual patient. Seventh, prognostic accuracy of hs-cTnT for all-cause mortality within one year was moderate-to-high and not affected by the circadian rhythm.

Our findings extend and corroborate recent pilot data suggesting a diurnal rhythm for hs-cTnT in a small group of male subjects with type 2 diabetes¹². As the circulating hs-cTn concentration is defined by the extent of release and clearance, this study does not allow to quantify the influence of both processes on the diurnal hs-cTnT rhythm. Nevertheless, the rhythmicity of this cardiac biomarker is in line with the circadian organization of the cardiovascular system^{26,27}. Synchronizing physiological functions to the natural 24h light-dark cycle, for example by upregulating body temperature and blood pressure before the time of awakening, anticipates to the demands of daytime activities. Circadian rhythmicity has in addition been described for many cardiovascular traits: heart rate, sympathetic activity, vascular resistance, the renin-angiotensin-aldosterone system, prothrombotic tendency, platelet aggregability and the fibrinolytic system²⁷. Identical to hs-cTnT concentrations, all these components exhibit peak activity in (early) morning hours. The physiological rhythmicity of these cardiovascular components is reflected in the increasing intensity of cardiovascular events in the morning hours. For example, the incidence of AMI, sudden cardiac death and stroke display a significant 24h variation, characterized by a higher frequency in morning hours²⁸. Even the clock time of AMI onset is associated with clinical course and survival, with the largest infarct sizes and higher mortality found among patients with AMI that commence in morning hours^{29,30}.

Potential limitations of our study merit consideration. First, study 1 enrolled 19 men compared to 5 women, which is a limitation given the difference in clinical hs-reference values between both genders. Second, study 2 was conducted with patients presenting to the emergency department with symptoms suggestive of AMI. Additional studies in patients presenting to a general practitioner, which in general have a much lower pretest-probability for AMI, are required to learn whether the diurnal hs-cTnT rhythm affects the diagnostic accuracy in this setting. Third, this diagnostic study required written informed consent. Therefore, invariably the rate of enrollment was lower than in registries. Fourth, we cannot comment on diurnal rhythms in patients with terminal kidney failure requiring dialysis, since such patients were excluded from our study.

In conclusion, rhythmic diurnal variation of hs-cTnT is a general phenomenon, that is not seen with hs-cTnI, and should trigger additional studies to better understand its pathophysiology. Fortunately, the rhythmic diurnal variation of hs-cTnT does not seem to affect the high diagnostic accuracy of hs-cTnT in the diagnosis of AMI. It should however be considered when using hs-cTnT as a screening tool, where much smaller differences exist between health and disease.

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Supplemental information

Supplemental Methods

Adjudication of final diagnoses

Adjudication of the final diagnosis was performed centrally in a core lab (University Hospital Basel)¹⁻⁵ and included two sets of cardiac troponin (cTn) measurements. Those performed clinically in each hospital at presentation and at 3-6h and possibly also at 12-24h according to current guidelines^{6,7}, as well as levels of high-sensitivity cardiac troponin T (hs-cTnT) measured from the study blood samples taken at presentation, at 1h, 2h, 3h, and 6h (if feasible) in order to take advantage of the higher sensitivity and higher overall diagnostic accuracy offered by hs-cTnT (this allows the additional detection of small acute myocardial infarctions [AMIs])^{1,2,8}.

AMI was defined and cTn levels interpreted as recommended in current guidelines^{6,7,9}. In brief, AMI was diagnosed when there was evidence of myocardial necrosis in association with a clinical setting consistent with myocardial ischemia. Myocardial necrosis was diagnosed by at least one cTn value above the 99th percentile together with a significant rising and/or falling^{6,7,9}. All other patients were classified as 'No NSTEMI' for this analysis, including in this group the categories of unstable angina (UA), non-cardiac chest pain (NCCP), cardiac but non-coronary disease (e.g. tachyarrhythmia, perimyocarditis), and symptoms of unknown origin with normal levels of cTn and hs-cTnT.

The following conventional cTn assays were used: For the Roche cTnT 4th generation assay, the 10% CV level is 0.035 µg/L. The laboratories of the participating sites reported only two decimals; therefore 0.04 µg/L was used as a cut-off for myocardial necrosis. In order to fulfil the criteria of a significant change (30% of 99th percentile or 10% CV level), a patient would e.g. need to have a level of <0.01 µg/L at presentation and 0.04 µg/L at 6h. A patient would also qualify if the first level is 0.02 µg/L and the second 0.04 µg/L. A patient would not fulfil the criteria if the first level is 0.03 µg/L and the second is 0.04 µg/L. If the first level is 0.04 µg/L, the second level needs to be at least 0.06 µg/L. For the Abbott AxSYM cTnI ADV, the 10% CV level is 0.16 µg/L. A patient having 0.16 µg/L at presentation would meet the criteria for significant change if the second was ≥0.21 µg/L. A patient having <0.12 µg/L at presentation (limit of detection) would qualify if the second is >0.16 µg/L. For the Beckmann Coulter Accu cTnI, the 10% CV level is 0.06 µg/L. A patient having 0.06 µg/L at presentation would qualify if the second is ≥0.08 µg/L. A patient having 0.05 µg/L at presentation would qualify if the second is 0.07 µg/L, but not 0.06 µg/L. A patient having undetectable cTnI (cTnI<0.01 µg/L) at presentation would qualify if the second is ≥0.06 µg/L.

For hs-cTnT, the 99th percentile (14 ng/L) was used as cut-off for myocardial necrosis. Absolute changes in hs-cTnT were used to determine significant changes based on the diagnostic superiority of absolute over relative changes. Based on studies of the biological variation of cTn as well as on data from previous chest pain cohort studies, a significant absolute change was defined as a rise or fall of at least 10 ng/L within six hours. In patients, in whom a 6 hour hs-cTnT level was not available, changes were assessed at earlier time points. In an assumption of linearity, an absolute change of 6 ng/L within three hours was considered.

Follow up

After hospital discharge, patients were contacted after 3, 12 and 24 months by telephone calls or in written form. Information regarding death was furthermore obtained from the national registry on mortality, the hospitals' diagnosis registry, and the family physicians' records.

Laboratory analysis

cTnT was measured by using the high-sensitivity cTnT assay of Roche Diagnostics (Cobas 8000); the 99th percentile among healthy subjects is 14 ng/L, with a 10% analytical variation at 13 ng/L¹⁰. Abbott high-sensitivity cTnI was measured with the STAT high-sensitivity troponin I assay (Architect, Abbott Diagnostics). According to the manufacturer, the 99th percentile is 26.2 ng/L with a corresponding coefficient of variation of <5%. The high-sensitivity cTnI assay of Siemens has a 99th percentile concentration of 9 ng/L and a coefficient of variation of 10% at 3 ng/L (according to the manufacturer). S-cTnI (Beckman Coulter) was measured by using the Access AccuTnI+3 Assay; with a 99th percentile limit of 40 ng/L with a corresponding 10% imprecision, as specified by the manufacturer. Cortisol, thyroid-stimulating hormone and testosterone were measured by using the assays of Roche Diagnostics (Cobas 8000).

Supplemental Figures

Supplemental figure 1. 25h distribution of high-sensitivity cardiac troponin T and I. Distribution of high sensitivity cardiac troponin T (hs-cTnT Roche Diagnostics) (A), high sensitivity cardiac troponin I (hs-cTnI Abbott Diagnostics) (B), and sensitive cardiac troponin I (s-cTnI Beckman Coulter) (C) in 24 subjects (8.30 AM to 9.30 AM the next day). Circles represent mean troponin values from each sampling point for a given individual. Participants 1, 2, 8, 12 and 21 are female subjects. Subjects were ranked on the basis of their visual range of hs-cTnT concentration: generally, high subject number correspond to a wider range in hs-cTnT concentration. The ranking used for Supplemental figure 1A is extended to Supplemental figures 1B, 1C, Table 1, Supplemental figure 2, 5, 6, allowing direct comparisons throughout the paper.

Supplemental figure 2. Individual diurnal high-sensitivity cardiac troponin T profiles and fitted cosine curves. Depicted are the mean of the duplicate measurements. The numbering is identical to Table 1, Supplemental figure 1, 5, 6 allowing direct comparisons throughout the paper. Subjects slept in a supine position between 11.30 PM and 7.00 AM (shaded area). Sampling in subjects 2, 3 and 16 was hindered throughout the night due to technical problems with the intravenous cannula.

Supplemental figure 3. Mean±SEM 25h high-sensitivity cardiac troponin T profile of men and women. Subjects slept in a supine position between 11.30 PM–7.00 AM (shaded area).

Supplemental figure 4. Mean±SEM 25h high-sensitivity cardiac troponin T profile of individuals with diabetes and normal glucose metabolism. Subjects slept in a supine position between 11.30 PM–7.00 AM (shaded area).

Supplemental figure 5. High-sensitivity cardiac troponin T fluctuations are most prominent in subjects with the highest troponin T concentrations. Correlation between the high sensitivity cardiac troponin T (hs-cTnT) value at 8.30 AM day 1 and the maximal intra-individual changes during the 25h test period (Pearson's R^2 0.70; $p < 0.001$).

Supplemental figure 6. Mean diurnal high sensitivity cardiac troponin T profile corrected for possible plasma volume changes and fitted cosine curve. Depicted are the mean $\pm$ SEM 25h high sensitivity cardiac troponin T (hs-cTnT) profile corrected for hemoglobin and hematocrit and fitted cosine curve. Subjects slept in a supine position between 11.30 PM and 7.00 AM (shaded area). The cosine curve is described as $Z(t) = 14.75 + 2.34 \cdot \cos(15t - 305.56)$, with a R^2 goodness of fit of 0.94 ($p < 0.001$).

Supplemental figure 7. Relation of the diurnal profile of high-sensitivity cardiac troponin T with cortisol, thyroid stimulating hormone and testosterone. A) Mean $25 \pm \text{SEM}$ profiles of cortisol, thyroid stimulating hormone (TSH) and testosterone. Subjects slept in a supine position between 11.30 PM and 7.00 AM (shaded area). B) Relation between the maximal intra-individual changes of high-sensitivity cardiac troponin T (hs-cTnT) with cortisol, thyroid-stimulating hormone (TSH) and testosterone. All correlations are non-significant. C) Relation between the time of high-sensitivity cardiac troponin T (hs-cTnT) peak with the time of cortisol, thyroid stimulating hormone (TSH) and testosterone. All correlations are non-significant.

Supplemental figure 8. Individual diurnal high sensitivity cardiac troponin I profiles. Depicted are the mean of the duplicate measurements. The numbering is identical to Table 1, Supplemental figure 1, 2, 6, allowing direct comparisons throughout the paper. Subjects slept in a supine position between 11.30 PM and 7.00 AM (shaded area). Sampling in subjects 2, 3 and 16 was hindered throughout the night due to technical problems with the intravenous cannula.

Supplemental figure 9. Individual diurnal sensitive cardiac troponin I profiles. Depicted are the mean of the duplicate measurements. The numbering is identical to Table 1 and Supplemental figures 1, 2 and 5, allowing direct comparisons throughout the paper. Subjects slept in a supine position between 11.30 PM and 7.00 AM (shaded area). Sampling in subjects 2, 3 and 16 was hindered throughout the night due to technical problems with the intravenous cannula.

Supplemental Tables

Supplemental table 1. Study 1: Participants' characteristics

	Study population
n	24
Male gender	19 (79%)
Age, years	72 ± 7
Type 2 Diabetes Mellitus ^a	7 (29%)
BMI, kg/m ²	27 ± 5
Systolic blood pressure ^b , mm Hg	140 ± 15
Diastolic blood pressure ^b , mm Hg	68 ± 8
Fasting glucose ^b , mmol/L	5.9 (5.2-6.7)
HbA1c ^b , %	5.7 (5.3-6.5)
Total cholesterol ^b , mmol/L	3.7 (4.8-5.4)
HDL cholesterol ^b , mmol/L	1.3 (1.6-1.9)
LDL cholesterol ^b , mmol/L	1.7 (2.5-3.0)
Triglycerides ^b , mmol/L	1.2 (0.8-1.8)
NT-proBNP ^b , pmol/L	10.9 (4.9-38.8)
Hs-cTnT – Roche ^b , ng/L	16.1 (10.7-21.1)
Hs-cTnI – Abbott ^b , ng/L	4.6 (3.0-7.0).
cTnI – Beckman Coulter ^b , ng/L	3.0 (1.0-4.5)
eGFR ^{b,c} , mL/min/1.73m ²	75 (61-92)
Oral blood glucose-lowering medication ^d	7 (29%)
Lipid-lowering medication ^d	12 (50%)
Anti-hypertensive medication ^d	10 (42%)

Data is presented as mean ± SD, median (IQR) or n (%). ^aDiabetes was defined as fasting plasma glucose ≥ 7.0 mmol/L and/or HbA1c ≥ 6.5%¹¹. ^bValue at 8.30 AM day 1. ^ceGFR, estimated glomerular filtration rate according to CKD-EPI Creatinine-Cystatin C equation¹². ^dMedication status was stable for at least three months prior to inclusion, and continued as normal during the study period. To convert concentrations from mmol/L to mg/dL, multiply glucose by 18.02, cholesterol by 38.67, and triglycerides by 88.54.

Supplemental table 2. Study 2: baseline characteristics of patients enrolled in the APACE trial.

	All (n=2782)	AMI (n=488)	No AMI (n=2294)	p-value ^a
Age, y	61 (49-74)	72 (59-80)	59 (47-72)	<0.001
Male gender, n (%)	1903 (68.4%)	360 (73.8%)	1543 (67.3%)	0.014
Body-mass index, kg/m ²	26 (24-30)	26 (24-29)	26 (24-30)	0.66
eGFR, mL/min/1.73 m ²	85 (69-101)	73 (56-94)	86 (71-102)	<0.001
Risk factors, n (%)				
Hypertension	1712 (61.5)	381 (78.1)	1331 (58.0)	<0.001
Hypercholesterolemia	1390 (50.0)	332 (68.0)	1058 (46.1)	<0.001
Diabetes Mellitus	477 (17.1)	126 (25.8)	351 (15.3)	<0.001
Current or previous smoking	1722 (61.9)	317 (65.0)	1405 (61.2)	0.11
Family history	767 (27.6)	158 (32.4)	609 (26.5)	0.008
History, n (%)				
Coronary artery disease	965 (34.7)	242 (49.6)	723 (31.5)	<0.001
Previous AMI	662 (23.8)	172 (35.2)	490 (21.4)	<0.001
Previous revascularization	775 (27.9)	183 (37.5)	592 (25.8)	<0.001
Peripheral artery disease	169 (6.1)	63 (12.9)	106 (4.6)	<0.001
Previous stroke	152 (5.5)	44 (9.0)	108 (4.7)	<0.001
ECG findings, n (%)				
Left bundle branch block	79 (2.8)	28 (5.7)	51 (2.2)	<0.001
ST-segment elevation	57 (2.0)	13 (2.7)	44 (1.9)	0.28
ST-segment depression	282 (10.1)	146 (29.9)	136 (5.9)	<0.001
T-wave inversion	359 (12.9)	120 (24.6)	239 (10.4)	<0.001
Medication at presentation, n (%)				
ASA/thienopyridine	1111 (39.9)	268 (54.9)	843 (36.7)	<0.001
b-Blockers	966 (34.7)	214 (43.9)	752 (32.8)	<0.001
ACEIs/ARBs	1069 (38.4)	253 (51.8)	816 (35.6)	<0.001
Calcium antagonists	411 (14.8)	100 (20.5)	311 (13.6)	<0.001

Data is presented as median (IQR) or n (%). ^aComparisons between AMI and no-AMI patients.

Supplemental table 3. Multivariate analysis of baseline high-sensitivity cardiac troponin T values

hs-cTnT Roche	univariate (95%CI)	p-value	multivariate (95%CI)	p-value
gender	8.14 (-8.97-25.3)	0.35		
age	1.38 (0.88-1.87)	<0.001	0.77 (0.93-1.44)	0.26
history of CAD	19.08 (2.34-35.81)	0.025	1.178 (-18.37-20.73)	0.91
history of hypertension	24.09 (7.72-40.46)	0.004	-0.003 (-20.27-20.26)	1.0
positive family history	26.88 (8.76-45.00)	0.004	26.57 (8.34-44.80)	0.004
eGFR	-1.00 (-1.31-0.69)	<0.001	-0.65 (-1.04—0.27)	0.001
admission time	2.50 (0.31-4.96)	0.047	2.59 (0.08-5.10)	0.04

Supplemental table 4. Early-morning and evening absolute changes of high-sensitivity cardiac troponin T and I according to final diagnosis

Δ 1h (median, IQR)	hsTnT Roche			hsTnI Siemens			hsTnI Abbott		
	evening	early morning	p	evening	early morning	p	evening	early morning	p
AMI	12 (5.7-36)	9 (3.3-33.0)	0.2	49.4 (9.9-325.8)	73.6 (11.4-287.2)	0.5	39.3 (8.3-271.2)	47.4 (6.4-229.7)	0.7
UA	1 (0.1-10)	1 (0.2-1.3)	0.2	1 (0.6-2.0)	1 (0.3-1.7)	0.8	0.8 (0.3-1.5)	0.7 (0.7-1.5)	0.4
cardiac non-coronary	0.75 (0-2.0)	1 (0.2-3)	0.08	0.6 (0.3-2.1)	1.4 (0.4-9.7)	0.4	0.9 (0.4-2.7)	1.1 (0.4-6.2)	0.5
non-cardiac	0.2 (0-1)	0.5 (0-1)	0.1	0.3 (0.1-0.8)	0.3 (0.1-0.9)	0.2	0.4 (0.2-0.8)	0.4 (0.1-0.7)	0.4
unknown	0.9 (0-1.5)	1 (0-1.8)	0.9	0.7 (0.2-1.3)	0.7 (0.3-1.4)	0.8	0.5 (0.2-1.2)	0.6 (0.3-1.2)	0.6
Δ 2h (median, IQR)	hsTnT Roche			hsTnI Siemens			hsTnI Abbott		
	evening	early morning	p	evening	early morning	p	evening	early morning	p
AMI	19 (6-55)	15 (5-48)	0.4	79 (15-299)	124 (23-462)	0.5	56 (18-263)	63 (10-261)	0.8
UA	1 (1-2)	1 (0-1)	0.9	1 (1-2)	1 (1-3)	0.4	1 (0-2)	1 (0-2)	0.6
cardiac non-coronary	1 (0-2)	2 (1-4)	0.04	1 (0-2)	2 (1-16)	0.1	1 (0-2)	2 (0-13)	0.1
non-cardiac	0 (0-1)	0 (0-1)	0.5	0 (0-1)	1 (0-1)	0.8	0 (0-1)	0 (0-1)	0.1
unknown	1 (0-1)	1 (0-1)	0.6	1 (0-2)	1 (0-2)	0.7	1 (0-1)	0 (0-1)	0.9
Δ 3h (median, IQR)	hsTnT Roche			hsTnI Siemens			hsTnI Abbott		
	evening	early morning	p	evening	early morning	p	evening	early morning	p
AMI	33 (15-81)	18 (7-63)	0.2	120 (39.2-774)	159 (29-1058)	0.8	127 (44-396)	79 (15-591)	1
UA	1 (1-2)	1 (0-2)	0.6	2 (1-3)	1 (0-3)	0.8	1 (0-2)	1 (0-1)	0.6
cardiac non-coronary	1 (0-2)	2 (1-6)	0.02	2 (0-12)	4 (1-17)	0.2	1 (0-4)	2 (0-17)	0.1
non-cardiac	1 (0-1)	1 (0-1)	1	1 (0-2)	1 (0-2)	0.5	1 (0-1)	0 (0-1)	0.8
unknown	1 (0-2)	1 (0-2)	1	1 (0-2)	1 (0-2)	0.4	1 (1-2)	1 (0-1)	0.3
Δ 6h (median, IQR)	hsTnT Roche			hsTnI Siemens			hsTnI Abbott		
	evening	early morning	p	evening	early morning	p	evening	early morning	p
AMI	31 (8-76)	63 (10-261)	0.8	127 (12-410)	215 (46-1764)	0.8	174 (58-2141)	137 (29-1221)	0.7
UA	1 (1-2)	1 (0-2)	0.4	1 (1-2)	2 (1-4)	0.1	1 (0-2)	1 (0-1)	0.2
cardiac non-coronary	1 (0-2)	2 (1-9)	0.02	2 (0-9)	3 (1-48)	0.3	1 (0-4)	2 (0-21)	0.3
non-cardiac	0 (0-1)	1 (0-2)	0.4	1 (0-2)	1 (0-2)	0.2	1 (0-1)	1 (0-1)	0.5
unknown	1 (0-2)	1 (0-2)	0.8	1 (0-3)	1 (0-3)	0.7	1 (0-1)	0 (0-1)	0.7

evening = 2:00 P.M. – 11:00 P.M; early morning = 11:00 P.M. – 2:00 P.M.

Supplemental table 5. Diagnostic accuracy of absolute changes in high-sensitivity cardiac troponin T and I in a) early-morning and evening presenters and in b) day and night presenters.

a)

hs-cTnT Roche	early morning	evening	p-value
Δ1h	0.90 (0.88-0.92)	0.95 (0.93-0.97)	0.01
Δ2h	0.92 (0.90-0.93)	0.95 (0.92-0.97)	0.8
Δ3h	0.96 (0.94-0.97)	0.97 (0.94-0.99)	0.5
Δ6h	0.94 (0.91-0.96)	0.97 (0.92-0.99)	0.3
hs-cTnI Siemens	early morning	evening	p-value
Δ1h	0.93 (0.91-0.94)	0.95 (0.92-0.97)	0.4
Δ2h	0.93 (0.92-0.95)	0.96 (0.94-0.98)	0.2
Δ3h	0.93 (0.91-0.95)	0.97 (0.93-0.99)	0.2
Δ6h	0.92 (0.88-0.95)	0.97 (0.91-0.99)	0.2
hs-cTnI Abbott	early morning	evening	p-value
Δ1h	0.91 (0.90-0.94)	0.94 (0.92-0.96)	0.2
Δ2h	0.93 (0.92-0.95)	0.95 (0.92-0.97)	0.5
Δ3h	0.93 (0.90-0.95)	0.97 (0.92-0.99)	0.2
Δ6h	0.94 (0.91-0.97)	0.99 (0.94-1.00)	0.1

b)

hs-cTnT Roche	day (8 AM to 8 PM)	night (8PM to 8AM)	p-value
0h	0.93 (0.92-0.94)	0.93 (0.87-0.96)	1
1h	0.95 (0.94-0.96)	0.94 (0.89-0.97)	0.8
Δ1h	0.92 (0.90-0.93)	0.92 (0.87-0.96)	0.9
Δ2h	0.93 (0.91-0.94)	0.88 (0.81-0.93)	0.3
Δ3h	0.96 (0.94-0.97)	0.98 (0.92-1.00)	0.5
Δ6h	0.94 (0.91-0.96)	0.98 (0.91-1.00)	0.2
hs-cTnI Siemens	day (8 AM to 8 PM)	night (8PM to 8AM)	p-value
0h	0.92 (0.90-0.93)	0.96 (0.92-0.99)	0.2
1h	0.94 (0.93-0.95)	0.97 (0.92-0.99)	0.3
Δ1h	0.93 (0.92-0.95)	0.94 (0.88-0.98)	0.9
Δ2h	0.94 (0.93-0.96)	0.94 (0.87-0.97)	0.9
Δ3h	0.94 (0.92-0.96)	0.95 (0.87-0.99)	0.9
Δ6h	0.93 (0.90-0.95)	0.93 (0.83-0.98)	1
hs-cTnI Abbott	day (8 AM to 8 PM)	night (8PM to 8AM)	p-value
0h	0.93 (0.92-0.94)	0.94 (0.89-0.97)	0.9
1h	0.94 (0.93-0.95)	0.96 (0.91-0.99)	0.8
Δ1h	0.93 (0.91-0.94)	0.93 (0.87-0.97)	0.9
Δ2h	0.94 (0.92-0.95)	0.93 (0.86-0.97)	0.8
Δ3h	0.94 (0.92-0.96)	0.74 (0.60-0.85)	0.2
Δ6h	0.96 (0.93-0.98)	0.94 (0.83-0.99)	0.8

Supplemental table 6. Prognostic accuracy of high-sensitivity cardiac troponin T in early-morning and evening presenters for the prediction of all-cause mortality within one year.

	Early-morning 11.00 PM –2.00 PM	Evening 2.00 PM–11.00 PM	
	AUC (95% CI)	AUC (95% CI)	p-value
All patients	0.74 (0.71-0.77)	0.82 (0.81-0.84)	0.22
AMI	0.57 (0.48-0.66)	0.66 (0.37-0.62)	0.65
Unstable angina	0.49 (0.37-0.62)	0.74 (0.67-0.80)	0.28
Cardiac non-coronary	0.81 (0.72-0.88)	0.79 (0.74-0.84)	0.90
Non-cardiac	0.78 (0.73-0.82)	0.85 (0.83-0.87)	0.53
Unknown	Insufficient data	Insufficient data	

Supplemental Table 7. Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value of baseline values, 1h values and changes in hs-cTnT and hs-cTnI in early-morning and evening presenters.

	Early-morning 11.00 PM– 2.00 PM	Evening 2.00 PM– 11.00 PM	Early-morning 11.00 PM– 2.00 PM	Evening 2.00 PM– 11.00 PM
	Sensitivity (95%CI)	Sensitivity (95%CI)	Specificity (95%CI)	Specificity (95%CI)
<i>hs-cTnT Roche</i>				
0h	99.4 (97.9-99.9)	100 (97.0-100)	28.3 (26.1-30.5)	35.0 (31.2-39.0)
1h	98.9 (96.9-99.8)	100 (96.5-100)	27.8 (25.4-30.2)	35.6 (31.3-40.1)
Δ1h	78.2 (72.8-82.9)	86.4 (78.3-92.4)	93.1 (91.6-94.4)	94.1 (91.6-96.1)
0h+Δ1h	99.7 (98.4-100.0)	100 (97.0-100)	65.9 (63.3-68.3)	69.6 (65.4-73.6)
<i>hs-cTnI Siemens</i>				
0h	99.1 (96.7-99.9)	100 (94.9-100)	14.7 (12.7-17.0)	15.7 (12.4-19.5)
1h	98.9 (95.9-99.9)	100 (94.1-100)	12.6 (10.5-14.9)	13.1 (9.7-17.0)
Δ1h	91.3 (86.0-95.0)	86.9 (75.8-94.2)	81.3 (78.6-83.8)	86.5 (82.4-89.9)
0h+Δ1h	98.1 (95.3-99.5)	100 (94.9-100)	54.1 (50.9-57.2)	59.0 (53.9-64.0)
<i>hs-cTnI Abbott</i>				
0h	100 (98.8-100)	100 (96.6-100)	19.8 (17.7-22.0)	19.0 (15.6-22.7)
1h	99.6 (97.8-100)	100 (96.1-100)	18.5 (16.2-20.9)	19.0 (15.2-23.2)
Δ1h	85.2 (80.1-89.5)	91.1 (83.2-96.1)	87.0 (84.8-88.9)	88.8 (85.2-91.8)
0h+Δ1h	97.4 (94.9-98.9)	100 (96.6-100)	57.5 (54.6-60.3)	59.0 (54.1-63.8)
	PPV (95% CI)	PPV (95% CI)	NPV (95% CI)	NPV (95% CI)
<i>hs-cTnT Roche</i>				
0h	22.8 (20.7-25.0)	23.8 (20.2-27.8)	99.6 (98.5-100.0)	100 (98.3-100)
1h	22.1 (19.8-24.5)	25.2 (21.1-29.7)	99.2 (97.7-99.8)	100 (97.8-100)
Δ1h	70.0 (64.6-75.1)	76.1 (67.3-83.5)	95.4 (94.1-96.5)	97.0 (95.0-98.3)
0h+Δ1h	41.4 (38.1-44.9)	43.8 (37.8-50.0)	99.9 (99.4-100)	100 (99.0-100)
<i>hs-cTnI Siemens</i>				
0h	18.5 (16.3-20.9)	16.3 (13.0-20.2)	98.8 (95.7-99.9)	100 (94.6-100)
1h	17.4 (15.1-20.0)	16.6 (13.0-20.8)	98.3 (94.1-99.8)	100 (92.3-100)
Δ1h	47.7 (42.2-53.3)	53.0 (42.8-63.1)	98.0 (96.8-98.9)	97.4 (95.0-98.9)
0h+Δ1h	31.6 (28.1-35.3)	31.1 (25.1-37.6)	99.3 (98.1-99.8)	100 (98.4-100)
<i>hs-cTnI Abbott</i>				
0h	21.8 (19.7-24.1)	21.1 (17.6-24.9)	100 (98.7-100)	100 (96.2-100)
1h	21.2 (18.9-23.7)	22.8 (18.8-27.2)	99.5 (97.3-100)	100 (95.1-100)
Δ1h	58.9 (53.5-64.2)	66.1 (57.1-74.4)	96.4 (95.1-97.5)	97.7 (95.4-99.0)
0h+Δ1h	36.9 (33.6-40.3)	38.8 (33.0-44.8)	98.9 (97.8-99.5)	100 (98.5-100)

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Twenty-four-hour biological variation profiles of cardiac troponin I in individuals with or without chronic kidney disease

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Abstract

Background

Correct interpretation of serial cardiac troponin measurements is of profound importance for the diagnosis of acute myocardial infarction, especially in populations with a high prevalence of persistently elevated cardiac troponin levels. To gain more insight in physiological cardiac troponin I fluctuations, we performed a biological variation study in which we examined and compared the hour-to-hour biological variation in cardiac troponin I over 24-hours in subjects with and subjects without chronic kidney disease.

Methods

During 24h, hourly blood samples were collected from 20 subjects with and 24 subjects without chronic kidney disease. Cardiac troponin I was determined in duplicate. Analytical variation (CVA), within-subject biological variation (CVI) and between-subject biological variation (CVG) were calculated for 1-hour, 3-hour and 6-hour intervals in both groups.

Results

Baseline cardiac troponin I levels were higher in subjects with chronic kidney disease compared to those without (6.8 [3.5–9.2] ng/L vs. 4.7 [2.8–6.9] ng/L; $p=0.09$). The within-day variation showed no diurnal pattern. Variation components remained constant for all time-intervals. Whereas CV_G (49-95%) and CV_I (8-9%) were not significantly different between both groups, CVA was significantly lower in subjects with chronic kidney disease (6% vs. 10%).

Conclusions

Cardiac troponin I concentrations fluctuate at random during the day. Except from the analytical variation, variation components were of the same magnitude in subjects with or without CKD. Our findings imply that the variation in cardiac troponin I levels is not affected by the presence of chronic kidney disease. Interpretation of serial high-sensitivity troponin (hs-cTn) measurements for the diagnosis of acute myocardial infarction (AMI) assumes random fluctuation of hs-cTn around an individual's homeostatic set-point. The aim of this study was to challenge this diagnostic concept.

Introduction

The interpretation of cardiac troponin concentrations at presentation and their dynamics over time is a key aspect in the diagnostic workup of acute myocardial infarction in the absence of characteristic ECG-abnormalities^{1,2}. Whereas subjects with very low or high cardiac troponin concentrations at presentation can immediately be ruled-out or ruled-in respectively, serial measurements are particularly indicated in patients with moderately elevated cardiac troponin concentrations³⁻⁶. A majority of these patients does not have an acute myocardial infarction, but shows persistently elevated cardiac troponin concentrations which are associated with the presence of underlying (subclinical) disease like diabetes, coronary artery disease, hypertension and chronic kidney disease (CKD)⁷⁻⁹. Especially in these patients it is of profound importance to distinguish pathology associated changes from physiological fluctuation in serial cardiac troponin measurements.

Biological variation studies provide insight in the magnitude of physiological variation. On the basis is the assumption that the biomarker concentrations fluctuate around an individual set point, and that the totally observed variation can be subdivided in a between-subject, a within-subject and an analytical component¹⁰. These individual components facilitate the estimation of the reference change value (RCV), the required threshold for a change between serial measurements to be significantly different from a physiological variation. Additionally, it is possible to calculate the index of individuality (II), which represents the variation between the individual set points¹¹⁻¹³.

During the last decade, the duration of the diagnostic workup for acute myocardial infarction shortened, which was reflected in the recent introduction of the 0h/1h-algorithm^{1,14,15}. Since most biological variation studies focus on long(er)-term variation in cardiac troponin I (cTnI)¹⁶⁻¹⁹, less is known about the hour-to-hour variation. Furthermore, all previous reported studies were limited to a certain time interval and it is therefore not clear whether the derived values apply to both day and night, or may be affected by a diurnal rhythm²⁰. The present biological variation study was the first to examine and compare the hour-to-hour biological variation in cTnI over 24-hours in subjects without and subjects with CKD.

Methods

This study was carried out according to the principles of the Declaration of Helsinki, approved by the Institutional Review Board and Ethics Committee of Maastricht University Medical Center, and registered at clinicaltrial.gov (NCT02091427 and NCT02210897). All participants provided written informed consent. The current study meets the critical appraisal checklist criteria for studies of biological variation as proposed by Bartlett et al.²¹.

Study population

The study population consisted of 44 adult subjects, 24 subjects (19 males and 5 females) without CKD and 20 subjects (14 males and 6 females) with CKD. The subjects without CKD were previously described in more detail²⁰. Subjects with CKD stage 3 or higher (estimated glomerular filtration rate (eGFR) < 59 mL/min/1.73m²)²² were included at the nephrology outpatient department of the Maastricht University Medical Center (MUMC), and suffered from various types of kidney disease (glomerular (n=7), tubulointerstitial (n=4), vascular (n=8) and congenital kidney disease (n=1)). Exclusion criteria were: history of acute myocardial infarction less than 12 months prior to exclusion, (current) dialysis regimen, active cardiac disease (angina pectoris, cardiomyopathy and myocarditis) and anemia (Hb < 10.5 g/dL). To monitor whether the degree of renal impairment was stable in CKD subjects, we examined kidney function (estimated glomerular filtration rate (eGFR)) one week and one month after the initial test day.

Study design

After an overnight fast, subjects arrived at 8 a.m. at the laboratory by car or public transport. For 25h, from 8.30 A.M. till 9.30 A.M. the next day, subjects were restricted to the laboratory environment, and samples were collected every hour from an antecubital venous catheter. Extension lines for blood sampling were used to prevent disturbance of participants' sleep during the night. Meals were consumed at 8.30 A.M., 12.30 P.M. and 6.00 P.M. (breakfast, lunch and dinner, respectively). Subjects went to bed at 11.30 P.M., lights were off between

11.35 P.M. and 7.00 A.M., and they slept in a supine position. Participants were asked to refrain from exhaustive physical activities and exercise training, two days before the test day.

Laboratory measurements

Blood samples were collected in ethylenediaminetetraacetic acid containing (EDTA) tubes. Samples were centrifuged and the plasma was stored at -80°C until analysis. cTnI was measured with the STAT high-sensitivity troponin I assay (Architect i2000 SR, Abbott Diagnostics). According to the manufacturer, the LoD is 1.1–1.9 ng/L, and 99th percentile of this assay is 26.2 ng/L with a corresponding coefficient of variation of <5%. To enable the determination of analytical variation, all samples were measured in duplicate. Creatinine and cystatin C were both measured on the COBAS 8000 analyzer. The eGFR was calculated according to the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula based on creatinine and cystatin C²³.

Statistical analysis

For the comparison between the baseline characteristics of both groups, continuous variables were compared with the Mann-Whitney U test, and categorical variables were compared by use of the Pearson χ^2 test. Data are expressed as median and interquartile range (median [IQR]) for non-normally distributed continuous variables and as numbers and percentages (n(%)) for categorical variables.

For the 24-hour analyses, we used data obtained between 10.30 AM (first day) till 9.30 (AM) (second day). As suggested by Fraser and Harris²⁴, we tested for homogeneity in the analytical- and within-subject biological variances using the method described by Cochran. Finally, the criteria of Reed were used to identify between-subject outliers¹⁰.

Two-fold nested ANOVA was used to calculate the coefficients of variation on a between-subject (CV_G), within-subject (CV_I) and an analytical (CV_A) level, including 95% confidence intervals^{25,26}. We calculated the reference change value (RCV) using the formula: $RCV = Z \cdot \sqrt{2 \cdot (CV_I^2 + CV_A^2)}$. In the RCV formula, Z represents the number of standard deviations appropriate for the desired level of statistical significance for a bidirectional change¹⁰. For

the RCV calculations in this study a Z-score of 1.96 was used. The index of individuality (II) was calculated using the formula: $II = \sqrt{(CV_I^2 + CV_A^2)} / CV_G^{13,27}$. All hypothesis testing was two-tailed, and values of $P < 0.05$ were considered statistically significant. Statistical analyses were performed with SPSS for Windows 23.0 (IBM Corp., Armonk, NY, USA).

Results

A total of 44 subjects, 20 with and 24 without CKD disease, participated in this biological variation study. We plotted the range in observed cTnI concentrations over 24h for all individual subjects in Figure 1.

Figure 1. Subject specific concentrations over 24h with hourly sampling. Absolute ranges (min – max) of cTnI concentrations over 24h in subjects without CKD (panel A) and with CKD (panel B).

Complete data sets were available for 40 subjects (subjects 1, 8, 15 and 23 had missing data). In addition, one subject had no measurable levels of cTnI (subject 36), and another subject became sick during the test day (subject 20). Two subjects (subjects 3 and 44) were identified as outliers and were therefore excluded from further analyses. A total of 36 subjects, 18 subjects with and 18 subjects without CKD, were included in the final data analyses. Figure 2 shows the in- and exclusion of subjects, including baseline characteristics. In subjects with CKD the eGFR remained stable for at least one month after the initial test day (data not shown).

Baseline cTnI levels were higher in subjects with CKD compared to those without CKD (6.8 [3.5–9.2] ng/L vs. 4.7 [2.8–6.9] ng/L; $p=0.09$). The within-day variation in these concentrations showed no diurnal pattern, and biological variation seemed to be constant over 24h in both groups (Figure 3, Supplemental Figure 1) (individual curves of subjects without CKD have been previously published²⁰; *chapter 3* of this thesis).

Figure 2. Flowchart of the study, including baseline characteristics. ^a eGFR, estimated glomerular filtration rate according to the CKD-EPI Creatinine-Cystatin C equation²³; ^b comparison between subjects with or without CKD.

Consecutively, we calculated the variation components of 1-hour, 3-hour and 6-hour intervals during the day, which are presented in Table 1. We detected no significant differences in the components of variation for the different time intervals. When the variation components were compared between subjects without and subjects with CKD, CV_G and CV_I were not significantly different. In contrast, we observed significant differences in CV_A between both groups: CV_A was significantly lower in subjects with CKD (the group with

Figure 3. No diurnal pattern in cTnI variation in subjects with or without CKD. We calculated the deviation (in %) from the mean concentration (over 24h) of each individual on every time point in subjects with (panel A) or without CKD (panel B). This variation is random and is not subject to a circadian pattern.

Table 1. Hour-to-hour biological variation in subjects with or without chronic kidney disease

	1-hour		3-hour ^c		6-hour ^d	
	No CKD n=18	CKD n=18	No CKD n=18	CKD n=18	No CKD n=18	CKD n=18
<i>Variance components</i>						
CV _G	49.4 (37.1 – 74.2)	62.4 (46.8 – 93.5)	48.0 (35.9 – 71.1)	61.6 (46.2 – 92.4)	48.6 (36.2 – 73.1)	61.6 (46.1 – 92.5)
CV _I	8.6 (7.6 – 9.7)	7.7 (7.1 – 8.4)	9.4 (7.6 – 11.5)	8.7 (7.5 – 10.2)	9.2 (6.2 – 12.4)	8.9 (7.1 – 11.3)
CV _A ^a	10.0 (9.4 – 10.8)	5.6 (5.2 – 6.0)	9.9 (8.8 – 11.2)	5.6 (5.0 – 6.3)	10.1 (8.7 – 12.1)	5.9 (5.0 – 7.0)
<i>RCV^b</i>						
Normal	36.7	26.4	37.9	28.6	37.5	29.5
Log-normal	-30.6; 44.0	-23.16; 30.15	-31.4; 45.8	-24.8; 33.0	-31.4; 45.7	-25.5; 34.2
<i>Index of individuality</i>						
	0.27	0.15	0.28	0.17	0.28	0.17

Values are % (95% CI); CI = confidence interval; CV_A = analytical coefficient of variation; CV_G = between-subject coefficient of variation; CV_I = within-subject biological coefficient of variation; RCV = reference change value ^a on the basis of duplicate measurements; ^b on the basis of a Z-score of 1.96; ^c 3-hour intervals: 10:30 AM-01:30 PM, 01:30 PM-04:30 PM, 04:30 PM-07:30 PM, 07:30 PM-10:30 PM, 01:30 AM-4:30 AM and 4:30 AM-7:30 AM; ^d 6-hour intervals: 10:30 AM-04:30 PM, 04:30 PM-10:30 PM and 10:30 PM-04:30 AM

the higher cTnI concentrations). As a result of the lower CV_A , the RCV is also lower among these subjects. Besides, we observed a trend towards a higher between-subject variation and a lower index of individuality in this group.

Discussion

This study examined the hour-to-hour biological variation of cTnI, and calculated its variation components over 24h in subjects with or without CKD. We report four major findings: First, we observed randomly fluctuating cTnI levels over the day. In the absence of a circadian rhythm, we calculated the 1-hour, 3-hour and 6-hour variation over the day without any additional transformations. The fact that the CV_I remained constant (8-9%) during all time-intervals over 24h, supports our observation of random variation. Second, the CV_I was not significantly different in subjects with or without CKD. This seems to be in line with the claim that for the majority of analytes CV_I values are in the same order in health and (chronically stable) disease^{10,28}. Third, we identified a substantial amount of variation between individuals ($CV_G = 48-62\%$). This finding corresponds to that of previous reported studies^{16-18,29,30} and, since the CV_I is rather limited, it produces a low index of individuality (<0.3). The observation that the CV_G is slightly higher in subjects with CKD may be due to a high variety in comorbidities characteristic for this population³¹. Fourth, we showed that the CV_A is significantly higher in subjects without than in those with CKD (10% vs. 6%). This can be explained by the concentration-dependency of the CV_A . As a consequence of these differences in CV_A , the RCV is slightly lower in the group with the higher cTnI concentrations. From a clinical point of view, the finding of a substantial CV_G and a low index of individuality emphasizes the wide variety in cardiac troponin concentrations among the population, the limited utility of population-based reference intervals and the importance of the use of delta-change values^{13,32}. Nevertheless, it is important to consider that the ongoing progression towards increasingly short time intervals between serial measurements, will come to a stop at the point where the changes between consecutive concentrations are so small that there is no distinction anymore with the physiological variation within a person. This can only be partially overcome by increasing the sensitivity and precision of the assay: In the hypothetical

situation of a perfect assay (CV_A 0%) and a CV_I of approximately 8%, the calculated RCV is 22%. This implies that the RCV for cTnI values in the lower range may drop from 37% to 22%, but will never be lower than 22%.

Potential limitations of our study merit consideration. First, most subjects included in the final data analyses have relatively low concentrations of cTnI. It is therefore hard to extrapolate our findings to higher concentration ranges. However, when cTnI values at presentation are much higher, serial measurements have limited additional value^{1,3}. Second, since the prevalence of comorbidities in both populations is relatively high²⁰, we cannot make an unbiased comparison between health and disease. Therefore, we were only able to examine the effect of chronic kidney disease.

In conclusion, with this biological variation study we were the first to examine the hour-to-hour biological variation in cTnI over a full time span of 24h. We showed a random variation over the day. Except from the analytical variation, variation components were of the same magnitude in subjects with or without CKD. Our findings imply that the within-subject biological variation of cardiac troponin I is not affected by the presence of chronic kidney disease.

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Supplemental information

Supplemental Figures

Supplemental Figure 1. Individual diurnal cardiac troponin I profiles. cTnI concentrations over the day in subjects with chronic kidney disease. Depicted are the mean of the duplicate measurements. The numbering is identical to Figure 1. Subjects slept in a supine position between 11:30 P.M. and 7:00 A.M. (shaded area) (curves of non-CKD subjects are shown in chapter 3).

Origin of cardiac troponin T elevations in chronic kidney disease

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Abstract

Background

Elevated cardiac troponin concentrations are common among patients with impaired renal function. Their origin is unknown, but may involve the heart, by increased release, or the kidneys, by decreased renal elimination.

Methods

We employed the diurnal rhythm in troponin T as a model to assess the contribution of impaired renal function to elevated troponin levels in subjects with impaired renal function. We obtained blood samples over 24 hours from subjects with (n=24) and without (n=20) impaired renal function. Diurnal rhythms of cardiac troponin T were analysed by fitting data to a cosine curve. Expected fading of rhythmicity upon a progressively decreasing renal elimination rate was modelled using the cosine curves of subjects without impaired renal function, and compared with the amplitudes in subjects with impaired renal function.

Results

Baseline cardiac troponin T levels were significantly higher in the group with, than in the group without impaired renal function (median 20.5 [18.0–24.6] vs. 14.1 [10.4–20.6] ng/L, $p=0.009$). Concentrations of cardiac troponin T over the day fitted a cosinor model in all subjects (range R^2 0.23–0.91; all $p\leq 0.001$). A progressively decreasing renal elimination was mathematically predicted to decrease the amplitude of the diurnal troponin T rhythm. However, the actual amplitudes of the diurnal rhythm in subjects with chronic kidney disease followed a similar pattern, and were not reduced in amplitude compared to subjects without CKD (11.4 ± 0.5 vs. 12.0 ± 0.5 %; $p=0.53$; mathematically predicted amplitude 8%). In contrast, the amplitude of cystatin C was significantly faded among subjects with decreased renal function compared with the reference group. $1.7\pm 0.2\%$ vs. $3.9\pm 0.2\%$; $p<0.001$; mathematically predicted amplitude 2%).

Conclusions

Whereas a severely decreased elimination of cardiac troponin T would lead to accumulation of cardiac troponin T and fading of the diurnal rhythm, our results suggest that impaired renal elimination itself is not the main cause of elevated cardiac troponin T levels in subjects with chronic kidney disease.

Introduction

Plasma concentrations of cardiac troponins, the preferred biomarkers for the diagnosis of acute myocardial infarction¹⁻³, are often persistently elevated in patients with chronic kidney disease (CKD)⁴. Epidemiological cohort studies support this clinical observation and show robust associations between a deteriorating glomerular filtration rate and plasma troponin elevations⁵⁻⁷. The origin of elevated cardiac troponin concentrations in these patients is unknown: Is it the heart, by increased release, or the kidneys, by decreased renal elimination⁷⁻⁹? CKD is strongly associated with an increased incidence of cardiovascular disease^{10,11}, supporting increased cardiac vulnerability of these patients. Nevertheless, by some, elevated plasma troponin levels in CKD patients are regarded as the prototype example of troponin elevations without cardiovascular etiology^{8,12}. In clinical practice, this equivocal view on troponin elevations in patients with reduced glomerular clearance underlies countless clinical discussions among physicians and may delay rapid initiation of adequate treatment when these patients present with chest pain^{4,13}.

In the present study we sought to discriminate between 'increased cardiac release' or 'reduced renal elimination' as the main process underlying this phenomenon. Specifically, we employed the recently demonstrated rhythmic diurnal oscillation pattern of troponin T, characterized by gradually decreasing concentrations throughout daytime and rising concentrations during night-time, as a model to assess the contribution of impaired renal elimination to persistently elevated troponin levels in patients with CKD^{14,15}. We hypothesized that if decreased renal clearance, and not increased production, is the main driver behind the elevated levels of cardiac troponins in patients with CKD, the subsequent accumulation of cardiac troponins will fade the diurnal rhythm of cardiac troponin T. Therefore, by comparing the amplitude of diurnal oscillations in cardiac troponin T concentrations in subjects with or without CKD, we evaluated the role of reduced renal elimination on elevated basal cardiac troponin T concentrations¹⁶.

Methods

To test the hypothesis that impaired renal elimination is the main driver behind elevated cardiac troponin concentrations, and that the diurnal rhythm in cardiac troponin T levels is affected by the prolonged terminal elimination time in subjects with severely decreased renal function, we performed hourly blood sampling over one full day (24 h), in 20 reference subjects (estimated glomerular filtration rate (eGFR) above $60 \text{ ml}\cdot\text{min}^{-1}\cdot 1.73\text{m}^{-2}$) and in 24 subjects with decreased renal function (eGFR below $60 \text{ ml}\cdot\text{min}^{-1}\cdot 1.73\text{m}^{-2}$)¹⁷. Two consecutive analyses were performed: First, to examine how the rhythm in cardiac troponin T would change due to a decreased elimination rate, we applied a mathematical model, that quantifies the effect of decreased elimination on the resulting diurnal cosine curve, to the circadian rhythm that was observed in the reference subjects¹⁵. Second, we assessed whether the predicted effect of prolonged elimination time on the troponin amplitude matched with the actual results obtained in subjects with impaired renal function.

The study was carried out according to the principles of the Declaration of Helsinki, approved by the Institutional Review Board and Ethics Committee of Maastricht University Medical Centre, and registered at clinicaltrials.gov (NCT02091427 and NCT02210897). All participants provided written informed consent.

Study participants

A total of 44 adult subjects, 20 reference subjects (16 males, 4 females, median eGFR $82.8 [68.0\text{-}92.7] \text{ ml}\cdot\text{min}^{-1}\cdot 1.73\text{m}^{-2}$), and 24 subjects with impaired renal function (eGFR below $60 \text{ ml}\cdot\text{min}^{-1}\cdot 1.73\text{m}^{-2}$) (17 males, 7 females, median eGFR $18.3 [14.2\text{-}32.2] \text{ ml}\cdot\text{min}^{-1}\cdot 1.73\text{m}^{-2}$)¹⁵, were consecutively included. Exclusion criteria were: history of acute myocardial infarction less than 12 months prior to exclusion, (current) dialysis regimen, active cardiac disease (angina pectoris, cardiomyopathy and myocarditis) and anemia ($\text{Hb} < 10.5 \text{ g/dL}$). To facilitate the detection of a rhythm in cardiac troponin T levels, all included subjects were selected for their elevated basal cardiac troponin T levels.

Day and night hourly blood sampling

After an overnight fast, subjects arrived at the laboratory by car or public transport. For 25 h, from 8.30 AM till 9.30 AM the next day, subjects were restricted to the laboratory environment, and samples were collected every hour from an antecubital venous catheter. Extension lines for blood sampling were used to prevent disturbance of participants' sleep during the night. Meals were consumed at 8.30 AM, 12.30 PM and 6.00 PM (breakfast, lunch and dinner, respectively). Subjects went to bed at 11.30 PM, lights were off between 11.35 PM and 7.00 AM, and they slept in a supine position. Participants were asked to refrain from exhaustive physical activities and exercise training, two days before the test day. A schematic overview of the test day is shown in Figure 1.

Figure 1. Hourly blood sampling protocol in subjects with or without chronic kidney disease. Between 8.30 AM till 9.30 AM the next day, subjects were restricted to the laboratory environment, and samples were collected every hour. The lights were off between 11.35 PM and 7.00 AM (grey part), and subjects slept in a supine position. Meals - breakfast, lunch and dinner - were consumed at 8.30 A.M., 12.30 PM and 6.00 PM.

Laboratory measurements

Blood samples were collected in ethylenediaminetetraacetic acid containing (EDTA) tubes. Samples were centrifuged and plasma was stored at -80°C until analysis. Cardiac troponin T was measured with the high-sensitivity cardiac troponin T assay (Roche) on a COBAS 6000 analyser. The 99th percentile among healthy subjects is 14 ng/L, with a 10% analytical variation at 13 ng/L¹⁸. N-terminal prohormone of brain natriuretic peptide (NT-proBNP), creatinine and cystatin C were also measured on the COBAS analyser (all with assays from Roche). The estimated glomerular filtration rate (eGFR) was calculated according to the

Chronic Kidney Disease Epidemiology Collaboration formula based on creatinine and cystatin C¹⁹.

Statistical analysis

Data are expressed as median and interquartile range (mean [IQR]) for non-normally distributed continuous variables, mean and standard deviation (mean±sd) for normally distributed continuous variables and as numbers and percentages (n(%)) for categorical variables. Non-normally distributed continuous variables were compared with the Mann-Whitney U test, categorical variables with the Pearson χ^2 test.

Diurnal rhythms of cardiac troponin T and cystatin C were analysed by fitting the data to a cosine curve by using the method of cosinor rhythmometry^{14-16,20,21}. The cosinor model is described as: $Z(t) = M + A \cdot \cos(\omega t + \varphi) + e(t)$; where $Z(t)$ represents the measured concentration at a given time (t), M the mesor (value about which oscillation occurs), A the amplitude (half the difference between the peak and the nadir value), ω the angular frequency (degrees per unit time with 360° representing a complete cycle), φ the acrophase (timing of maximal value in degrees), and $e(t)$ the error between the cosinor model and the measurement. Evidence of a diurnal rhythm was indicated by an amplitude significantly greater than 0.

A validated mathematical framework on the dynamics of diurnal rhythms based on cosine functions was applied to examine the hypothetical effect of prolonged elimination-times due to impaired renal function on the rhythmic oscillation of cardiac troponin T and cystatin C. This model is extensively discussed elsewhere, and briefly described in the Supplemental Methods¹⁶.

Cosine curves were expressed as deviation (%) from the 24h mesor concentration. Given a mean relative cardiac troponin T amplitude of 14±6% in the reference group¹⁵ and assuming a mean decrease in glomerular filtration rate of 80% in the subjects with impaired renal function, the inclusion of 18 subjects per group would afford 80% power at a significance level of 5% to detect a significantly decreased amplitude. The relative amplitudes between both groups were compared using an unpaired T-test. Additionally, a Wald test was

performed to examine differences in the amplitudes after plotting cosine curves on group level. To test an overall correlation between renal function and the relative amplitude, we performed a Pearson correlation.

All hypothesis testing was two-tailed, and p-values less than 0.05 were considered statistically significant. Statistical analyses were performed with SPSS for Windows 23.0 (IBM Corp.) and R version 3.3.1 and the R-package Cosinor version 1.1 (R-project.org)²¹.

Results

Prolonged elimination time is predicted to blunt the rhythmic oscillations

We mathematically modelled the effect of decreased elimination on the amplitude of the diurnal rhythm in cardiac troponin T that was detected in subjects without CKD (individual curves of subjects without CKD have been published previously^{14,15}). As depicted in Figure 2, restricted elimination of cardiac troponin T would increase elimination time and fade the circadian rhythm: The relative amplitude of 12% would diminish to 11%, 8% and 4% in case of a 60%, 80% and 90% decrease in elimination of cardiac troponin T, respectively.

Figure 2. Predicted effect of decreased elimination on relative amplitudes and circadian rhythm of cardiac troponin T. Fitted cosine curves of 25-hour profile of the percentile deviation from the mesor 24h cardiac troponin T (cTnT) concentration (in %) of 20 reference subjects (panel A) and the predicted curves for decreased elimination rate (panels B-D).

Diurnal rhythm of cardiac troponin T remains intact in patients with decreased renal function

To examine whether the elimination rate of cardiac troponin T is decreased, and thus the predicted effect is evident in subjects that truly have decreased renal elimination rates, the

diurnal rhythm in cardiac troponin T concentrations was analysed in 24 participants with decreased renal function. Participants' baseline characteristics (8.30 AM day 1) are shown in Table 1. Baseline eGFR was by definition lower (median eGFR 18.3 [14.2 – 32.2] vs 82.8 [68.0 – 92.7] ml/min/1.73m²), whereas baseline cardiac troponin T was significantly higher in the group with CKD, than in the group without CKD disease (median cardiac troponin T 20.5 [18.0 – 24.6] vs. 14.1 [10.4 – 20.6] ng/L, $p=0.009$).

As shown in the Supplemental Table 1, cardiac troponin T concentrations over the day fitted a cosinor model with an amplitude significantly larger than 0 in all subjects with decreased renal function (range R^2 0.54-0.90, all $p \leq 0.001$) with similar patterns to those that were observed in the reference population: Gradually decreasing concentrations throughout daytime and rising concentrations during night-time. The observed diurnal rhythm was not related to changes in blood pressure, nor abrogated by the use of loop diuretics (Supplemental Figures 1 and 2).

Table 1. Baseline characteristics

	Reference n= 20	Decreased renal function n= 24	p-value*
Age, y	72 [67 – 78]	69 [61 – 77]	0.25
Male sex	16 (80)	17 (71)	0.49
Antihypertensive medication	8 (40)	16 (67)	0.08
Lipid lowering medication	10 (50)	16 (58)	0.27
Diabetes Mellitus	6 (30)	8 (33)	0.82
Body-mass index, kg/m ²	26.3 [24.7 – 29.7]	26.8 [24.8 – 28.5]	0.94
eGFR, ml.min ⁻¹ .1.73m ²	82.8 [68.0 – 92.7]	18.3 [14.2 – 32.2]	< 0.001
Creatinine, µmol/L	83.5 [68.0 – 89.8]	256.5 [155.0 – 329.3]	< 0.001
Cystatin C, mg/L	0.94 [0.82 – 1.10]	2.75 [1.84 – 3.51]	< 0.001
Cardiac troponin T, ng/L	14.1 [10.4 – 20.6]	20.5 [18.0 – 24.6]	0.009
NT-proBNP, pmol/L	9.8 [4.4 – 24.4]	61.5 [17.9 – 102.7]	<0.001

eGFR denotes estimated glomerular filtration rate according to the Chronic Kidney Disease-EPI Creatinine-Cystatin C equation¹⁹; NT-proBNP, N-terminal prohormone of brain natriuretic peptide. Data is presented as median [IQR] or n (%). *Comparisons between reference and subjects with decreased renal function.

The amplitude of cardiac troponin T is not dependent on renal function

The mean relative amplitude was not significantly different between both groups (reference: 13.2 [9.1 – 20.5] %, decreased renal function: 11.6 [9.0 – 15.2] %, $p=0.45$). Also when data was plot and compared on group level, we observed no significant difference between the subjects with or without CKD (Figure 3) (amplitude 12.0 ± 0.5 vs. 11.4 ± 0.5 %, $p=0.53$). If an impaired renal function, expressed as a low eGFR, would decrease the elimination rate of cardiac troponin T, a significant positive correlation between the relative amplitude and eGFR is predicted. However, this is not supported by a combined analysis of both studies ($R\ 0.21$, $p=0.16$) (Supplemental Figure 3).

Figure 3. The amplitudes in diurnal troponin T (cTnT) oscillations in subjects with and without decreased renal function. A) All individual data points and cosine curve on the group level of the reference population (CKD ($Z(t) = 1.00+0.120.\cos(0.262t+25.51)$). All individual data points and cosine curve on the group level for the group with decreased renal function (CKD ($Z(t) = 1.00+0.224.\cos(0.262t+22.02)$).

Meanwhile, the amplitude of cystatin C, an analyte that also has a diurnal rhythm and with an established renal elimination route²², was significantly faded among subjects with decreased renal function compared with the reference group. Its mathematically predicted amplitude in subjects with impaired renal function matched the observed amplitude in these subjects, supporting the validity of the mathematical model (amplitude cystatin c reference group, $3.9\pm0.2\%$ vs $1.7\pm0.2\%$ in subjects with impaired renal function ($P<0.001$), mathematically predicted amplitude, 2%).

Discussion

This study examined the diurnal rhythm in cardiac troponin T and its relation to the estimated glomerular filtration rate in subjects with or without CKD. We report three major findings.

First, cardiac troponin T concentrations exhibit a similar diurnal rhythm in all subjects, with or without CKD. This rhythm is characterized by gradually decreasing concentrations throughout the day and rising concentrations during night-time, and is in line with the circadian pattern of several processes in the cardiovascular system, for example reflected by an increased intensity of cardiovascular events in the morning hours^{23,24}.

Second, and most important, there is no fading of the circadian rhythm in subjects with impaired renal function. Decreased elimination of cardiac troponin T and the consecutive prolongation of the terminal elimination time should lead to a faded circadian rhythm with a substantially decreased relative amplitude, as shown by our mathematical model (Figure 2). Whereas previous studies have shown that terminal elimination times of several, endogenous and exogenous, compounds can be prolonged up to more than ten times in patients with CKD^{25,26}, we expected to find an obvious decrease in relative amplitude, like we did for cystatin C, if impaired renal clearance would be the main driver behind the elevated cardiac troponin concentrations. The fact that we did not find a difference strongly suggests that the elevated cardiac troponin concentrations in subjects with a decreased renal function are not only explained by decreased elimination and consecutive accumulation of cardiac troponin T. Therefore, our observations do not support the hypothesis that impaired renal elimination is the main driver behind elevated cardiac troponin T levels in subjects with decreased renal function.

Third, we showed a relative amplitude in the diurnal variation of cardiac troponin T of approximately 12%, irrespective of glomerular filtration rate and basal cardiac troponin T concentration. Although in a previous study the diurnal variation in cardiac troponin T did not seem to affect the diagnostic accuracy for acute myocardial infarction in general¹⁵, the rhythm can cause substantial absolute troponin changes in CKD subjects with very high chronic troponin levels, and awareness of the rhythm may benefit accurate interpretation²⁷.

Since we did not detect an effect of decreased renal function on the circadian rhythm of cardiac troponin T, we disprove a common misconception in clinical practice, i.e. that impaired renal clearance of cardiac troponins is the main determinant of their accumulation in subjects with CKD^{7-9,28}. Accordingly, our findings strongly suggest that other factors than renal elimination contribute to the increase in cardiac troponins in subjects with a decreased renal function, such as increased release of cardiac troponins from the heart due to (subclinical) myocardial injury^{29,30}. This is in line with epidemiological evidence, showing a strong association between decreased renal function and cardiovascular risk¹⁰. However, we cannot fully exclude impaired renal clearance of cardiac troponin T in subjects with CKD³¹.

From a clinical perspective our findings are of profound importance for the interpretation of cardiac troponin T levels in patients with decreased renal function. Since our results indicate that elevated levels of cardiac troponin T cannot simply be attributed to only decreased renal elimination, an extensive diagnostic work-up is indicated in all patients with elevated cardiac troponin concentrations irrespective of their estimated glomerular filtration rate⁴.

Potential limitations of our study merit consideration. First, the origin of the rhythmic oscillations we observed is still unknown. We showed that the rhythm is independent of sex, glucose metabolism status, or the use of loop diuretics on the diurnal rhythm. Besides, the circadian pattern of cardiac troponin T is not correlated with blood pressure, levels of cortisol, thyroid stimulating hormone and testosterone¹⁵. Second, we assumed continuous, non-rhythmic, elimination of cardiac troponin T when modelling the predicted effect of a decreasing glomerular filtration rate on the troponin T amplitude. However, even in the case of rhythmic renal elimination, decreased elimination would lead to accumulation of cardiac troponins and fading of the diurnal pattern¹⁶. Third, an alternative, but unlikely, scenario that may explain the unchanged amplitude in CKD, is that troponin T oscillations on itself are a tightly regulated phenomenon to maintain organism homeostasis. Such a scenario would allow unchanged amplitudes in CKD even if clearance rate is decreased, if compensated by increased oscillation amplitudes in the production rate in the heart and/or clearance.

In conclusion, cardiac troponin T exhibits a similar diurnal rhythm in subjects with or without CKD. Whereas a severely decreased elimination of cardiac troponin T would lead to

accumulation of cardiac troponin T and fading of the diurnal rhythm, our results suggest that the elimination of cardiac troponin T is not substantially affected in subjects with CKD. Therefore, it is very unlikely that impaired renal elimination is the main cause of persistently elevated cardiac troponin T levels.

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Supplemental information

Supplemental Methods

Mathematical modeling

To study the effect of prolonged elimination-times on the observed rhythm of diurnal variation we applied a mathematical framework on the dynamics of diurnal rhythms. Since this model is extensively discussed elsewhere¹⁶, we just explain it briefly in this section. The applied model assumes a cosine-shaped function underlying the circadian rhythm (an assumption that is met for cardiac troponin T¹⁵), described as: $Z(t) = M + A \cdot \cos(\omega t - \varphi)$, in which $Z(t)$ represents the measured concentration of cardiac troponin T at a given time (t), M the mesor (the average value on cosine curve fitted to the data), A the amplitude (half the difference between the peak and the nadir value), ω the angular frequency (degrees per unit time, with 360° representing 24h) and φ the acrophase (timing of maximal value in degrees).

When the observed diurnal rhythm is considered as the resultant of production and degradation, these observed diurnal variation can be expressed as combination of two cosine-shaped functions: $\frac{dx}{dt} = \kappa(1 + A_{\text{prod}} \cos(\omega t - \varphi_{\text{prod}})) - \gamma(1 + A_{\text{deg}} \cos(\omega t - \varphi_{\text{deg}}))x(t)$, where κ represents the mean production coefficient, and γ the mean degradation coefficient. This model leads to the abundance: $x(t) \approx M_x \cdot (1 + A_x \cdot \cos(\omega t - \Delta\varphi_x))$. In case of constant degradation ($A_{\text{deg}} = 0$), $M_x = \kappa/\gamma$, $A_x = \gamma \cdot A_{\text{prod}}/\sqrt{\gamma^2 + \omega^2}$ and $\Delta\varphi_x = \arctan(\omega/\gamma)$, are the solutions for mean, amplitude and phase, respectively. This formula for the amplitude shows that when the mean degradation rate γ decreases, the amplitude in x decreases. It can be shown¹⁶ that this relationship holds qualitatively also when the degradation rate itself is rhythmic ($A_{\text{deg}} > 0$).

Supplemental Figures

Supplemental figure 1. Individual diurnal cardiac troponin T profiles and fitted cosine curves. Cosine curves for subjects with impaired renal function. Depicted are the mean of the duplicate measurements. The numbering is identical to Table S1. Subjects slept in a supine position between 11.30 PM and 7.00 AM (shaded area). *indicates the use of loop diuretics.

Supplemental Figure 2. Individual diurnal cardiac troponin T profiles and mean arterial pressure. Cosine curves for subjects with decreased renal function. Depicted are the mean of the duplicate measurements. The numbering is identical to Table S1. Subjects slept in a supine position between 11.30 PM and 7.00 AM (shaded area). Arterial pressure data is not available for subjects 21-24.

Supplemental figure 3. Correlation between relative amplitudes and kidney function (eGFR) . No significant correlation between eGFR and relative amplitude of cardiac troponin T (Pearson’s R 0.21, p=0.16).

Supplemental Tables

Supplemental table 1. The individual cosinor models for subjects with decreased renal function

Participant	Mesor (ng/L)	Amplitude (ng/L)	Acrophase (h)	R ²	P-value
1	20.5	2.7	11.00 AM	0.84	<0.001*
2	15.5	2.3	7.59 AM	0.73	<0.001*
3	11.1	1.7	9.46 AM	0.88	<0.001*
4	15.9	2.2	9.46 AM	0.75	<0.001*
5	13.8	2.1	7.09 AM	0.77	<0.001*
6	4.4	1.6	6.52 AM	0.83	<0.001*
7	35.7	2.5	10.39 AM	0.83	<0.001*
8	20.2	1.9	12.19 PM	0.69	<0.001*
9	19.7	2.8	8.47 AM	0.63	<0.001*
10	14.4	1.6	8.00 AM	0.81	<0.001*
11	24.8	2.9	9.25 AM	0.75	<0.001*
12	5.5	0.5	4.11 AM	0.72	<0.001*
13	15.4	2.5	8.02 AM	0.86	<0.001*
14	47.4	8.8	8.29 AM	0.83	<0.001*
15	19.3	1.7	10.37 AM	0.74	<0.001*
16	18.1	1.5	7.44 AM	0.60	<0.001*
17	16.9	1.3	9.12 AM	0.82	<0.001*
18	118.6	23.9	10.14 AM	0.90	<0.001*
19	57.7	3.9	8.00 AM	0.85	<0.001*
20	19.4	1.5	3.05 AM	0.54	<0.001*
21	16.3	1.9	10.26 AM	0.57	<0.001*
22	19.4	2.2	4.35 AM	0.86	<0.001*
23	17.8	1.9	5.29AM	0.79	<0.001*
24	18.3	2.4	8.19 AM	0.82	<0.001*

The cosinor model is described as: $Z(t) = M + A \cdot \cos(\omega t + \varphi) + e(t)$, where $Z(t)$ represents the measured cardiac troponin T concentration at a given time (t). M the mesor (value about which oscillation occurs). A the amplitude (half the difference between the peak and the nadir value). ω the angular frequency (degrees per unit time, with 360° representing a complete cycle). φ the acrophase (timing of maximal value in degrees) and $e(t)$ the error between the cosinor model and the measurement. *Significant after Bonferroni correction for multiple testing. $p=0.0025$ ($0.05/20$).

Mass spectrometric identification of cardiac troponin T in urine of patients suffering from acute myocardial infarction

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**equal contribution*

Abstract

Background

Due to its high cardiospecificity, cardiac troponin T (cTnT) is one of the biomarkers of first choice to diagnose acute myocardial infarction (AMI). cTnT is extensively fragmented in serum of patients suffering from AMI. However, it is as of yet unknown whether all cTnT is completely degraded in the body or if some cTnT fragments can leave the body via the urine. The aim of the present study is to develop a method for the detection of cTnT in urine and to examine whether cTnT is detectable in patient urine.

Methods

Proteins in urine samples of twenty patients were precipitated using a cTnT-specific immunoprecipitation technique and a non-specific acetonitrile protein precipitation. After in-solution digestion of the precipitated proteins, the resulting peptides were separated and analysed using high-performance liquid chromatography and mass spectrometry using a targeted selected ion monitoring assay with data-dependent tandem-MS (t-SIM/dd-MS2).

Results

The t-SIM/dd-MS2 assay was validated using a synthetic peptide standard containing ten specific cTnT peptides of interest and with purified human intact cTnT spiked in urine from healthy individuals. Using this assay, a total of six different cTnT-specific peptides were identified in urine samples from three different patients, all suffering from AMI.

Conclusions

We show here for the first time that cTnT can be present in the urine of patients suffering from AMI using a targeted LC-MS/MS assay. Whether the presence of cTnT in urine reflects a physiological or rather a pathophysiological process still needs to be elucidated.

Introduction

Cardiac troponin T (cTnT) is one of the preferred biomarkers to establish the diagnosis of acute myocardial infarction (AMI) without characteristic electrocardiogram changes in addition to a clinical presentation and physical examination¹⁻³. Nevertheless, the interpretation of cardiac troponin concentrations can be challenging, especially in subjects with persistently elevated cardiac troponin levels^{4,5}, like patients suffering from chronic kidney disease. The high prevalence of persistently elevated cardiac troponin concentrations among subjects with impaired renal function has raised the question if, and if so how, the kidneys are involved in the terminal elimination of cardiac troponins⁶⁻⁸. A better understanding of the final elimination of cardiac troponins might help us in correctly interpreting cardiac troponin concentrations.

Previous studies have shown that the initial release of cTnT in response to cardiomyocyte damage^{9,10} is followed by a prolonged increase in cTnT levels that can persist for 7-14 days¹⁰⁻¹². In addition, it has been shown that cTnT is highly degraded in serum of patients suffering from AMI in a time-dependent manner^{12,13}. However, the next step in the elimination of these cTnT fragments is still unclear. One possible hypothesis is that the smallest cTnT fragments are further degraded in the circulation until they are no longer detectable by the clinical assay. Another hypothesis is that cTnT fragments are eliminated via the kidneys^{12,14}. Based on their size and charge, the cTnT fragments are susceptible to glomerular filtration¹⁵, and should normally be degraded and reabsorbed by the proximal tubule^{16,17}. These filtered cTnT fragments may also be excreted via the urine, for example due to dysfunction or overload of the tubuli as a result of proteinuria¹⁸.

Recently, we have developed a gel-based targeted mass spectrometry assay for the identification of cTnT and its fragments in serum¹⁹⁻²¹. In the present study, we have modified and validated this assay for the analysis of urine samples. We explored two different sample workup methods and applied them on urine samples of patients with acute myocardial injury.

Methods

Reagents, synthetic peptide standard, and positive control

UPLC-grade water, acetonitrile (ACN), formic acid (FA), and trifluoroacetic acid (TFA) for sample preparation and mass spectrometry were obtained from Biosolve (Valkenswaard, the Netherlands). Ammonium bicarbonate (AMBIC), $\geq 99.5\%$, was from Sigma-Aldrich (St. Louis, MO, USA). Trypsin Gold and ProteaseMAX™ surfactant were from Promega (Madison, WI, USA).

Ten tryptic cTnT peptides of interest were synthesised by Pepscan (Lelystad, the Netherlands) with an average purity $> 95\%$. These peptides were dissolved in 20% ACN and subsequently pooled in 2.0% ACN, 0.01% TFA at a concentration of 40 nmol/L to create a synthetic peptide standard. This standard was used as a quality control sample throughout the experiment and was also used to schedule the targeted MS experiments.

Validation experiments were performed with a positive control consisting of purified human intact cTnT (cat# 8T13, HyTest LTD, Turku, Finland) spiked in both 50 mM AMBIC and fresh urine from a healthy volunteer in various concentrations.

Urine samples

Twenty urine samples from different patients suffering from acute myocardial injury (ST-elevation myocardial infarction (STEMI) (n=9), non ST-elevation myocardial infarction (NSTEMI) (n=4), myocarditis (n=5), cardiac arrest (n=1) or miscellaneous (n=1)) submitted for routine diagnostic analysis were collected over the course of one year when a concomitant cTnT-measurement in the serum of that patient was > 1000 ng/L. Supplemental table 1 shows the relevant patient characteristics. Urine samples were stored for a maximum of 7 days at 4 °C, after which they were aliquoted and stored at -80 °C until analysis. After thawing of samples, urine was first centrifuged at 2,000 g for 10 min and subsequently treated as described below. Patient samples were randomised and treated according to the code of proper secondary use of human tissue in the Netherlands (www.fmwv.nl). As a control, 19 urine samples were collected from presumably healthy volunteers who did not report

symptoms related to acute myocardial infarction neither had a medical history of cardiovascular disease.

Sample workup; protein concentration and digestion

In this study, all samples were analysed using both immunoprecipitation and ACN precipitation to concentrate cTnT from urine. Both methods were followed by in-solution digestion and mass spectrometry. Immunoprecipitation of cTnT in 400 μ L urine was performed by the addition of 50 μ L M-270 Streptavidin Dynabeads (Invitrogen, Carlsbad, CA, USA) coupled to the biotinylated anti-cTnT antibody M11.7 (Roche Diagnostics, Basel, Switzerland) ²². After incubation for 1 hour at room temperature, immunoprecipitated proteins were eluted in 50 mM AMBIC, pH 10, for 15 min at 56 °C, followed by titration to pH 8. As an alternative to immunoprecipitation, ACN precipitation was performed by the addition of 1400 μ L ice-cold (-20 °C) ACN to 400 μ L urine and incubation at -20 °C overnight. Samples were centrifuged at 12,000 g for 30 min at 4 °C and the supernatant was decanted. Pellets were washed in 1 mL ice-cold ACN, air dried, and redissolved in 50 μ L AMBIC (50 mM, pH 8). The digestion of all samples was performed with 1 μ g Trypsin Gold in the presence of 0.03 % ProteaseMAX™ surfactant for 3 hours at 37 °C. TFA was added to a final concentration of 0.5% before LC-MS/MS analysis.

Mass spectrometric analysis of urine samples

Identification of cTnT presence in urine was performed using an established targeted mass spectrometry assay^{19,20} on a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer, connected to a UHPLC Dionex Ultimate 3000 (ThermoFisher Scientific, Waltham, MA, USA). Solvent A consisted of 0.1% formic acid (FA) in 100% water, solvent B consisted of 0.08% FA, 20% water, and 80% ACN. Peptides were first trapped on an Acclaim PepMap 100, 100 μ m x 2 cm, C18, 5 μ m, 100 A trap column in 0.1% TFA, 2% ACN and 98% water. Next, peptides were separated on an Acclaim PepMap RSLC, 75 μ m x 15 cm, C18, 2 μ m, 100 A analytical column by a 30 min gradient of 4-55% solvent B. Washout was performed by a 1 min gradient of 55-90% solvent B and a constant flow of 90% solvent B for 4 min at 300 nL/min.

Selected ion monitoring (SIM) scans were acquired in the Orbitrap with a width of 2.0 Th at a resolution of 70,000 full width at half maximum (FWHM) at 200 m/z, automated gain control (AGC) of 10^5 , and a maximum injection time of 250 ms. SIM scans were scheduled based on the retention times of different peptides obtained using a full scan of the synthetic standard. Selected m/z values were based on the observed peptide masses from the synthetic standard. As a second scan event, a maximum of 10 most intense precursor ions within each SIM scan were selected for higher-energy collisional dissociation (HCD) with an isolation window of 1.0 Th and a normalised collision energy (NCE) that was optimized for each peptide. Product ions were detected in the range of 250-1500 m/z at a resolution of 17,500 FWHM at 200 m/z, AGC target of $2 \cdot 10^5$, maximum injection time of 200 ms, and a dynamic exclusion window of 30 s.

cTnT immunoreactivity determination

cTnT-specific immunoreactivity within the collected urine samples was determined with the 5th generation (high sensitivity) cTnT assay on the Cobas® 6000 instrument by Roche Diagnostics; the 99th percentile among healthy subjects is 14 ng/L, with an interassay CV between 1% and 5% above 30 ng/L²³.

The use of the Roche immunoassay in urine was validated by creating a serial dilution series of the positive control spiked in a urine sample of a healthy individual, in triplicate.

Data analysis

Product ion spectra were searched against the Uniprot Human database dated 13 november 2013 (39,690 entries) using the database search engine SEQUEST with Proteome Discoverer, version 1.8 (ThermoFisher). Precursor and product ion mass tolerances were set to 10 ppm and 0.1 Da, respectively. Enzyme specificity was set to semi-tryptic with 2 missed cleavages. Fixed value PSM validator was used using default Xcorr cut-offs. All accepted spectra were manually evaluated. Statistical significance of cTnT concentrations in urine as measured with the 5th generation Roche assay was assessed with a two-tailed Mann-Whitney test where $p < 0.05$ was considered statistically significant, using GraphPad Prism, version 5.03.

Results

Optimization of sample workup and LC-MS/MS

The synthetic peptide standard, containing synthesised peptides specific to cTnT, was first analysed using a full scan with data-dependent MS/MS (dd-MS2). Figure 1 shows a representative precursor ion chromatogram of this measurement. Every peptide present in the synthetic standard was positively identified using dd-MS2. Even though all peptides were pooled in equimolar amounts, it can be observed that the measured abundance of each peptide differs (for example due to ionisation differences). This analysis was repeated regularly to assess the mass accuracy of the instrument and retention time drift between experiments.

Figure 1. Selected ion chromatogram of the precursor ions of all peptides present in the synthetic peptide standard. The synthetic peptide standard consisted of ten synthesized peptides spiked in equimolar amounts. All labelled peaks were identified with MS/MS. Methionine residues contained within some peptides can be oxidised, designated with: '(ox)'.

Both the immunoprecipitation and the ACN precipitation protocols were validated by serially diluting the positive control in urine from a healthy volunteer and subjecting this dilution series to both workflows. Table 1 lists the peptides identified using MS/MS in both dilution experiments. The listed cTnT concentrations are calculated based on the added amount of

cTnT and the dilution factors used in both experiments. As shown, both protocols were able to identify cTnT spiked in low concentrations in urine. However, some peptides are identified in lower concentrations than others, while others may not be identified at all. In addition, some of the peptides (like ¹⁰⁸DLNE...FENR¹²³ and ²²⁸VLAI...DQLR²⁴⁰) are not detected at all using ACN precipitation, but can be identified in very low concentrations using immunoprecipitation. In general, it can be shown that the limit of identification is lower when using immunoprecipitation than using ACN precipitation.

Table 1. dd-MS2 identification of cTnT peptides of interest in dilution series of positive control samples.

Workflow	ACN precipitation				Immunoprecipitation			
Concentration (ng/L)	3000	1500	750	300	5000	2500	1000	500
⁵³ AEED...EEAK ⁶³	No	No	No	No	Yes	No	No	No
⁵³ AEED...PKPR ⁷⁸	Yes	Yes	No	No	Yes	No	No	No
⁶⁴ EAED...PKPR ⁷⁸	Yes	Yes	No	No	Yes	Yes	No	No
⁷⁹ SFMP...VPPK ⁸⁸	Yes	Yes	No	No	Yes	Yes	Yes	Yes
⁹⁵ VDFDDIHR ¹⁰²	Yes	Yes	No	No	Yes	Yes	Yes	Yes
¹⁰⁸ DLNE...FENR ¹²³	No	No	No	No	Yes	Yes	Yes	Yes
¹²⁴ KKEE...VSLK ¹³⁴	No	No	No	No	Yes	No	No	No
¹⁸⁷ ALSN...YIQK ²⁰⁰	Yes	Yes	No	No	Yes	Yes	Yes	No
²²⁸ VLAI...DQLR ²⁴⁰	No	No	No	No	Yes	Yes	Yes	Yes
²⁶⁹ YEINVLR ²⁷⁵	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Purified human intact cTnT was dissolved in urine and serially diluted. Both dilution series were then subjected to either acetonitrile (ACN) precipitation or immunoprecipitation followed by in-solution digestion and mass spectrometry. ‘Yes’ indicates that the peptide was identified using dd-MS2, ‘No’ indicates that no such identification was made. Amino acid numbering is based on the canonical adult human isoform of cTnT (CTNT-3, P45379-6).

Troponin identification in patient urine

Both protocols were applied to the 20 clinical urine samples from patients with acute myocardial injury. When using immunoprecipitation, cTnT specific peptides were identified in two of the analysed samples. In one sample, four peptides could be identified (figure 2a). In the other sample, only one peptide was identified (figure 2b). When ACN precipitation was

used, cTnT peptides were identified in a single additional sample (figure 2c). The identification of cTnT in these three samples was independently verified using fresh aliquots in a repeat experiment. No cTnT peptides could be detected with LC-MS/MS in the other 17 urine samples. All peptides were identified with high confidence ($xcorr > 1.9$ and $\Delta Cn = 0$ for all peptides; table 2).

In addition to the LC-MS/MS identifications, cTnT immunoreactivity was measured in all urine samples using the 5th generation Roche assay. Figure 3a shows the measured cTnT immunoreactivity of a dilution series of the positive control in urine of a healthy volunteer ($R^2=0.9981$), indicating that the assay responds linearly to a serial dilution within the relevant measuring range in urine. Figure 3b shows that the immunoreactivity is significantly higher in urine samples from patients suffering from myocardial injury (median = 17.9 ng/L) than in urine samples from healthy individuals (median = 3.54 ng/L, $p<0.0001$). There is no significant correlation between the measured cTnT concentrations in serum and in urine (Spearman's $R^2=0.095$, $p=0.186$, not shown). Supplemental table 1 shows the relevant laboratory measurements of each individual patient included in the study.

Table 2. Cross-correlation ($xcorr$) and delta correlation (ΔCn) values of identified cTnT peptides in patient urine.

Patient	Identified peptide	Xcorr	ΔCn
a	⁷⁹ SFMP...VPPK ⁸⁸	2.29	0
a	⁹⁵ VDFDDIHR ¹⁰²	2.58	0
a	¹⁰⁸ DLNE...FENR ¹²³	5.65	0
a	¹²⁴ KKEE...VSLK ¹³⁴	4.55	0
b	¹²⁴ KKEE...VSLK ¹³⁴	4.45	0
c	⁵³ AEED...EEAK ⁶³	2.08	0
c	⁶⁴ EADG...PKPR ⁷⁸	3.48	0

Xcorr and ΔCn scores are listed for all the spectra in figure 3. Xcorr cutoff value was set to 1.9, maximum ΔCn score allowed was 0.05.

Figure 2. Product ion spectra of cTnT peptides present in patient urine samples. A) Four peptides, located at the centre region of cTnT, were identified in patient (a) using immunoprecipitation. B) One peptide, located at the centre region of cTnT, was identified in patient (b) using immunoprecipitation. C) Two peptides, located at the N-terminus of cTnT, were identified in patient (c) using acetonitrile precipitation.

Figure 3. Immunoreactivity in urine samples as measured with the 5th generation clinical cTnT immunoassay by Roche Diagnostics. A. Serial dilution series of the positive control spiked in urine of a healthy individual. $R^2 = 0.9981$, $n = 3$. B. Twenty urine samples of patients suffering from acute myocardial injury and of 19 healthy controls. Error bars indicate median and interquartile range, $p < 0.0001$. Open dots indicate urine samples where cTnT was positively identified using mass spectrometry, labelled a, b, and c, respectively, as in figure 2, and table 2.

Discussion

In this study, a targeted LC-MS/MS assay and two different proteomic workflows were used to detect the presence of cardiac troponin T in urine. Using this method, we are the first to provide conclusive evidence that cTnT can be present in the urine of patients with high serum concentrations of cTnT. We were able to show the presence of cTnT-specific peptides in urine in three out of twenty patients suffering from acute myocardial injury. All three of these patients suffered from AMI, with concomitant proteinuria.

While urine is a biological matrix that can be conveniently and non-invasively acquired in large quantities, proteomics-based identification of proteins in urine is a challenge. Urine proteomics is complicated by high interfering concentrations of salts and urea, a broad pH spectrum, and relatively low protein concentrations²⁴. Since our primary interest was the identification of cTnT in urine, we selectively captured cTnT from urine using an adapted immunoprecipitation technique based on a successful protocol for cTnT isolation from serum^{12,19,22}. This technique removes salts, urea, and other interfering components, but is

very selective in its binding to the central region of cTnT. The peptides identified with this workflow (figures 2a and 2b) indeed do originate from this central region (figure 4). Since cTnT is subject to proteolytic degradation in the blood circulation^{12,13,21,25}, it is possible that this methodology is too selective and that cTnT fragments that do not contain this central region might be missed. For this reason, we repeated the experiment using a non-selective workflow based on the precipitation of proteins using an organic solvent^{24,26-29}. This resulted in the identification of two peptides originating from the N-terminus (which removal is considered to be the first step in the degradation of cTnT^{12,21}) in an additional sample (figure 2c, figure 4). The peak serum concentration of cTnT in this particular patient was very high (31,199 ng/L, supplemental table 1), which may explain why we identified cTnT peptides in the urine of this patient, despite the fact that the measured cTnT immunoreactivity in his urine was much lower (125 ng/L). The other two patients in which cTnT peptides were identified had a very high cTnT-specific immunoreactivity in urine (13,000 and 7,365 ng/L; red dots in figure 3b), supporting the hypothesis that the limit of identification of the t-SIM/dd-MS2 assay was insufficient to detect cTnT peptides in the other urine samples. Another possibility is that the cTnT present in urine is even more progressively degraded compared to serum. When the arginine and lysine rich centre region is the only part of cTnT that remains in urine, it may become completely destroyed during tryptic digestion and will be missed with the t-SIM/dd-MS2 assay, but possibly not with the immunoassay, where digestion is not performed. Because we utilized leftover samples from patients routinely analysed in our laboratory, no protease inhibitors or other stabilizers were added to the urine upon collection. Therefore, it is possible that any fragmentation of cTnT continues in urine. For the same logistic reason, the urine samples were not collected on standardized time points and no serial sampling was performed. Future research should take these suggestions into account.

Figure 4. Annotated sequence of the canonical isoforms of cardiac troponin T. Green highlights indicate peptides that were successfully identified using mass spectrometry in patients (a), (b), and (c), as depicted. Red highlights indicate targeted peptides that were not identified in any of the samples. The orange highlighted area indicates a region that is spliced in the adult human cTnT isoforms (isoforms 6, cTnT-3). The purple highlights indicate the epitopes of the Roche M7 detector and M11.7 catcher antibodies.

It is important to note that we were only able to identify cTnT in three samples, and that in one of these three samples only a single cTnT peptide was identified, violating commonly used guidelines for the identification of proteins³⁰. However, these guidelines are often focused on intact proteins making the requirement of identifying more than one peptide more feasible than in this specific case, where we are most likely dealing with protein fragments. Considering the high confidence of this match, the quality of the MS/MS spectrum, and the fact that a very abundant cTnT-specific immunoreactivity was also determined, we deemed this match convincing. While it could be possible that the low number of identifications was because of the limit of identification of the assay (Table 1) or because of extensive progressive cTnT degradation (possibly continuing in urine), it cannot be excluded that this effect only occurs in specific pathophysiological conditions. The single selection criterion of our study was a routinely obtained urine sample from a patient with high cTnT concentration in serum (>1000 ng/L), resulting in a wide range of pathophysiological conditions in the selected patients. In addition to AMI, all three patients where cTnT was identified with LC-MS/MS additionally suffered from proteinuria, possibly causing filtered cTnT fragments to end up in the urine as a result of protein overload of the proximal tubule.

Previously, Ziebig et al. also detected cTnT in urine of patients suffering from AMI or undergoing heart surgery using the clinical cTnT immunoassay³¹. Patients included in the work of Ziebig also suffered from mild to severe proteinuria, however, their methodology was not intended to be used in urine and was not properly validated for that matrix. This makes it hard to draw firm conclusions, as positive results can be caused by matrix effects or other disturbances. After all, while immunoassays may have relatively low limits of detection, their limit of identification is more questionable. Immunoassays will require multiple epitopes in order to reach the same level of identification certainty as tandem mass spectrometry. In this study, we were able to confirm the findings by Ziebig et al. using the 5th generation cTnT immunoassay (whereas Ziebig used the 2nd generation immunoassay, both by Roche Diagnostics). The thus measured cTnT-specific immunoreactivity was considerable in most patient samples and differed significantly from those measured in healthy controls (figure 3b). However, due to the urinary matrix, the identity of this immunoreactivity is uncertain. Therefore, it is important to interpret figure 3b, as well as the study by Ziebig et al.³¹, with caution.

Lastly, a recent study from our group showed that impaired renal clearance is not the main driver behind persistently elevated cardiac troponin concentrations⁸. While the results presented here may seem to be at odds with that conclusion, it is important to consider that the current demonstration of cardiac troponin in urine is limited to subjects suffering from acute myocardial infarction with high cTnT concentrations in serum (>1000 ng/L) and concomitant proteinuria. Involvement of the kidneys in the elimination of cTnT (be it physiological or pathophysiological) does not automatically imply that reduced renal clearance leads to aspecific troponin elevations in the absence of myocardial damage.

In conclusion, we show here, using a targeted mass spectrometry assay, that the highly cardiospecific biomarker cTnT can be present in urine of patients suffering from AMI. Further study to the extent and implications of these observations are required, as well as determining whether this is a non-specific effect caused by concomitant proteinuria, or if this is a more general elimination pathway of cTnT.

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Supplemental information

Supplemental Tables

Supplemental Table 1. Characteristics and laboratory values of patients included in the study

Pat. No. ^a	Sex, age	Diagnosis	cTnT _{serum} (ng/L)	cTnT _{urine} (ng/L)	eGFR ^b (ml/min/1.73m ²)	Urine albumin ^c (g/L)
a	M, 80	NSTEMI	2274	13000	12	1
b	M, 64	STEMI	3494	7365	13	0.3
c	M, 93	STEMI	31199	125	70	n/a
1	M, 71	STEMI	5443	27	39	1
2	F, 90	Miscellaneous	1180	7	42	1
3	M, 82	STEMI	2556	355	27	0.3
4	M, 85	NSTEMI	1220	27	39	0.3
5	F, 70	STEMI	2770	15	93	0.3
6	M, 54	Myocarditis	7891	8	65	0.3
7	M, 52	STEMI	1203	38	65	n/a
8	F, 81	STEMI	1792	6	58	Negative
9	M, 71	NSTEMI	1551	19	21	Trace
10	M, 34	Myocarditis	8497	17	112	Negative
11	M, 56	Myocarditis	3803	706	17	0.59
12	M, 77	STEMI	5967	17	34	0.3
13	F, 91	STEMI	2681	14	25	n/a
14	F, 84	NSTEMI	3783	50	17	Trace
15	M, 65	Myocarditis	1225	<3.00	82	Negative
16	F, 55	Cardiac arrest	1715	5	80	0.3
17	M, 25	Myocarditis	1688	12	122	0.3

^a Patient numbers a-c belong to patients with MS/MS identification of cTnT and correspond to the numbers in table 2 and the figures in the main article. Numbers 1-17 belong to patients without MS/MS identifications.

^b Estimated glomerular filtration rate (eGFR) was calculated by the CKD-EPI formula as proposed by Levey et al., Ann Intern Med 2009; 150(9):604-612., as follows: $eGFR = 141 \times \min(Scr/\kappa, 1)^\alpha \times \max(Scr/\kappa, 1)^{-1.209} \times 0.993^{Age} \times 1.018$ [if female] $\times 1.159$ [if black], where Scr is serum creatinine, κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, min indicates the minimum of Scr/ κ or 1, and max indicates the maximum of Scr/ κ or 1.

^c Urine albumin levels were used as indicators of proteinuria. n/a indicates that albumin levels were not available, negative indicates albumin levels < 0.05 g/L, and trace means an albumin level between 0.05 and 0.1 g/L.

Large variation in measured cardiac troponin T concentrations after standard addition in serum or plasma of different individuals

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Introduction

During acute myocardial infarction (AMI), cardiac troponin T (cTnT) is released from the damaged myocardium. One would expect a strong correlation between concentrations of cTnT measured in the blood after AMI and the extent of myocardial damage. However, various studies have shown a rather moderate correlation between infarct size and cTnT concentrations^{1,2}. Frequently considered explanations for this phenomenon are methodological, or focused on physiological factors associated with differences in release and elimination of cTnT². In contrast, less attention has been paid to the possible presence of inherent factors in the blood, such as (auto)antibodies or proteases, that might modify cTnT or interfere with the assay. We hypothesized that these factors may be ubiquitously present in the general population, and might affect the measured concentrations of cTnT in individuals.

Methods

We measured cTnT, cardiac troponin I (cTnI), creatine kinase MB isoenzyme (CK-MB) and myoglobin concentrations in sera from 24 healthy volunteers (age range 22 – 54 years) before and after the addition of serum from a patient suffering from AMI (age 59 years, cTnT $\approx$ 19.5 μ g/L). All participants gave written informed consent and leftover material was used in accordance with the code of proper secondary use of human tissue in the Netherlands. Spiking of AMI-patient serum (40 μ L in 760 μ L of volunteer serum) was performed in triplicate. cTnT (hs-cTnT: limit of detection (LoD) 5 ng/L; 10%-CV at 13 ng/L), CK-MB (LoD of 0.3 μ g/L, 20%-CV at 1 μ g/L), and myoglobin (LoD 21 μ g/L) were determined on the COBAS (Roche Diagnostics), and cTnI (hs-cTnI: LoD 1.1–1.9 ng/L, 10%-CV at 4.7 ng/L) on the Architect (Abbott Diagnostics) (information based on product inserts). Biomarker concentrations were corrected for their baseline concentrations, and outliers (Grubbs test) were excluded. To make the results of different biomarkers easily comparable, the subject-specific deviation was calculated. The subject-specific deviation was defined as the percentage of deviation of a measured biomarker concentration in an individual subject compared to the mean

concentration of that biomarker across all samples after spiking of a fixed amount of material.

Results

The subject-specific deviation of cTnT after spiking varied between -54% and +22% (Fig. 1). Next, the inter-subject coefficient of variation (CV_g) was calculated for cTnT (14.6%), which was significantly higher than the CV_g for cTnI (6.9%), CK-MB (2.6%), and myoglobin (3.0%) (all $p < 0.001$) measured in the same samples. In contrast, no significant difference was shown in the coefficient of variation between the triplicate measurements of the different cardiac biomarkers ($\pm 1.5\%$, not shown). Interestingly, when this experiment was repeated in serum from the same individuals that was deproteinated by perchloric acid and neutralized to pH 7.4, the CV_g for cTnT concentration significantly decreased from 14.6% to 6.5% ($p < 0.001$). These findings were confirmed in an independent duplicate experiment using purified human cTnT (cat#8T13, HyTest LTD, Turku, Finland) instead of AMI-patient serum.

Figure 1. Subject-specific deviation (%) of cardiac biomarkers added to serum samples of healthy individuals. Boxplots showing quartiles and range (min-max) of the subject-specific deviations for cardiac biomarkers in 24 healthy volunteers (mean of triplicates). We detected two outliers (one for cTnT in deproteinated serum and one for CK-MB) within triplicate measurements and excluded these single measurements. * Significant ($p < 0.001$) difference in the inter-subject coefficient of variation of a biomarker compared to that of cTnT in unprocessed serum samples. CK-MB, creatine kinase MB isoenzyme; cTnI, cardiac troponin I; cTnT, cardiac troponin T; CV_g , inter-subject coefficient of variation; deprot, deproteinated serum; Myo, myoglobin.

The wide inter-subject variation in cTnT concentrations observed in these first experiments was validated in a consecutive experiment in which 2.5 μL of purified human cTnT (HyTest) was spiked in leftover heparin plasma samples (250 μL) from 119 patients. Again, concentrations were corrected for baseline values. In this population, we observed a subject-specific deviation between -39% and +37% that was normally distributed (D'Agostino-Pearson omnibus test, $p=0.210$). The CV_g of 15.8% in this experiment was comparable to that in our previous experiment.

Conclusion

Thus, we showed a substantial and specific variation in cTnT concentrations after the standard addition of equal amounts of cTnT to both serum and plasma of several individuals. This inter-subject variation decreased significantly when serum was deproteinated. Currently, we can only speculate about the nature of the observed variations. Since the effect diminished after the removal of proteins from serum, a protein factor could be responsible. Individual differences in proteolytic degradation of cTnT cannot be excluded as the responsible factor, but seems to be unlikely since the cTnT present in the serum sample used for spiking will already be fragmented and the observed pattern remains similar when purified human cTnT was used³. Also, the involvement of heterophilic antibodies, like human anti-mouse antibodies (HAMA) and rheumatoid factor, is unlikely since the variation is ubiquitously present. A remaining possible explanation could be the blocking of epitopes or alternative folding of cTnT due to the interaction with specific (auto)antibodies⁴. More research is needed to identify the exact factor(s) responsible for the observed variation, including their mechanism of action, to ascertain whether or not these differences also occur with non-foreign cTnT *in vivo*, and to determine the exact clinical implications including the determination of infarct size.

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Combining cardiac troponin I and cardiac troponin T in the early diagnosis of acute myocardial infarction

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Abstract

Background

Combining two signals of cardiomyocyte damage, cardiac troponin I (cTnI) and T (cTnT), might overcome some individual pathophysiological and analytical limitations and thereby increase diagnostic accuracy for acute myocardial infarction (AMI) with a single blood draw. We aimed to evaluate the diagnostic performance of combinations of cTnI and cTnT for the early diagnosis of AMI.

Methods

The diagnostic performance of combining cTnI and cTnT (simple mathematical derivations and a combination algorithm) was evaluated in a large prospective multicenter diagnostic study of patients with suspected AMI. The optimal thresholds were externally validated in a second large multicenter diagnostic study.

Results

Combining cTnI and cTnT did not consistently increase overall diagnostic accuracy as compared to the individual isoforms. In contrast, the combination seemed to improve the very early rule-out, which at the guideline-recommended cut-offs is limited (6-11%) and assay dependent. Application of optimized cut-off values using the sum (9 ng/L) and the product (18 ng²/L²) of cTnI and cTnT led to a significant increase in rule-out to 34-41% in the original (sum: negative predictive value (NPV) 100% (95%CI: 99.5-100%); product: NPV 100% (95%CI: 99.5-100%) and in the external validation cohort (sum: NPV 99.6% (95%CI: 99.0-99.9%); product: NPV 99.4% (95%CI: 98.8-99.8%)) as in comparison to the ESC 2015 algorithm. The use of a combination algorithm (cTnI <4 ng/L and cTnT <9 ng/L) showed comparable results (rule out: 40-43%; NPV original cohort 99.9% (95%CI: 99.2-100%); NPV external validation cohort 99.5% (95%CI: 98.9-99.8%)).

Conclusions

New strategies combining cTnI and cTnT measurements may significantly increase the number of patients eligible for very early and safe rule-out, but do not seem helpful for the rule-in of AMI.

Introduction

Approximately 10% of all patients seeking medical attention at the emergency department (ED) report chest discomfort, a complaint that reflects many potential etiologies including acute myocardial infarction (AMI)¹. Rapid identification of patients with AMI is of profound clinical importance for fast initiation of medical treatment and management². In addition, rapid rule-out of patients without AMI can overcome prolonged patient anxiety, unnecessary resource use and overcrowding in the ED³⁻⁹. Despite major improvements in diagnostic accuracy due to the introduction of high-sensitivity cardiac troponin (hs-cTn) assays and data-driven optimized diagnostic algorithms, rapid, accurate and safe rule-out based on a single measurement of hs-cTn is still possible only in a minority of patients^{2,3,10}.

Current guidelines recommend to measure one of the cardiac specific isoforms of the cardiac troponin (cTn) complex: cTnI or cTnT^{2,11,12}. Despite differences in biochemical characteristics and release kinetics^{13,14}, a recent direct comparison between high-sensitivity cTnI (hs-cTnI) and T (hs-cTnT) showed similar, high diagnostic accuracy for AMI emphasizing the similarities between both isoforms¹⁵. Based on the observation of an imperfect correlation between blood concentrations of cTnT and cTnI in chronic and acute disorders^{16,17}, and in analogy to the quantification of renal function using creatinine and cystatin C, where the combination of two parameters associated with the same pathophysiological process but influenced by distinct factors lead to a more precise and accurate indicator¹⁸, we hypothesize that combining cTnI and cTnT will overcome independent pathophysiological, pre-analytical and analytical differences of the individual molecules, and might therefore have higher diagnostic accuracy for AMI than either cTnI or cTnT. This hypothesis was tested in two large prospective multicenter diagnostic studies.

Methods

Patients and setting

The combination of cTnI and cTnT for the diagnosis of AMI was investigated in two studies; The primary cohort was the Advantageous Predictors of Acute Coronary Syndrome Evaluation (APACE) study^{3,15,19,20}, and the secondary (external validation) cohort was the 2-Hour Accelerated Diagnostic Protocol to Assess Patients With Chest Pain Symptoms Using Contemporary Troponins as the Only Biomarker (ADAPT) study²¹.

APACE is an ongoing prospective international multicenter diagnostic study that enrolls patients presenting to the ED with acute chest discomfort with an onset of peak within the last 12 hours. Patients are enrolled regardless of their renal function. Only patients with terminal kidney failure on chronic dialysis are excluded. This analysis contains data of patients enrolled between April 2006 and May 2013 who had a final diagnosis adjudicated by two independent cardiologists (n=3029). For this analysis, patients were excluded if hs-cTnI or hs-cTnT blood concentrations at presentation were not available (n=661), if the final adjudicated diagnosis was ST-elevation myocardial infarction (STEMI) (n=74), or if the final diagnosis remained unclear after adjudication and at least one cTn level was elevated (possibly indicating the presence of AMI) (n=69).

In the ADAPT cohort (4 cohorts combined: ADAPT-Brisbane, ADAPT-Christchurch, EDACS-RCT, ADAPT-RCT), patients with at least 5 min of symptoms consistent with acute coronary syndrome²², but without ST-segment elevation, were enrolled at two EDs in Brisbane, Australia and Christchurch, New Zealand between November 2007 and February 2011.

Both studies were carried out according to the principles of the Declaration of Helsinki, approved by the local ethics committees, and registered at clinicaltrial.gov (APACE: NCT00470587) or at the Australia-New Zealand Clinical Trials Registry (ADAPT: ACTRN12611001069943). Written informed consent was obtained from all patients.

Routine clinical assessment

In both cohorts, patients underwent routine clinical assessment that included medical history, physical examination, standard blood tests including serial measurements of local

(hs)-cTn, 12-lead ECG, chest radiography, continuous ECG rhythm monitoring and pulse oximetry. Management of patients was left to the discretion of the attending physician.

Adjudicated final diagnosis

In the APACE cohort, adjudication of the final diagnosis was performed by two independent cardiologists at the core laboratory (University Hospital Basel) applying the universal definition of AMI²³ using two sets of data: first, all available medical records obtained during clinical care including history, physical examination, results of laboratory testing (including serial clinical (hs)-cTn levels, radiologic testing, ECG, echocardiography, cardiac exercise test, lesion severity and morphology in coronary angiography - pertaining to the patient from the time of ED presentation to 90-day follow up; second, study-specific assessments including detailed chest pain characteristics using 34 predefined criteria, serial hs-cTnT blood concentrations obtained from study samples, and clinical follow-up by telephone and/or mail. In situations of disagreement about the diagnosis, cases were reviewed and adjudicated in conjunction with a third cardiologist. These procedures were comparable to those in the ADAPT cohort, where the adjudication of the final diagnosis was performed by two independent cardiologists blind to results of the index-test biomarkers under investigation, but with knowledge of the clinical record, ECG, and serial cTnI results from routine care (details of adjudication are given in the Supplementary Data).

In both cohorts, AMI was defined and (hs-)cTn interpreted as recommended in the current guidelines^{2,24,25}. In brief, AMI was diagnosed when there was evidence of myocardial necrosis in association with a clinical setting consistent with myocardial ischemia. Myocardial necrosis was diagnosed by at least one cTn value above the 99th percentile (or for the conventional cTn assays above the 10% imprecision value if not fulfilled at the 99th percentile) together with a significant rise and/or fall. The criteria used to define a rise and/or fall in conventional cTn and hs-cTnT are described in detail in the method section in the data supplement. All other patients were classified in the categories of unstable angina (UA), Non Cardiac Chest Pain (NCCP), cardiac but non-coronary disease (e.g. tachyarrhythmias, perimyocarditis), and symptoms of unknown origin with normal levels of hs-cTnT.

Measurement of hs-cTnT and hs-cTnI

After centrifugation, serum was frozen at -80°C until measurement with hs-cTn assays. Hs-cTnI was measured by using the ARCHITECT High Sensitive STAT Troponin I assay (Abbott Laboratories). According to the manufacturer, the 99th percentile concentration is 26.2 ng/L with a corresponding coefficient of variation (CV) of <5%²⁶. Hs-cTnT was measured with the Roche hs-cTnT assay. The 99th percentile among healthy subjects is 14 ng/L, with a 10% analytical variation at 13 ng/L²⁷. Data presented here were not affected by the 2010–2012 hs-cTnT low-end shift in APACE and appropriately corrected in ADAPT^{28–30}. Calculation of the glomerular filtration rate (eGFR) was performed using the abbreviated Modification of Diet in Renal Disease (MDRD) formula³¹.

Statistical analysis

We evaluated the diagnostic accuracy and performance of the combined hs-cTnI and hs-cTnT measurement in two different ways: First, we examined simple clinically easily interpretable mathematical derivations (sum, product, ratio). Second, we tested a combination algorithm of hs-cTnI and hs-cTnT. Data are expressed as median $\pm$ interquartile range (IQR) for continuous variables and as numbers (n) and percentages (%) for categorical variables. Continuous variables were compared with the Mann-Whitney U test, and categorical variables were compared by use of the Pearson χ^2 test. Cohen's kappa statistic was performed to examine the agreement between rule-in and rule-out at presentation based on hs-cTnT and hs-cTnI according to the two diagnostic algorithms recommended with a class I recommendation in the current European Society of Cardiology (ESC) guidelines: the 0/3h-hs-cTn-algorithm and the 0h/1h-hs-cTn-algorithm². Mathematical derivations were calculated from raw data after the correction of undetectable low concentrations into 0.1ng/L. Binary logistic regression analyses were used to calculate predicted probabilities for combined test variables.

Receiver-operating characteristics (ROC) curves were constructed to assess diagnostic performance at presentation and 1h after initial presentation including the absolute change value. Diagnostic accuracy was reported as the area under the ROC curve (AUC) and the

corresponding 95% confidence intervals (95% CI). The comparison of dependent and independent AUCs was performed as recommended by Hanley and McNeil^{32,33}. 0h and 0h/1h serial sampled hs-cTn blood concentrations were combined to represent the current gold standard of clinical care as suggested in the 2015 ESC guidelines². Furthermore, integrated discrimination improvement (IDI) and category-free net reclassification improvement (cf-NRI) were calculated.

For the determination of optimal cut-off values for mathematical derivations (minimal negative predictive value (NPV) of 99.6% and a positive predictive value (PPV) of 75.0%, respectively, to match the performance of the 0h/1h-hs-cTn-algorithm^{2,34-36}, the cohort was randomly divided in a derivation (80% of patients) and a validation sub-cohort (20% of patients).

For the cut-off values in the combination algorithm, we determined the optimal combination of hs-cTn thresholds (maximized specificity and a minimum sensitivity of 99%) based on a smoothed average of 500 bootstraps of the original cohort, in which we varied the hs-cTn threshold for each troponin assay in steps of 0.1 ng/L.

All hypothesis testing was two-tailed, and values of $p < 0.05$ were considered statistically significant. We did not adjust for multiple testing. Statistical analyses were performed with SPSS for Windows 23.0 (SPSS Inc), MedCalc 9.6.4.0 (MedCalc software) and R version 3.2.4 (with packages 'boot' v1.3-18 and 'fields' v8.10).

Results

Distribution of hs-cTn concentrations at presentation in patients with suspected AMI

Baseline characteristics of 2225 patients in the APACE cohort presenting to the ED with suspected AMI are shown in Supplementary Table 1. The adjudicated final diagnosis was AMI (NSTEMI) in 18% of patients (85% had type I and 15% type II AMI), UA in 10%, cardiac but not coronary artery disease in 14%, NCCP in 54%, and symptoms of unknown origin in 5%. AMI patients had significant higher levels of hs-cTnI and hs-cTnT at presentation compared with the no-AMI group (hs-cTnI median 115.2 ng/L (IQR: 21.7–632.9) vs. 3.5 ng/L (IQR: 2.2–7.2) $P<0.001$; hs-cTnT median 64.1 ng/L (IQR: 28.0–152.4) vs. 7.0 ng/L (IQR: 4.0–12.4) $P<0.001$; Supplementary Table 3 and Supplementary Figure 1). The correlation between hs-cTnI and hs-cTnT concentrations at presentation was moderate (Figure 1).

Figure 1. Bland Altman plot of hs-cTnI and hs-cTnT at presentation in the APACE cohort. Bland Altman plot displaying hs-cTnI and hs-cTnT concentrations at presentation in the APACE cohort (n=2225).

Application of the ESC 0h/3h-algorithm

AMI could be ruled-out in 157 (7.1%, sensitivity 99.2%, specificity 8.4%, NPV 98.1%) and 133 (6.0%, sensitivity 99.7%, specificity 7.2%, NPV 99.2%) patients with a single blood draw at presentation, using hs-cTnI or hs-cTnT, respectively. The agreement on patient allocation between hs-cTnI and hs-cTnT for rule-out at presentation was good ($\kappa=0.90$) (Figure 3, Supplementary Table 4a).

Application of the ESC 0h/1h-algorithm

AMI could be ruled-out in 149 (6.7%, sensitivity 100%, specificity 8.1%, NPV 100%) and 235 (10.6%, sensitivity 100%, specificity 12.9%, NPV 100%) patients after a single blood draw at presentation, using hs-cTnI or hs-cTnT, respectively. Direct rule-in could be achieved in 331 (14.9%, sensitivity 62.8%, specificity 95.6%, PPV 75.5%) and 273 (12.3%, sensitivity 57.8%, specificity 2.4%, PPV 84.2%) subjects, using hs-cTnI or hs-cTnT, respectively. The agreement on patient allocation at presentation between hs-cTnI and hs-cTnT was moderate for rule-out ($\kappa=0.42$) and good for rule-in ($\kappa=0.79$) (Figure 3, Supplementary Tables 4b and 4c).

Diagnostic performance of combined hs-cTnI and hs-cTnT measurement at presentation

The diagnostic accuracy in the APACE cohort, as quantified by AUC was evidently lower for ratio than for the other combined measurements and individual isoforms (Table 1 and Figure 2). Addition of a second isoform to 0h hs-cTn led to numerically small, but statistically significant improvements in diagnostic accuracy of hs-cTnT, but not hs-cTnI. Furthermore, addition of a combined measurement at presentation to the 0h and 0h/1h change concentrations led to a numerically small, but statistically significant improvement in diagnostic accuracy of hs-cTnI, but not of hs-cTnT (Supplementary Table 5). Reclassification statistics (IDI and cf-NRI) did not uniformly show incremental value of combining cardiac troponins at presentation when applied to the entire APACE cohort (Supplementary Tables 6a and 6b).

Table 1. Diagnostic accuracy of hs-cTnI, hs-cTnT, the combination and athematical derivations their (sum, product and ratio) for the diagnosis of AMI at presentation.

Parameters	AUC (95% CI)	Compared with hs-cTnI (p-value)	Compared with hs-cTnT (p-value)
hs-cTnI	0.93 (0.92 – 0.94)		0.714
hs-cTnT	0.93 (0.92 – 0.94)	0.714	
hs-cTnI & hs-cTnT	0.93 (0.92 – 0.94)	0.789	0.002
Sum	0.94 (0.93 – 0.95)	0.053	0.114
Product	0.94 (0.93 – 0.95)	0.007	0.078
Ratio	0.79 (0.78 – 0.81)	<0.001	<0.001

Figure 2. ROC curves of the diagnostic performance of high-sensitivity cTn and their ratio, sum and product for NSTEMI in the APACE cohort. Diagnostic performance of high-sensitive cTn for non-ST segment myocardial infarction at presentation to the emergency department with acute chest pain. Receiver-operating-characteristic curves show the diagnostic accuracy of high-sensitive cardiac troponins I and T, their ratio, sum and product.

Table 2a. Performance of cut-off values for rule-out and rule-in of sum and product at presentation in the original validation cohort (n=2225; 398 AMI, 1827 NO AMI).

Rule-out	Sum < 9 ng/L	Product < 18 ng ² /L ²	hs-cTnI < 4 ng/L AND hs-cTnT < 9 ng/L
AMI	0 (0.0 %)	0 (0.0 %)	1 (0.3%)
No AMI	746 (40.8 %)	782 (42.8 %)	885 (48.4%)
NPV	100% (99.5% - 100%)	100% (99.5% - 100%)	99.9% (99.2% - 100%)
Rule-in	Sum > 99 ng/L	Product > 1608 ng ² /L ²	
AMI	249 (62.2 %)	261 (65.6 %)	
No AMI	75 (4.1 %)	79 (4.3 %)	
PPV	76.9% (71.8% - 81.3%)	76.8% (71.9% - 81.2%)	

Table 2b. Performance of cut-off values for rule-out and rule-in of sum and product at presentation in the external validation cohort (n=2537; 408 AMI, 2129 NO AMI).

Rule-out	Sum < 9 ng/L	Product < 18 ng ² /L ²	hs-cTnI < 4 ng/L AND hs-cTnT < 9 ng/L
AMI	4 (1.0%)	6 (1.5%)	5 (1.2%)
No AMI	984 (46.2%)	1041 (48.9%)	1083 (50.9%)
NPV	99.6% (99.0% - 99.9%)	99.4% (98.8% - 99.8%)	99.5% (98.9% - 99.8%)
Rule-in	Sum > 99 ng/L	Product > 1608 ng ² /L ²	
AMI	273 (66.9%)	294 (72.1%)	
No AMI	39 (1.8%)	43 (2.0%)	
PPV	87.5% (83.3% - 91.0%)	87.2% (83.2% - 90.6%)	

Early allocation based on simple mathematical derivations of hs-cTnI and hs-cTnT

We examined the use of simple mathematical derivations, sum and product, on the allocation of patients at presentation. In a randomly selected derivation cohort of 1799 patients (313 AMI, 1486 no AMI), thresholds for rule-out and rule-in achieving a NPV of at least 99.6% and a PPV of 75.0%, respectively, were: rule-out cut-off for the sum of 9 ng/L and for the product of 18 ng²/L² (NPV both 100% (95% CI, 99.4–100%), and a rule-in cut-off for the sum of 99 ng/L and for the product of 1608 ng²/L² (PPV sum 75.1% (95% CI 69.3% – 80.3%), PPV product 75.1% (95% CI 69.5%–80.1%)). When these cut-off values were applied to the internal validation cohort of 426 patients (85 AMI, 341 no AMI), we found comparable results for sum (rule-out: sensitivity 100%, specificity 41.3%, NPV 100%; rule-in: sensitivity 65.9%, specificity 96.8%, PPV 83.6%) and product (rule-out: sensitivity 100%, specificity 42.2% NPV 100%; rule-in: sensitivity 65.9%, specificity 96.8%, PPV 83.6%). Application of

these cut-off values in the original cohort (APACE) would cause a 3-to-5-fold increase in the number of rule-outs at presentation (Table 2a, Figure 3) as compared to the 2015 ESC algorithms. Applying these cut-off values in the external validation cohort (ADAPT) (patient characteristics are shown in Supplementary Table 2), would lead to an early rule-out of more than 45% in subjects without AMI (NPV sum: 99.6%; NPV product: 99.4%; Table 2b).

Early rule-out based on a combination algorithm consisting of hs-cTnI and hs-cTnT

Examining rule-out based on a combination algorithm, an optimal cut-off value (NPV 99.6%) was found for the combination of hs-cTnT < 9.8 ng/L and hs-cTnI < 4.8 ng/L. From a pragmatic point of view, we rounded these cut-off values down to hs-cTnT < 9 ng/L and hs-cTnI < 4 ng/L. Applying this combination algorithm in the original cohort (APACE) would lead to a 4-to-6-fold increase in the number of rule-outs at presentation (Table 2a, Figure 3) (sensitivity 99.7%, specificity 48.8%, NPV 99.9%). Application of this algorithm in the external validation cohort (ADAPT), enables early rule-out in >50% of subjects without AMI (sensitivity 98.8%, specificity 50.9%, NPV 99.5%) (Table 2b).

Discussion

We evaluated the combination of cTnI and cTnT for the early diagnosis of AMI in two large prospective diagnostic multicenter studies, and report three major findings.

First, the number of direct rule-outs at presentation using the algorithms of the current ESC guidelines² is limited (7-13% of subjects without an AMI) and, especially in case of the 0h/1h algorithm, assay-dependent. Second, we showed that the difference in diagnostic accuracy between the combined and single cTn measurements is numerically small (except for ratio). In addition, the results of the reclassification statistics indicated that the application of two cTn isoforms at presentation may add incremental value, but that this is not the case for the mathematical derivations when applied to the whole cohort. Third, combining cardiac hs-cTnI and hs-cTnT, using the mathematical derivations sum and product or a combination algorithm, may overcome limitations of the individual assays and lead to a 3-to-6-fold increase in the number of rule-outs after a single blood draw (Figure 3).

Figure 3. Percentage of patients without AMI in which AMI can be ruled-out after a single blood draw at presentation in the APACE cohort

The findings from this study corroborate and extend previous work aiming to further improve the safety and efficacy of the rule-out and rule-in of AMI among patients presenting with acute chest discomfort to the ED^{2-10,23,37}. To the best of our knowledge this work is the first systematic approach testing the clinical utility of mathematical combinations of hs-cTnI and hs-cTnT, the two most accurate biochemical signals in the early diagnosis of AMI¹⁻¹⁰. While there is broad agreement that hs-cTnI or hs-cTnT should be used as a key component in any AMI rule-out algorithm^{2,9,12,38,39}, it has remained unclear whether a second biochemical signature could provide enough incremental value to potentially justify routine clinical use. The novel concept investigated in this study was based on recent studies documenting at times remarkable differences between the cTnI and the cTnT signal, and the moderate agreement between clinical decisions made on these concentrations^{16,40,41}. We hypothesized that combining the two biochemical signals might overcome independent pathophysiological, pre-analytical and analytical differences between the individual molecules such as (auto)antibodies and suggested interference with troponin released from skeletal muscle^{15,42-46}, and might therefore have higher diagnostic accuracy for AMI than either cTnI or cTnT alone.

This study shows that combining hs-cTnI and hs-cTnT may contribute to a significant increase in the number of rule-outs at presentation. The small increase in false-negative results when the derived thresholds were applied in the external validation cohort raises the question what is considered a still acceptable number of false rule-ins and rule-outs⁴⁷. Furthermore, it illustrates the outlier-dependency of the determination of very low cut-off values, and advocates the use of extended (pooled) cohorts and the recalibration of cut-off values for the determination of more universally applicable decision rules⁴⁸. A second point that merits attention are the, at first sight contrary, unconvincing results of the diagnostic accuracy and reclassification statistics. These findings are of limited additional value for the whole population, whereas combining hs-cTnI and hs-cTnT might be especially valuable in patients with low hs-cTn concentrations at presentation. Another reason for this discrepancy might be the three-group (rule-out, observational, rule-in) approach that is used for the diagnosis of AMI and its outlier dependency.

The clinical implementation of a dual-marker approach combining cTnI and cTnT would likely be associated with substantial logistic obstacles since no diagnostic company currently is able to provide both hs-cTnT and hs-cTnI assays on the same laboratory platform. In addition, most hospitals currently do not have analyzers for both analytes running on a 24/7 basis. Therefore, the cost-effective clinical implementation of the dual-marker approach would require a collaboration among diagnostic companies for the provision of both hs-cTnT and hs-cTnI assays on the analyzer that is used for clinical chemistry routine. Nevertheless, rapid and safe clinical decision making based on a single hs-cTn measurement at presentation seems to be approaching its limits, and the exploration of new diagnostic strategies including combinations of biomarkers or imaging seems to be indicated¹⁰. From this point of view, overcoming these logistic obstacles by close collaboration between diagnostic companies, hospital laboratories, medical doctors and researchers would be able to provide substantial medical value for patients and physicians, and economic value for hospitals and the health care system in general. Future studies are necessary to identify the best strategy and to better quantify the possible clinical benefit associated with the combination of cTnI and cTnT. Considering the relevant unmet clinical need as quantified by the high percentage of rule-

out mismatches, the substantial increase in early rule-outs compared to the current ESC 0h/1h-algorithm and the substantial cost savings associated with reductions in the length of stay in the ED⁴⁹, dedicated economic analyses can be expected to show substantial reductions in time to decision, time to discharge, and therefore treatment costs. Furthermore, it is important to highlight that despite the very high diagnostic accuracy, hs-cTn and their combinations will always have to be used clinically only in conjunction with full clinical assessment including detailed patient history, physical examination, and the ECG².

Some limitations of this study merit consideration. First, the central adjudication by two independent cardiologists based on the clinical dataset including cardiac imaging and serial measurements of the local (hs)-cTn and the study-specific dataset including 34 chest pain characteristics, serial measurements of hs-cTnT and follow-up in the APACE study represents the highest quality possible in a diagnostic study. However, it possibly introduced a very small but unavoidable disadvantage for hs-cTnI regarding diagnostic accuracy. This is at large counterbalanced by the use of s-cTnI for the adjudication in ADAPT, as this possibly introduced a very small but unavoidable disadvantage for hs-cTnT regarding diagnostic accuracy. Second, we cannot exactly quantify the cost-effectiveness of this novel approach. Third, patients with terminal kidney failure on chronic dialysis were excluded from APACE. Accordingly, we cannot comment on the possible clinical utility of the combination approach in these vulnerable patients.

In conclusion, diagnostic strategies combining cTnI and cTnT measurements, mathematical derivations and a combination algorithm, may significantly increase the number of patients eligible for very early and safe rule-out, but does not seem helpful for the rule-in of AMI.

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Supplemental information

Supplemental Methods

Adjudication of final diagnoses in APACE cohort

The cTn assays used clinically in most of the participating institutions changed during the study from a conventional cTn assay to the hs-cTnT assay. In order to take advantage of the higher sensitivity and higher overall diagnostic accuracy offered by the hs-cTnT assay, patients were adjudicated using the hs-cTnT values in all patients. In patients in whom clinically a conventional cTn assay was used, the conventional cTn values and the hs-cTnT values were available for the adjudication. In patients in whom clinically the hs-cTnT assay was used, only the hs-cTnT values were available for the adjudication.

The following conventional cTn assays were used: For the Roche cTnT 4th generation assay, the 10% CV level is 0.035ug/l. The laboratories of the participating sites reported only two decimals; therefore 0.04ug/l was used as a cut-off for myocardial necrosis. In order to fulfil the criteria of a significant change (30% of 99th percentile or 10% CV level), a patient would e.g. need to have a level of <0.01ug/l at presentation and 0.04ug/l at 6h. A patient would also qualify if the first level is 0.02ug/l and the second 0.04ug/l. A patient would not fulfil the criteria if the first level is 0.03ug/l and the second is 0.04ug/l. If the first level is 0.04ug/l, the second level needs to be at least 0.06ug/l.

For the Abbott Axsym cTnI ADV, the 10% CV level is 0.16ug/l. A patient having 0.16ug/l at presentation would meet the criteria for significant change if the second was ≥ 0.21 ug/l. A patient having <0.12ug/l at presentation (limit of detection) would qualify if the second is >0.16ug/l.

For the Beckmann Coulter Accu cTnI, the 10% CV level is 0.06ug/l. A patient having 0.06ug/l at presentation would qualify if the second is ≥ 0.08 ug/l. A patient having 0.05 at presentation would qualify if the second is 0.07ug/l, but not 0.06ug/l. A patient having undetectable cTnI (cTnI<0.01ug/l) at presentation would qualify if the second is ≥ 0.06 ug/l.

For hs-cTnT the 99th percentile (14ng/l) was used as cut-off for myocardial necrosis^{1,2}. Absolute changes in hs-cTnT were used to determine significant changes based on the diagnostic superiority of absolute over relative changes^{3,4}. Based on studies of the biological variation of cTn^{5,6} as well as on data from previous chest pain cohort studies^{7,8}, a significant absolute change was defined as a rise or fall of at least 10ng/l within six hours. In patients, in whom a 6 hour hs-cTnT level was not available, changes were assessed at earlier time points. In an assumption of linearity, an absolute change of 6ng/l within three hours was considered.

Adjudication of final diagnoses in ADAPT cohort

Outcomes were adjudicated independently by local cardiologists using predefined standardized reporting guidelines. Cardiologists had knowledge of the clinical record, ECG, troponin results and objective testing from standard care. A second cardiologist conducted a blind review of all ACS cases and 10% of non-ACS cases. In cases of disagreement, endpoints were agreed by consensus.

Diagnosis of AMI was according to international guidelines and based on evidence of myocardial necrosis and ischemia. Evidence of ischaemia included at least one of ECG changes or positive imaging results from exercise tolerance testing, myocardial perfusion scan, stress echocardiography, computed tomographic coronary angiography or coronary angiography during catheterization. Necrosis was diagnosed based on a rise or fall of cardiac troponin concentration over at least six hours with at least one value above the 99th percentile of the normal reference range at a level of assay imprecision near to 10%. If the troponin was greater than the reference range but no rise or fall was recorded, other causes of raised troponin were considered. If no alternative cause for the troponin rise was apparent and if the clinical presentation was suggestive of ACS, an adjudicated diagnosis of AMI was made.

Emergency revascularization was defined as PCI or CABG in a symptomatic patient where the clinical status includes either 1) ischaemic dysfunction (ongoing ischaemia despite maximal medical therapy, acute evolving myocardial within 24 hours before intervention or pulmonary oedema requiring intubation) or 2) mechanical dysfunction (shock with or without circulatory support). Urgent revascularisation included PCI or CABG that did not meet the emergency criteria above but was required during the same hospitalization to minimize chance of further clinical deterioration. Elective revascularization, or those procedures that could be deferred without increased risk of compromised cardiovascular outcome, were not included in the endpoint.

Assumption of linearity of absolute changes of hs-cTnT within the first hours

The assumption of linearity of absolute changes within the first hours is based on unpublished internal data as well as recent data from Ola Hammarsten et al. showing a near-linear increase in levels of hs-cTnT with increasing time from symptom onset in their NSTEMI cohort 9.

Determination of cut-offs for the combination algorithm

We aimed to determine the safe and optimal combination of hs-cTnI concentration and hs-cTnT concentration. A test was considered positive if either hs-cTn concentration was greater than or equal to an hs-cTn threshold for that troponin assay. We varied the hs-cTn threshold for each troponin assay in steps of 0.1 ng/L across the range from the LoD to 26 ng/L. At each combination of hs-cTn thresholds

we calculated the sensitivity and specificity for the diagnosis of AMI. We created 500 bootstraps of the data, and to average the results we then fitted a smooth surface (thin plate spline) for sensitivity on the hs-cTnI threshold – hs-cTnT threshold grid from which we could determine the 99% sensitivity contour and contours for specificity. The optimal threshold was defined as the combination with 99% sensitivity and maximized specificity.

Supplemental Figures

Supplementary Figure 1. hs-cTn concentrations at presentation in the APACE cohort. The boxes represent median and inter-quartile ranges, the whiskers 1st-99th percentile of A) cardiac troponin T (hs-cTnT), B) cardiac troponin I (hs-cTnI), C) sum, D) product and E) ratio. hs-cTn denotes high-sensitivity cardiac troponin; hs-cTnI, high-sensitivity cardiac troponin I; AMI, acute myocardial infarction; UA, unstable angina; CNC, cardiac non-coronary disease; NCCP, non-cardiac chest pain.

Supplemental Tables

Supplementary Table 1. Baseline characteristics of patients in the APACE cohort

	All (n=2225)	AMI (n=398)	No AMI (n=1827)
Age, y	62 (49 – 75)	72 (61 – 80)	60 (47 – 73)
Male sex, n (%)	1511 (68)	290 (73)	1221 (67)
eGFR, ml·min ⁻¹ ·1.73m ⁻²	85 (69 – 101)	73 (55 – 93)	87 (71 – 103)
Body-mass index, kg/m ²	26 (24 – 30)	26 (24 – 29)	26 (24 – 30)
Risk factors, n (%)			
Hypertension	1382 (62)	318 (80)	1064 (58)
Hypercholesterolemia	1110 (50)	272 (68)	838 (46)
Diabetes Mellitus	384 (17)	108 (27)	276 (15)
Current or previous smoking	1366 (61)	253 (64)	1113 (61)
Family history	549 (25)	117 (29)	432 (24)
History, n (%)			
Coronary Artery Disease	776 (35)	202 (51)	574 (31)
Previous AMI	513 (23)	136 (34)	377 (21)
Previous revascularisation	612 (28)	147 (37)	465 (26)
Peripheral artery disease	141 (6)	56 (14)	85 (5)
Previous stroke	122 (6)	39 (10)	83 (5)
ECG changes, n (%)			
ST segment depression	232 (10)	121 (30)	111 (6)
T wave inversion	296 (13)	100 (25)	196 (11)
LBBB	25 (1)	11 (3)	14 (1)
Medication at presentation, n (%)			
ASA	808 (36)	199 (50)	609 (33)
Vitamin K antagonist	191 (9)	41 (10)	150 (8)
β-blockers	770 (35)	174 (44)	596 (33)
Statins	777 (35)	178 (45)	599 (33)
ACEIs/ARBs	842 (38)	206 (52)	636 (35)
Calcium antagonists	321 (14)	82 (21)	239 (13)
Nitrates	257 (12)	85 (21)	172 (9)

APACE denotes Advantageous Predictors of Acute Coronary Syndrome Evaluation; AMI, acute myocardial infarction; ECG, electrocardiogram; eGFR, estimated glomerular filtration rate; LBBB, left bundle branch block; ASA, acetyl salicylic acid; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker. Values are expressed in percentage or medians ± IQR.

Supplementary Table 2. Baseline characteristics of patients in the ADAPT cohort

	All (n=2537)	AMI (n=408)	No AMI (n=2129)
Age, y	60 (51 – 71)	59 (49 – 69)	69 (59 – 78)
Male sex, n (%)	1535 (61)	292 (72)	1243 (58)
Body-mass index, kg/m ²	27 (24 – 31)	28 (24 – 31)	27 (24 – 31)
Risk factors, n (%)			
Hypertension	1316 (52)	254 (62)	1062 (50)
Hypercholesterolemia	1309 (52)	228 (56)	1081 (51)
Diabetes Mellitus	377 (15)	81 (20)	296 (14)
Current smoking	462 (18)	72 (18)	390 (18)
Family history of CAD	1393 (55)	245 (60)	1148 (54)
History, n (%)			
Previous AMI	627 (25)	128 (31)	499 (23)
Previous PCI	525 (21)	85 (21)	440 (21)
Previous CABG	219 (9)	55 (13)	164 (8)
Peripheral artery disease	94 (4)	25 (6)	69 (3)
Previous stroke	205 (8)	39 (10)	166 (8)

ADAPT denotes Accelerated Diagnostic Protocol to Assess Patients With Chest Pain Symptoms Using Contemporary Troponins as the Only Biomarker; AMI, acute myocardial infarction; CABG, coronary artery bypass grafting; PCI, percutaneous coronary intervention. Values are expressed in percentage or medians ± IQR.

Supplementary Table 3. hs-cTn concentrations at presentation in the APACE cohort

	hs-cTnI (ng/L)	hs-cTnT (ng/L)	Sum (ng/L)	Product (ng ² /L ²)	Ratio
AMI (n=398)	115.2 (21.7 – 632.9)	64.1 (28.0 – 152.4)	184.2 (52.4 – 812.8)	7258 (596 – 91526)	1.84 (0.82 – 4.72)
No AMI (n=1827)	3.5 (2.2 – 7.2)	7.0 (4.0 – 12.4)	10.6 (6.5 – 20.0)	23 (9 – 86)	0.57 (0.38 – 0.90)
UA (n=216)	6.4 (3.6 – 12.1)	10.7 (6.9 – 16.0)	17.5 (11.1 – 29.6)	63 (25 – 199)	0.65 (0.42 – 1.12)
Cardiac, non-coronary (n=307)	8.4 (3.4 – 28.6)	13.4 (7.0 – 29.0)	10.6 (23.3 – 59.5)	112 (24 – 727)	0.70 (0.45 – 1.31)
NCPP (n=1202)	3.0 (1.9 – 5.1)	6.0 (3.9 – 9.7)	8.9 (5.9 – 15.1)	17 (8 – 47)	0.52 (0.36 – 0.81)
Unknown (n=102)	2.9 (2.3 – 4.8)	5.8 (3.0 – 9.4)	8.8 (5.8 – 14.9)	17 (8 – 48)	0.58 (0.39 – 0.76)
<i>p-value (AMI compared with no AMI)</i>	<i>p<0.001</i>	<i>p<0.001</i>	<i>p<0.001</i>	<i>p<0.001</i>	<i>p<0.001</i>
Median (IQR)					

Supplementary Table 4a: Agreement on rule-out at presentation using the 0h/3h-algorithm in the APACE cohort

		hs-cTnT	
hs-cTnI		rule-out*	no rule-out
	rule-out*	132 (5.9%)	25 (1.1%)
	no rule-out	1 (0.0%)	2067 (92.9%)

** if hs-cTnT < 14 ng/L or hs-cTnI < 26.2 ng/L, >6h after onset of symptoms and painfree, and GRACE score < 140*

Supplementary Table 4b: Agreement on rule-out at presentation using the 0h/1h-algorithm in the APACE cohort

		hs-cTnT	
hs-cTnI		rule-out*	no rule-out
	rule-out*	98 (4.4%)	51 (2.3%)
	no rule-out	137 (6.2%)	1939 (87.1%)

** if hs-cTnT < 5 ng/L or hs-cTnI < 2 ng/L and >3h after onset of symptoms*

Supplementary Table 4c: Agreement on rule-in at presentation using the 0/1h-algorithm in the APACE cohort

		hs-cTnT	
hs-cTnI		rule-in*	no rule-in
	rule-in*	246 (11.1%)	85 (3.8%)
	no rule-in	27 (1.2%)	1867 (83.9%)

** if hs-cTnT > 52 ng/L or hs-cTnI > 52 ng/L*

Supplementary Table 5. Diagnostic accuracy of hs-cTnT and hs-cTnI after the addition of the alternative signal or sum, product or ratio at presentation in the APACE cohort

	Time point	Single	Combination	p-value	Sum	p-value	Product	p-value	Ratio	p-value
hs-cTnT	0h*	0.93 (0.92 – 0.94)	0.94 (0.93 – 0.95)	0.002	0.94 (0.93 – 0.95)	0.006	0.94 (0.93 – 0.95)	0.002	0.94 (0.93 – 0.95)	0.002
	0h + 1h [†]	0.96 (0.95 – 0.97)	0.96 (0.95 – 0.97)	0.795	0.96 (0.95 – 0.97)	0.838	0.96 (0.95 – 0.97)	0.795	0.96 (0.95 – 0.97)	0.795
	0h + 0h/1h-delta [‡]	0.95 (0.93 – 0.96)	0.95 (0.94 – 0.96)	0.142	0.95 (0.94 – 0.96)	0.217	0.95 (0.94 – 0.96)	0.142	0.95 (0.94 – 0.96)	0.142
	0h	0.93	0.94	0.118	0.94	0.157	0.94	0.118	0.94	0.118
hs-cTnI	0h*	0.93 (0.92 – 0.94)	0.94 (0.93 – 0.95)	0.118	0.94 (0.93 – 0.95)	0.007	0.96 (0.95 – 0.97)	0.006	0.96 (0.95 – 0.97)	0.006
	0h + 1h [†]	0.95 (0.94 – 0.96)	0.96 (0.95 – 0.97)	0.006	0.96 (0.95 – 0.97)	0.021	0.95 (0.94 – 0.96)	0.014	0.95 (0.94 – 0.96)	0.014
	0h + 0h/1h-delta [‡]	0.94 (0.93 – 0.95)	0.95 (0.94 – 0.96)	0.014	0.95 (0.94 – 0.96)	0.021	0.95 (0.94 – 0.96)	0.014	0.95 (0.94 – 0.96)	0.014
	0h	0.93	0.94	0.118	0.94	0.157	0.94	0.118	0.94	0.118

*2225 patients, [†]1786 patients, [‡]1752 patients

Supplementary Table 6a. Absolute IDI at presentation in the APACE cohort

	Combination	p-value	Sum	p-value	Product	p-value	Ratio	p-value
hs-cTnI	0.214	< 0.001	0.038	< 0.001	-0.100	1.00	-0.083	1.00
	(0.183; 0.244)		(0.011; 0.065)		(-0.128; -0.073)		(-0.113; -0.052)	
hs-cTnT	0.016	0.23	-0.159	1.00	-0.298	1.00	-0.280	1.00
	(-0.011; 0.043)		(-0.188; -0.130)		(-0.328; -0.267)		(-0.311; -0.248)	

dedicates hs-cTnT resp. hs-cTnI alone (old model) versus the mathematical combinations of hs-cTnT and hs-cTnI (new model)

Supplementary Table 6b. Category-free NRI at presentation in the APACE cohort

	Combination	p-value	Sum	p-value	Product	p-value	Ratio	p-value
hs-cTnI	121.1%	<0.001	127.5%	<0.001	-100.6%	1.00	-15.8%	1.00
hs-cTnT	105.1%	<0.001	-123.0%	1.00	-131.7%	1.00	-133.0%	1.00

dedicates hs-cTnT resp. hs-cTnI alone (old model) versus the mathematical combinations of hs-cTnT and hs-cTnI (new model)

Supplemental references

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The effect of a six-month resistance-type exercise training program on the course of high sensitive cardiac troponin T levels in (pre)frail elderly

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Introduction

Cardiac troponin is the preferred biomarker for diagnosing non ST-segment elevation myocardial infarction¹. With the introduction of high-sensitivity assays, cardiac troponin levels became also detectable in apparently healthy subjects. This has expanded its role from acute cardiac care to risk stratification and prognostic medicine². Meanwhile, high sensitive cardiac troponin (hs-cTn) has acquired the status of a prognostic biomarker that predicts cardiovascular risk, in addition to established Framingham risk factors such as age, sex, blood pressure and dyslipidemia³. Despite the remarkable consistency in literature on the association between cardiac troponin levels and increased risk for cardiovascular morbidity and mortality, it is unclear whether preventative measures such as lifestyle intervention programs can influence the course of basal troponin levels. Suggestive evidence in favor of this hypothesis was provided by an observational study by deFilippi et al, showing that higher physical activity levels in elderly subjects were associated with both lower basal cardiac troponin T (cTnT) levels, and a lower probability of a significant increase in cTnT concentrations between consecutive visits⁴. However, no intervention study has evaluated the effect of an exercise program on the concentration of cardiac troponin. The present trial was conducted to investigate the hypothesis that a 24-week resistance-type training program modulates the course of basal plasma cTnT levels in (pre)frail elderly subjects. Coronary artery disease is common in the (pre)frail elderly population. While elderly in general are prone to cardiovascular disease, the subgroup of (pre)frail elderly is a subset of patients with highest vulnerability to adverse outcomes and all-cause mortality⁵. We therefore postulated that this particular patient group is characterized by high baseline levels of cTnT, and may be most receptive to the potential effects or benefits of an exercise intervention.

Methods

Elderly subjects (≥ 65 years), who previously had not participated in any structured exercise training program over the past 2 years and were considered (pre)frail according to the Fried criteria⁶, were eligible. Subjects diagnosed with chronic obstructive pulmonary disease

(COPD), malignancies, muscle disease, type 2 diabetes (glucose concentrations $\geq 7\text{mmol/L}$), renal dysfunction ($\text{eGFR} < 60\text{mL/min/1.73m}^2$) or unable to perform the exercise regimen were excluded. The Medical Ethics Committee of Wageningen University approved this study and all subjects gave their written informed consent. Subjects participated either in a 24-week supervised resistance-type exercise training program, or were followed up during a parallel non-interventional control period. Training was performed under personal supervision, and consisted of a 5-minute warming up on a cycle ergometer, 4 sets on leg-press and leg-extension machines and 3 sets on chest press, lat pulldown, pecdec and vertical row machines (Technogym, Rotterdam, the Netherlands). This program was scheduled twice per week and workload was increased during the 24-week period. Blood samples were collected before and after 12 and 24 weeks of intervention. Assuming a mean baseline cTnT concentration of 11.00 ng/L , a 24-week increase of $3\%^4$ (estimated baseline correlation and decay rate 0.90 and 0.25, respectively), and an expected reduction of basal troponin levels after 24 weeks of 15% , the inclusion of 23 subjects per group would afford 80% power at a significance level of $5\%^7$. We anticipated a dropout rate of approximately 25% , and therefore recruited 62 subjects for this study (31 per group). Plasma cTnT was measured using the high-sensitivity cTnT assay on the Cobas-6000 analyzer (Roche Diagnostics). This study is part of a greater trial examining the impact of protein supplementation and training on skeletal muscle mass in (pre)frail elderly as primary outcome. The effects of the training program on muscle mass, muscle strength and physical performance were published recently^{8,9}.

Results

Ten subjects withdrew from the study; 6 in the intervention group and 4 in the control group, leaving 52 subjects for follow-up analyses. Table 1 shows the clinical characteristics of the participants. All cTnT values measured during the study period (range $3.78 - 105.70\text{ ng/L}$) were above the detection limit of the assay (3ng/L), and 40 subjects (65%) had at least one measurement above the 99th percentile of 14 ng/L . Table 2 shows the baseline characteristics and the 12-week and 24-week follow-up data of both groups. Figure 1 shows the course of cTnT levels over time in both groups. Using mixed linear model analyses, we

did not observe a significant difference in the course of basal cTnT levels between the intervention and the control group (intention-to-treat analysis $p=0.38$, per-protocol analysis $p=0.16$).

Table 1. Clinical characteristics

Variable	Control (n=27)	Intervention (n=25)
Demographics		
Age (years), mean (SD)	80.2 (7.4)	78.6 (6.8)
Women, n (%)	14 (52)	17 (68)
BMI (kg/m ²), mean (SD)	26.6 (3.3)	28.7 (4.9)
Medical history		
Systolic BP (mm Hg), mean (SD)	151 (24)	142 (18)
Diastolic BP (mm Hg), mean (SD)	75 (9)	72 (9)
Current smoking, n (%)	1 (4)	1 (4)
Laboratory data		
Fasting glucose (mmol/L), mean (SD)	5.2 (0.5)	5.3 (0.5)
Total cholesterol (mmol/L), mean (SD)	5.4 (1.1)	5.2 (1.5)
Triglycerides (mmol/L), mean (SD)	1.2 (0.6)	1.3 (0.5)
HDL cholesterol (mmol/L), mean (SD)	1.5 (0.4)	1.3 (0.3)
LDL cholesterol (mmol/L), mean (SD)	3.4 (0.9)	3.2 (1.3)
Creatinine (μmol/L), mean (SD)	77.7 (12.1)	74.4 (14.1)
eGFR (ml/min/1.73 m ²), mean (SD)	77 (11)	78 (13)
NT-proBNP (pg/mL), median (IQR)	19.1 (8.0 – 46.3)	14.6 (6.2 – 25.3)
Medication use		
Antihypertensives, n (%)	14 (52)	11 (44)
Statins, n (%)	3 (11)	9 (36)

SD, standard deviation; n, number; IQR, interquartile range; BP, blood pressure; eGFR, estimated glomerular filtration rate.

Table 2. Baseline and follow-up concentrations of cTnT

Variable	Control (n=27)	Intervention (n=25)
Baseline hs-cTnT (ng/L), median (IQR)	14.7 (10.0 – 19.7)	10.3 (7.8 – 17.5)
12-week hs-cTnT (ng/L), median (IQR)	14.6 (12.0 – 23.3)	13.9 (8.9 – 20.5)
24-week hs-cTnT (ng/L), median (IQR)	14.8 (11.4 – 25.5)	14.3 (8.5 – 20.5)

Figure 1. Median (interquartile range) concentrations of cTnT in the control and intervention group of (pre)frail elderly.

Conclusion

The finding that the majority of participants had cTnT levels above the 99th percentile confirms our hypothesis that chronically elevated cTnT concentrations are highly prevalent among (pre)frail elderly subjects. However, despite the elevated basal levels of cTnT and the fact that subjects had not previously participated in any structured exercise program, we found no evidence for an effect of a 24-week resistance-type exercise training program on the course of cTnT levels. A possible limitation of our study is that the exercise program was based on resistance-type exercise training, and that a more endurance-type based training may have a greater effect on cTn levels. Another limitation might have been the physical state of the subjects, which precluded a higher training frequency than the biweekly program that was applied in this study. The biweekly schedule was however sufficient to induce substantial improvements of muscle strength and physical performance in this group^{8,9}. In conclusion, we demonstrated that prolonged resistance-type exercise training in (pre)frail elderly does not lead to changes in the course of cTnT levels. Future studies will reveal whether more intensive and more prolonged interventions can beneficially affect the course of basal troponin levels over time.

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The effect of exercise training on the course of cardiac troponin T and I levels: three independent training studies

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Abstract

With the introduction of high-sensitive assays, cardiac troponins became potential biomarkers for risk stratification and prognostic medicine. Observational studies have reported an inverse association between physical activity and basal cardiac troponin levels. However, causality has never been demonstrated. This study investigated whether basal cardiac troponin concentrations are receptive to lifestyle interventions such as exercise training. Basal high-sensitive cardiac troponin T (cTnT) and I (cTnI) were monitored in two resistance-type exercise training programs (12-week (study 1) and 24-week (study 2)) in older adults (≥ 65 years). In addition, a retrospective analysis for high sensitive troponin I in a 24-week exercise controlled trial in (pre)frail older adults was performed (study 3). In total, 91 subjects were included in the final data analyses. There were no significant changes in cardiac troponin levels over time in study 1 and 2 (study 1: cTnT -0.13 ($-0.33 - +0.08$) ng/L/12-weeks, cTnI -0.10 ($-0.33 - +0.12$) ng/L/12-weeks; study 2: cTnT -1.99 ($-4.79 - +0.81$) ng/L/24-weeks, cTnI -1.59 ($-5.70 - +2.51$) ng/L/24-weeks). Neither was there a significant interaction between training and the course of cardiac troponin in study 3 ($p=0.27$). In conclusion, this study provides no evidence that prolonged resistance-type exercise training can modulate basal cardiac troponin levels.

Introduction

The development of increasingly sensitive assays for cardiac troponin has now reached the point where cardiac troponin concentrations can be accurately assessed in the majority of subjects from a healthy reference population¹. Parallel to the development of assays with increasing sensitivity, interest in cardiac troponin has expanded from acute cardiac care to risk prediction and risk stratification². Studies in various patient groups and asymptomatic individuals have provided compelling evidence that baseline cardiac troponin levels predict outcome³. Interestingly, the observed risk gradient is not restricted to elevations above the 99th percentile, but is even apparent within the 'healthy' population reference interval, and independent of traditional risk factors such as age, sex and diabetes⁴⁻⁹. In the Framingham heart study, the basal cardiac troponin I concentration adds prognostic value to standard risk factors for predicting death, and cardiovascular disease¹⁰. Furthermore, temporal increases in cardiac troponin concentrations over time confer additional cardiovascular risk, and are inversely related to the level of physical fitness^{7,11}. These observations form the basis of the hypothesis that cardiac troponin is a modifiable parameter, which may be receptive to lifestyle interventions such as an exercise training program. In a recent study with (pre)frail subjects we tested the hypothesis that a supervised resistance-type exercise training program can influence the course of cardiac troponin levels over time¹². However, this 24-week supervised bi-weekly exercise training program conferred no beneficial effect on the course of cardiac troponin T levels, despite substantial improvements of these subjects at the level of physical performance¹². This negative study was conducted in frail and pre-frail subjects, characterized by substantially elevated baseline cardiac troponin levels, and therefore theoretically most amenable to the potential benefits of an exercise intervention. A possible drawback however, was the limited training load that could be imposed due to the level of frailty of these older subjects. Nevertheless, despite these limitations, our results were similar to those of other recent reports: 14-week endurance training in untrained adults¹³, 17-week half-marathon training in previously sedentary men¹⁴ and 3-month exercise training in heart failure patients (NYHA class II-IV)¹⁵ did not result reduce basal cardiac troponin T concentrations. However, limitations of these studies were the low basal

cardiac troponin concentrations¹³ and the use of conventional instead of high-sensitive cardiac troponin T assays^{14,15} which reduced the power to detect an effect. Another issue that merits attention is that the effect of training intervention on cardiac troponin I has never been assessed. Since cardiac troponin T and I are two different proteins of the cardiac troponin complex with different biochemical characteristics¹⁶ and distinct release patterns¹⁷, both cardiac troponins may also respond differently to lifestyle interventions.

The aim of the current study is 1) to overcome the limitations of previous studies, and 2) to examine the effect of exercise training on basal cardiac troponin I levels. Therefore, we now present two additional exercise training studies to the effect of a more intense training regimen on the course of high-sensitive cardiac troponin T and I levels in older adults, a population that often exhibits elevated basal cardiac troponin concentrations. Additional high sensitive troponin I was measured in our previously conducted study in (pre)frail subjects in order to present a complete and comprehensive overview to the effects of three supervised training programs.

Methods

To investigate the effect of resistance-type exercise training on cardiac troponin T (cTnT) and I (cTnI) in older adults, two independent studies were conducted: a study to the effect of 12-week resistance-type exercise training in older men (≥ 65 years) (study 1) and a second study to the effect of a 24-week resistance-type exercise training older men and women (≥ 65 years) (study 2). In addition, we performed a retrospective analysis for cardiac troponin I in our recently published study to the effect of a 24-week resistance-type exercise training program on the course of troponin T levels in (pre)frail subjects (≥ 65 years) (study 3).

All studies complied with the principles of the Declaration of Helsinki and were approved by the local institutional review boards and the ethics committees of Wageningen University or Maastricht University Medical Center. Participants were recruited by advertisements in local newspapers (study 1 and 2) or by approaching elderly living in apartment houses and in homes for the elderly (study 3). All participants provided written informed consent. Subjects

were only eligible for inclusion when they had not participated in any structured exercise training program over the past 2 years.

Study 1: 12-week training in older men

In total 14 subjects were included in study 1. Inclusion criteria were age (65 - 85 years), male gender and the ability to understand and perform study procedures. Exclusion criteria were (silent) cardiac or peripheral vascular disease, orthopedic limitations (self-reported) and type 2 diabetes (based on an oral glucose tolerance test). A training session consisted of a 5-minute warming up on a cycle ergometer, four sets on leg-press and leg-extension machines and a 5-minute cooling down on the cycle ergometer. The workload started at 60% of 1 repetition maximum (1-RM) (10-15 repetitions per set) and was increased to 75-80% of 1-RM (8-10 repetitions per set). The typical duration of a training session was approximately 45 minutes, and the work:rest ratio was 1:4. This supervised resistance-type exercise training program was performed three times per week during 12 weeks. There was at least a resting period of 2 days between consecutive training sessions. Blood samples were obtained by a venipuncture after an overnight fast, at least three days after the last training session, before the start of the training program (week 0) and after 4, 8 and 12 weeks of intervention. This study is a substudy of a more extensive trial examining the effects of resistance-type exercise training in older men on muscle strength, body composition and muscle fiber type-specific characteristics as primary outcome¹⁸.

Study 2: 24-week training in older men and women

In total 27 subjects were included in study 2. Inclusion criteria were age (65 – 90 years) and the ability to understand and perform study procedures. Exclusion criteria were the same as in study 1. Training sessions consisted of a 5-minute warming up on a cycle ergometer, four sets on leg-press and leg-extension machines and three sets on the chest press and horizontal row; these four exercises were performed every training session. In addition, three sets of vertical lat pull and abdominal crunches were alternated with biceps curl and triceps extension between subsequent training sessions. The workload started at 60% of 1-RM (10-

15 repetitions per set) and was increased to 75-80% of 1-RM (8-10 repetitions per set). The typical duration of a training session was approximately 45 minutes, and the work:rest ratio was 1:4. This supervised resistance-type exercise training program was performed three times per week during 24 weeks. There was at least a resting period of 2 days between consecutive training sessions. Blood samples were obtained by a venipuncture after an overnight fast at least three days after the last training session before the start of the training program (week 0) and after 4, 8, 12, 16, 20 and 24 weeks of intervention. This is a substudy of a more extensive trial to the effects of an exercise training intervention in older adults on muscle strength, body composition and muscle fiber type-specific characteristics. In this current study we included only subjects from the control group who were not receiving dietary protein supplementation (as described in the original study protocol)^{19,20}.

Study 3: 24-week training in (pre)frail older men and women

Methodological details of study 3 have been described previously¹². Briefly, a total of 62 (pre)frail older men and women (≥ 65 years) were included and were equally distributed in an intervention and a control group. Frailty and prefrailty were defined according to the Fried criteria (subjects were considered prefrail when 1 or 2 criteria were applicable, and frail when 3 or more criteria were present): (1) unintentional weight loss, (2) weakness, (3) self-reported exhaustion, (4) slow walking speed, and (5) low physical activity²¹. The intervention group participated in a 24-week, biweekly, supervised resistance-type exercise training program. Blood samples were obtained by a venipuncture after an overnight fast before the start of the training program (week 0), after 12 and after 24 weeks of intervention. The effect of the training program on muscle mass, muscle strength, physical performance, and troponin T levels were published recently. In this current study we included only subjects from the control group who were not receiving dietary protein supplementation (as described in the original study protocol)^{12,22,23}.

Cardiac troponin T and I

Plasma and serum samples were stored at -80°C until analysis. Serum (study 1) and plasma (study 2 and 3) cardiac troponins were measured using the high sensitive cTnT assay (limit of blank 3.0 ng/L, limit of detection 5.0 ng/L, 99th percentile 14 ng/L²⁴) on the Cobas analyzer (Roche Diagnostics) and the high sensitive cTnI assay (according to the manufacturer, limit of blank 0.7 – 1.3 ng/L, limit of detection 1.1 – 1.9 ng/L, 99th percentile 26.2 ng/L and 10% CV 4.7 ng/L) on the ARCHITECT analyzer (Abbott Diagnostics).

Statistical analysis

Normally distributed parameters were expressed as mean $\pm$ standard deviation (SD), non-normally distributed parameters as median and interquartile range (IQR), and categorical variables as number (n) and percentage (%). Changes in basal cardiac troponin T and I concentrations over time were analyzed using mixed linear model analyses (random intercept) with the covariate time as fixed effect. The inclusion of 14 subjects in study 1 and 27 subjects in study 2 afforded at least 90% power at a significance level of 5% to detect a 10% reduction in cardiac troponin levels, anticipating a dropout rate of 25%. These post-hoc power calculations were based on the assumption of a mean baseline cTnT concentration of 8.0 ng/L and cTnI concentration of 6.0 ng/L for study 1 and a mean baseline cardiac cTnT concentration of 6.0 ng/L and a cTnI concentration of 4.0 ng/L for study 2 with older men and women (estimated baseline correlation and decay rate 0.90 and 0.25, respectively)²⁵. We used two-level design mixed linear model analyses (random intercept) with time and intervention as fixed effects to assess the differences in the course of cardiac troponin I between (pre)frail subjects in the control group and in the intervention group. Power calculation for study 3 has been described previously¹². All statistical analyses were performed using SPSS, version 20.0. $P \leq 0.05$ was considered statistically significant for all analyses.

Results

Compliance to the training program

Included subjects in study 1 and 2 demonstrated 97% and 90% compliance, respectively, to the scheduled, supervised training sessions. In each study one subject withdrew. Ten subjects (15%) withdrew from study 3. Subjects withdrew because of time constraints and medical reasons unrelated to the exercise training. In total, in the final data analysis we included: 13 older men participating in the 12-week training program (study 1), 26 older men and women participating in the 24-week training program (study 2), and 52 subjects in the retrospective troponin I analysis with (pre)frail older men and women (study 3). Table 1 shows the subjects' clinical characteristics at baseline. Baseline characteristics for the (pre)frail subjects have been published previously¹².

No decrease in cardiac troponin T and I levels during the exercise training program

Fig. 1 shows the course of cardiac troponin T and cardiac troponin I levels over time in study 1 and 2 (for individual data see Supplementary Fig. S1 and S2). Table 2 shows the corresponding baseline and the follow-up values for cTnT and cTnI. One subject in study 2 had cardiac troponin T concentrations below the limit of blank (3ng/L) at all time points, and was therefore excluded from the cardiac troponin T analysis. 98.6 % and 99.6 % of all other troponin T and I measurements respectively had concentrations above the limit of blank. Using mixed linear model analyses, we found no significant changes in cardiac troponin levels over time in older men participating in a 12-week resistance-type exercise training program (cTnT -0.13 (-0.33 – +0.08) ng/L/12-weeks (p=0.16), cTnI -0.10 (-0.33 – +0.12) ng/L/12-weeks (p=0.44)) and in older men and women participating in a 24-week resistance-type exercise training program (cTnT -1.99 (-4.79 – +0.81) ng/L/24-weeks (p=0.23), cTnI -1.59 (-5.70 – +2.51) ng/L/24-weeks (p=0.37)).

Table 1. Clinical Characteristics

	Older men 12-w intervention (n=13)	Older men and women 24-w intervention (n=26)	(pre)Frail older men and women 24-w intervention (n=25)	(pre)Frail older men and women control (n=27)
Age (years), mean (SD)	72.4 (5.4)	69.2 (4.1)	78.6 (6.8)	80.2 (7.4)
Women, n (%)	0 (0)	12 (46)	17 (68)	14 (52)
BMI (kg/m ²), mean (SD)	27.4 (3.8)	25.9 (2.0)	28.7 (4.9)	26.6 (3.3)
Medical history				
Systolic BP (mm Hg), mean (SD)	147 (14)	137 (15)	142 (18)	151 (24)
Diastolic BP (mm Hg), mean (SD)	82 (17)	77 (22)	72 (9)	75 (9)
Laboratory data				
Fasting glucose (mmol/L), mean (SD)	5.6 (0.7)	5.5 (0.5)	5.3 (0.5)	5.2 (0.5)
Total cholesterol (mmol/L), mean (SD)	5.6 (1.1)	6.2 (0.9)	5.2 (1.5)	5.4 (1.1)
Triglycerides (mmol/L), mean (SD)	1.4 (0.5)	1.3 (0.5)	1.3 (0.5)	1.2 (0.6)
HDL cholesterol (mmol/L), mean (SD)	1.3 (0.4)	1.6 (0.3)	1.3 (0.3)	1.5 (0.4)
LDL cholesterol (mmol/L), mean (SD)	3.6 (1.3)	4.3 (0.8)	3.2 (1.3)	3.4 (0.9)
Creatinine (μmol/L), mean (SD)	102.2 (7.5)	88.1 (17.4)	74.4 (14.1)	77.7 (12.1)
Cystatin C (mg/L), mean (SD)	1.07 (0.11)	0.90 (0.19)	1.00 (0.29)	1.08 (0.25)
eGFR (ml/min/1.73m ²), mean (SD)*	66.3 (7.2)	78.3 (15.2)	73.9 (15.4)	69.6 (13.9)
NT-proBNP (pg/mL), median (IQR)	12.1 (6.8 – 21.0)	7.6 (3.1 – 15.8)	14.6 (6.2 – 25.3)	19.1 (8.0 – 46.3)
Medication use				
Antihypertensives, n (%)	3 (25)	3 (12)	11 (44)	14 (52)
Statins, n (%)	1 (8)	1 (4)	9 (36)	3 (11)

*eGFR, estimated glomerular filtration rate based on the the CKD-EPI creatinine-cystatin C equation (2012)²⁹.

Fig. 1. Course of cardiac troponinT and I in older adults participating in 12- and 24-week training programs. Median (interquartile range) concentrations of cTn in older adults participating in a supervised resistance-type training program: A, course of cTnT in older men in a 12-week training program; B, course of cTnI in older men in a 12-week training program; C, course of cTnT in older men and women in a 24-week training program; D, course of cTnI in older men and women in a 24-week training program.

Table 2. Baseline and follow-up concentrations of cTn in older adults participating in 12-week and 24-week training programs

	hs-cTnT (ng/L) , median (IQR)		hs-cTnI (ng/L) , median (IQR)	
	12-w intervention (n=13)	24-w intervention (n=25)	12-w intervention (n=13)	24-w intervention (n=26)
Baseline	9.1 (6.0 – 12.1)	6.4 (5.0 – 8.7)	5.1 (4.0 – 7.4)	4.1 (3.2 – 5.2)
Week 4	9.3 (5.8 – 10.7)	7.4 (5.2 – 8.6)	5.9 (3.2 – 9.8)	3.9 (2.9 – 5.7)
Week 8	9.6 (5.9 – 10.8)	6.7 (5.6 – 9.4)	5.1 (3.4 – 7.1)	3.9 (3.3 – 5.1)
Week 12	7.8 (4.4 – 8.8)	6.5 (5.9 – 8.5)	4.6 (2.7 – 6.3)	4.0 (3.1 – 5.1)
Week 16		6.6 (5.3 – 9.2)		4.1 (3.1 – 5.2)
Week 20		5.9 (4.8 – 8.6)		3.6 (2.9 – 4.8)
Week 24		6.1 (4.5 – 8.0)		3.8 (2.9 – 4.8)

Table 3. Baseline and follow-up concentrations of hs-cTnI in (pre)frail older subjects in the control group and those participating in a 24-week resistance-type exercise training program

	(pre)Frail older subjects 24-w intervention (n=25)	(pre)Frail older subjects no intervention (n=27)
Baseline hs-cTnI (ng/L), median (IQR)	4.4 (3.3 – 6.2)	6.7 (4.7 – 7.7)
12-week hs-cTnI (ng/L), median (IQR)	4.6 (3.5 – 6.4)	6.7 (4.4 – 8.4)
24-week hs-cTnI (ng/L), median (IQR)	4.3 (3.6 – 8.4)	6.0 (3.4 – 7.6)

No effect of a 24-week resistance-type exercise training program on cardiac troponin I levels

Table 3 shows the baseline cTnI concentrations and the 12-week and 24-week follow-up data of (pre)frail older men and women. In the control group, all values were above the limit of blank of the assay (1.3 ng/L), 3 subjects had at least one value above the 99th percentile of 26.2 ng/L, which is used for the diagnosis of myocardial infarction. In the intervention group, one subject had one value below limit of blank of the assay, and one subject had at least one value above the 99th percentile. Mixed linear model analyses revealed no significant interaction between training and the course of cTnI levels between the intervention and the control group (intention-to-treat analysis $p=0.27$, per-protocol analysis $p=0.27$) (for individual data see Supplementary Fig. S2).

Discussion

In two independent supervised training studies of 12 and 24 weeks respectively, we found no evidence for a modifying effect of resistance-type exercise training on the course of cardiac troponin T and I levels in older subjects, nor did we find an effect of a 24-week training program on cardiac troponin I levels in (pre)frail subjects.

Our results do not support the idea that cardiac troponin levels might be modifiable through exercise. This hypothesis was postulated by observational studies, showing that higher physical activity levels in older subjects were associated with both lower basal cTnT levels, and a lower probability of a significant increase in cTnT concentrations between consecutive visits^{11,26}. To investigate whether a causal relationship underlies this association, we conducted a series of training studies in subjects where stable elevated cardiac troponin levels are common, and who are therefore – at least theoretically – most receptive to the favorable effects of an intervention. To ensure that basal cardiac troponin levels, rather than acute post-exercise effects were studied, we included a three day interval between the last training session and the blood sampling procedure²⁷. None of these studies showed a favorable effect of resistance-type exercise training on cardiac troponin levels. The non-responsive pattern of troponin T and I to an exercise program is in contrast with the beneficial changes observed for leg strength, muscle mass, insulin sensitivity, HbA1c, total cholesterol and LDL^{18-20,22,23}. The present results confirm and extend the results of our previous training intervention study in (pre)frail older adults¹², which was also characterized by favorable effects on physical performance, but a lack of effect on circulating cardiac troponin levels.

Although the absolute number of subjects in these training studies are relatively low, all studies afforded at least 90% power to detect a 10% reduction of cardiac troponin over time. We cannot exclude the possibility that training confers a smaller effect on the course of troponin levels that goes by undetected in the present study, but we feel that (much) smaller changes than specified in our power calculation would comprise limited clinical relevance. A possible limitation of our study relates to the type of exercise training intervention, comprising mostly resistance-type exercise. However the subjects' physical state precluded (intense) endurance training. Despite the physical and metabolic improvements found in our

studies^{18-20,22,23}, a valid question may be whether resistance-type exercise training is the most appropriate type of training to generate a favorable effect on cardiac troponin concentrations. In this respect the results of a recently conducted cardio-based exercise training intervention study in heart failure patients are interesting. Similar to our studies, no favorable effect was found of a supervised endurance exercise training program on cardiac troponin T levels¹⁵.

The supervision during the sessions contributed to the internal validity, but might also have led to increased safety, less adverse events, and a higher motivation among subjects. This may overestimate any beneficial effects of exercise training. Another point regarding the validity of our study is the participant recruitment process and study design. Since participation in the study is associated with a substantial time investment and a relatively high intensity training program, this might have attracted a specific, highly motivated subpopulation. Since our study was conducted in an elderly population we can only speculate about the effects of exercise training on basal cardiac troponin levels in younger subjects who may respond differently on exercise training and demonstrate higher cardiovascular plasticity²⁸. Nevertheless, we show that 12 and 24 weeks of supervised resistance-based exercise training does not reduce basal cardiac troponin T and I concentrations in older adults.

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Supplemental information

Supplemental Figures

Supplementary Figure 1. Individual troponin T profiles

Supplementary Figure 2. Individual troponin I profiles

Prognostic value of basal high-sensitive cardiac troponin levels on mortality in the general population: a meta-analysis

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Abstract

Background

Interest in the use of cardiac troponin T (cTnT) and cardiac troponin I (cTnI) has expanded from diagnosis of acute myocardial infarction to risk assessment for morbidity and mortality. Although cTnT and cTnI were shown to have equivalent diagnostic performance in the setting of suspected acute myocardial infarction, potential prognostic differences are largely unexplored. The aim of this study is to quantify and compare the relationship between cTnT and cTnI, and cardiovascular and all-cause mortality in the general population.

Methods

Medline, Embase and the Cochrane Library (from inception through October 2016) were searched for prospective observational cohort studies reporting on the prognostic value of basal high-sensitive cTnT and/or cTnI levels on cardiovascular and all-cause mortality in the general population. Data on study characteristics, participants' characteristics, outcome parameters and quality (according to the Effective Public Health Practice Project (EPHPP) 'Quality Assessment Tool For Quantitative Studies') were retrieved. Hazard ratios per standard deviation increase in basal cardiac troponin level (HR per 1-SD; retrieved from the included articles or estimated) were pooled using a random-effects model.

Results

On a total of 2585 reviewed citations, 11 studies, with data on 65019 participants, were included in the meta-analysis. Random effects pooling showed significant associations between basal cardiac troponin levels and hazard ratio for cardiovascular and all-cause mortality (HR per 1-SD 1.29 [95% CI, 1.20 to 1.38] and HR per 1-SD 1.18 [95% CI, 1.11 to 1.26], respectively). Stratified analyses showed higher hazard ratios for cTnT than cTnI (cardiovascular mortality: cTnT HR per 1-SD 1.37 [CI, 1.23-to-1.52]; and cTnI HR per 1-SD 1.21 [CI, 1.16-to-1.26]; all-cause mortality: cTnT HR per 1-SD 1.31 [CI, 1.13-to-1.53]; and cTnI HR per 1-SD 1.14 [CI, 1.06-to-1.22]). These differences were significant ($p < .01$) in meta-regression analyses for cardiovascular mortality but did not reach statistical significance for all-cause mortality.

Conclusions

Elevated, basal cTnT and cTnI show robust associations with increased risk of cardiovascular and all-cause mortality during follow-up in the general population.

Introduction

Cardiac troponins are the preferred biomarkers in the diagnostic work up for non-ST elevation myocardial infarction (NSTEMI)^{1,2}. Troponin assays target either cardiac troponin T or cardiac troponin I, both sarcomere components of the heart³. The release of cardiac troponin in the peripheral blood is strongly associated with myocardial injury⁴. The recent introduction of high-sensitive cardiac troponin assays has expedited the early diagnosis of NSTEMI, but also resulted in the detection of previously unnoticed cardiac troponin levels in various patient groups without acute cardiac injury such as chronic kidney disease, chronic heart failure, and even in apparently healthy subjects^{5,6}. Subsequently, several studies have shown that even these minimal increases of cardiac troponin T and I are associated with unfavorable outcomes in various patient groups⁶⁻¹⁰.

The current guidelines recommend either cardiac troponin T and I for the diagnosis of acute myocardial infarction, suggesting equivalent diagnostic performance of troponin T and I in the acute situation¹¹. In contrast, cardiac troponin T seems to have greater prognostic accuracy than cardiac troponin I in the acute setting¹². Potential differences in prognostic value between basal cardiac troponin T and I concentrations in subjects from the general population are largely unexplored. In addition, the quantitative relationship between elevated cardiac troponin levels, also below the 99th percentile, and the magnitude of risk for adverse events has not been systematically assessed.

To address these knowledge gaps, we conducted a systematic review and meta-analysis of the available evidence from published prospective cohort studies. Specifically, we quantified the relationship between basal levels of high-sensitive cardiac troponins and cardiovascular and all-cause mortality during follow-up in the general population. In addition, the prognostic performance of high-sensitive cardiac troponin T and I assays was separately assessed and compared.

Methods

Data sources and searches

Medline, Embase and the Cochrane Library were searched from inception through October 2016. Also the reference lists of all relevant articles and Web of Science (for prospective citations of key publications) were checked for any additional articles. The PubMed search terms were (mortality OR death OR 'Mortality'[Mesh]) AND (troponin OR 'Troponin'[Mesh]) AND (predictive OR prediction OR prognostic OR prognosis OR 'Prognosis'[Mesh] risk OR 'Risk'[Mesh]) AND (follow-up OR prospective OR cohort). We adapted this search strategy for searches of Embase and the Cochrane Library. The search was restricted to English, Dutch, French and German documents.

The protocol for this study was published on the International Prospective Register of Systematic Reviews, or PROSPERO. The registration number was CRD42014006964.

Study selection

Study selection was performed by two independent investigators (N.v.d.L. and L.K.). We included all prospective cohort studies that evaluated the prognostic value of basal high-sensitive cardiac troponin T and I levels in subjects from the general population (without any suspected acute event or surgery at the time of sampling) for cardiovascular and all-cause mortality during follow-up.

Studies were excluded from the meta-analysis when a) the duration of follow-up was less than one year, b) hazard ratios for cardiovascular or all-cause mortality were not provided (either as hazard ratio per stratum, or as hazard ratio (HR) per 1-standard deviation (SD) increase in logarithmic transformed cardiac troponin concentrations), or c) when hazard ratios were not adjusted for conventional cardiovascular risk factors (at least for age, sex, smoking, hypertension (or systolic blood pressure), diabetes mellitus (or glucose levels) and dyslipidemia (or levels of total and HDL cholesterol). When duplicate publications of data were encountered, only results from the most recent publication were considered. Discrepancies between reviewers were resolved in the presence of a third reviewer (S.M.).

Data Extraction and Quality Assessment

We extracted data using a customized and validated extraction form. Data on study characteristics (authors, publication year, journal, study design, sample size, country and duration of follow-up), participants' characteristics (age, sex, history of CVD, hypertension, dyslipidemia, diabetes), cardiovascular and all-cause mortality rate, cardiac troponin assay, hazard ratios for cardiovascular and all-cause mortality, the factors for which the hazard ratios had been adjusted for, and C-statistics and reclassification were retrieved. When multiple cardiac troponin assays of the same kind (cardiac troponin I or T) were presented, we used the data from the assay that was commercially available and most commonly used in clinical practice for our analyses. Two authors (N.v.d.L. and L.K.) performed the data-extraction separately. Discrepancies between reviewers were resolved in presence of a third reviewer (S.M.).

To assess study quality, each study was evaluated for selection bias, study design, confounders, blinding, data collection methods, and withdrawals/dropouts according to the Effective Public Health Practice Project (EPHPP) 'Quality Assessment Tool For Quantitative Studies'¹³. Two authors (N.v.d.L. and L.K.) performed quality assessment separately, disagreements were resolved by consensus in the presence of a third review author (S.M.)

Data Synthesis and Analysis

To quantitatively assess the relationship between basal cardiac troponin levels and cardiovascular and all-cause mortality during follow-up, we used hazard ratios HR per 1-SD increase in logarithmic transformed cardiac troponin levels. When hazard ratios were provided for stratified cardiac troponin concentrations only, we mathematically derived HR per 1-SD increase in logarithmic transformed cardiac troponin concentration. This estimation was based on the assumption that logarithmic transformed cardiac troponin levels are normally distributed in the population¹⁴⁻¹⁶, and consisted of the following three steps: 1) we extracted the cumulative distribution of the study population and calculated the associated z-scores based on the cumulative standard normal distribution, 2) we calculated the mean and standard deviation based on the z-scores ($z=(x-\mu)/\sigma$, where x is the log-transformed

cardiac troponin concentration acting as upper limit of the stratum (continuity correction was performed), μ is the mean, and σ is the standard deviation), and 3) we constructed a biomarker concentration-risk curve (x-axis: log-transformed medians for the strata, y-axis: associated hazard ratios) for which we calculated the slope (hazard ratio/log cardiac troponin) and transformed this into HR per 1-SD increase in logarithmic transformed cardiac troponin level. This method was validated in two studies that reported hazard ratios for both stratified data and log-transformed cardiac troponin as a continuous variable^{17,18}. We found a minimal differences between the reported and the calculated HR per 1-SD in these two studies (2-8% difference).

For the meta-analysis, we used full-model adjusted hazard ratios (at least adjusted for the conventional cardiovascular risk factors age, sex, smoking, hypertension (or systolic blood pressure), diabetes mellitus (or glucose levels), and dyslipidemia (or levels of total and HDL cholesterol). As a result of this approach the number of adjusting factors in the full-model differs between studies. Nevertheless, this method ensures that we correct for all significant confounders in the individual studies. Since cardiac troponins are strongly associated with cardiovascular disease, the set of adjusting factors mainly consists of cardiovascular risk factors. The adjusted HRs per 1-SD were pooled using a random-effects model and summarized using forest plots.

Examination of the impact of potential moderator variables on the study effect size was done by meta-regression analyses. Potential moderator variables included 1) type of cardiac troponin assay, 2) calculated versus reported HRs, 3) study quality, 4) geographic region, and 5) mean age of the population. A sensitivity analysis was applied to examine the effect of the inclusion of studies, that did not adjust for the minimal set of six conventional risk factors, on the robustness of the pooled results.

The MOOSE guidelines¹⁹ for meta-analysis of observational studies were followed and PRISMA criteria were performed for the search methodology (Figure 1). Publication bias was evaluated using Egger regression. Heterogeneity between studies was estimated using I^2 ²⁰. For all analyses, a 2-tailed p-value less than .05 was considered statistically significant. All statistical analyses were performed with the Stata software package (Stata/IC version 13.1).

Figure 1. Flow chart of the study selection.

Results

Study characteristics

The literature search protocol is depicted in Figure 1. Our initial search identified 2585 citations. Based on titles and abstracts, 41 articles were considered potentially eligible. After full text evaluation 29 articles were excluded: eight articles did not report hazard ratio's for high-sensitive cardiac troponin concentrations and survival, seven did not perform a survival analysis, four did not report on cardiovascular or all-cause mortality as outcome measures, two were not performed in the general population, and six articles reported data that were also published in more recent articles. In two studies the reported hazard ratios were not adjusted for the minimal list of conventional risk factors. Since the adjustment for these factors is one of the inclusion criteria, those studies were excluded. Hence, a total of 11 studies (12 articles) (65019 participants) were eventually included in the meta-analysis; seven studies (45956 participants) for cardiovascular mortality^{15,17,21-25} and ten studies

(48679 participants) for all-cause mortality^{15,17,18,22-24,26-29}. Five studies reported hazard ratios related to cardiac troponin T levels^{17,18,21-23,26}, another five studies reported on cardiac troponin I levels^{15,24,25,27-29}. Clinical and demographic characteristics are shown in Table 1. The median follow-up duration of all studies was between 3.8 and 20 years. The percentage of individuals with detectable cardiac troponin levels varied from 27 to 96%.

The study quality varied across studies. According to the EPHP quality assessment tool¹³ one study was of high methodological quality²⁷, eight studies were of moderate quality^{17,18,21-25,28,29} and two studies were of low methodological quality^{15,26}. The global rating 'weak' for study quality was due to lack of information about subject inclusion, study withdrawals, and dropouts. To ascertain vital status studies used death registries (n=5)^{17,22,24,26,29}, medical records (n=3)^{18,21,27,28}, the combination of medical records and death registries (n=2)^{15,25}, and the combination of interviews, medical records and a death register (n= 1)²³. All included studies provided hazard ratios adjusted for at least six conventional cardiovascular risk factors, described in the methods section. Quality assessment scores and additional adjustments made by the individual studies are presented in Supplemental Table 1.

Association between cardiac troponin concentration and mortality

We pooled adjusted HRs per 1-SD for all studies using a random-effects model. Pooling of the results from the seven included studies for cardiovascular mortality showed a significant association between increased basal cardiac troponin levels and an elevated hazard ratio for cardiovascular mortality (HR per 1-SD 1.29, 95% confidence interval CI 1.20 to 1.38). Cardiac troponin T was significantly stronger associated with cardiovascular mortality compared to cardiac troponin I (meta-regression analysis $p < .01$; HR per 1-SD 1.37, 95% confidence interval 1.23 to 1.52 and HR per 1-SD 1.21, 95% confidence interval 1.16 to 1.26, for cardiac troponin T and I, respectively). The forest plots of the random-effects pooled data for all-cause mortality are shown in Figure 2.

Table 1. Study characteristics

Study Name	Country	Year of publication	Participants		Follow-up			Cardiovascular risk factors						
			n, (% female)	Age, years	Detectable levels, %	Years	All-cause mortality, n	Cardiovascular mortality, n	BMI, kg/m ²	Diabetes, %	Hypertension, %	Dyslipidemia ^a , %	History of CVD, %	
hs-cTnT Roche														
ARIC ²³	USA	2013	11193 (56%)	64 ± 6	68	9.9	1909	358	NA	17	48	14	NA	
CHS ¹⁸	USA	2013	44311 (59%)	73 ± 6	54 ^b	13.1	NA	Not reported	27 ± 5	18	45	NA	NA	
CHS ²¹	USA	2010	4221 (60%)	73	66	11.8	2872	1103	27	18	51	NA	NA	
DHS ¹⁷	USA	2010	3546 (56%)	30–65 ^a	27	6.4	151	62	NA	12	34	11	NA	
PREDICTOR ²⁶	Italy	2015	1835 (47%)	73 ± 5	NA	3.8	135	Not reported	27 ± 4	17	58	43	29	
ULSAM ²²	Sweden	2013	940 (0%)	71 ± 1	93	8	265	115	26 ± 3	11	74	0	40	
hs-cTnI Singulex (Erenna)														
FOS ²⁸	USA	2012	3428 (53%)	59 ± 10	81	11.3	488	Not reported	28 ± 5	12	NA	NA	NA	
hs-TnI Abbott (Architect)														
AGES-R ²⁹	Ireland	2016	5691 (58%)	77 ± 5.9	100	8.9	2371	Not reported	27 ± 4	13	80	22	NA	
FINRISK ²⁷	Finland	2014	7899 (50%)	48 ± 22	82	14	810	Not reported	26 ± 6	5	45	NA	NA	
HUNT ²⁴	Norway	2015	9712 (54%)	50 ± 17	96	13.9	1689	708	NA	3	11	NA	NA	
PIVUS ¹⁵	Sweden	2013	1004 (50%)	70	96	8.0	111	37	27 ± 4	12	72	NA	NA	
SSHEC ²⁵	UK	2014	15340 (51%)	49 ± 9	75	20	Not reported	714	26 ± 4	2	NA	NA	NA	

AGES-R denotes Age, Gene/Environment Susceptibility-Reykjavik Study, ARIC Atherosclerosis Risk in Communities Study, CHS Cardiovascular Health Study, CVD cardiovascular disease, DHS Dallas Heart Study, FOS Framingham Offspring Study, HUNT The Northern Trøndelag Health Study, PIVUS Prospective Investigation of the Vascular Health in Uppsala Seniors, PREDICTOR Valutazione delle PREvalenze di Difunzione Cardiaca asintomatica e di scompenso cardiaco conclamato in un campione di popolazione di età ≥ 65 anni nel Lazio, SHHEC Scottish Heart Health Extended Cohort, and ULSAM Uppsala Longitudinal Study of Adult Men, a mean age not in article (age ranges between 30 and 65 years). b used a cut-off of 5 ng/L instead of 3 ng/L, like most studies that used hs-TnT. c defined as 1) elevated total or low-density lipoprotein (LDL) cholesterol levels, or low levels of high-density lipoprotein (HDL) cholesterol, or 2) the use or prescription of cholesterol lowering medication.

Pooling of the results from the nine included studies for all-cause mortality showed a significant association between increased basal cardiac troponin levels and an elevated hazard ratio for all-cause mortality (HR per 1-SD 1.18, 95% confidence interval CI 1.11 to 1.26). Considering the trend towards different effect sizes (HR per 1-SD) for cardiac troponin T and I shown by meta-regression analysis ($p = .09$), we performed stratified analyses for both isoforms of cardiac troponin. Basal cardiac troponin T levels translated to a higher hazard ratio for all-cause mortality ((HR per 1-SD 1.31, 95% confidence interval 1.13 to 1.53) versus (1.14, 1.06 to 1.22)). The forest plots of the random-effects pooled data for all-cause mortality are shown in Figure 3.

Heterogeneity and publication bias assessment

Our meta-analysis revealed substantial heterogeneity across studies (Figure 2 and 3). We used meta-regression analyses to identify the underlying factors. Univariable meta-regression analyses pointed out that estimated versus reported hazard ratio and study quality (strong/moderate versus weak global rating) were not statistically significant predictors, whereas the assay-type (high-sensitive cardiac troponin T or I) was statistically significant for cardiovascular mortality ($p < .01$) but did not reach significance for all-cause mortality ($p = .09$). Besides, age was a borderline significant factor in meta-regression analysis for cardiac troponin I and all-cause mortality ($p = 0.05$), indicating that older age is associated with a stronger correlation between troponin concentration and risk.

One SD increase in basal cardiac troponin T levels was associated with a higher HR for cardiovascular and all-cause mortality during follow-up than a similar increase in troponin I. Stratified analyses for cardiac troponin T and I decreased heterogeneity across troponin T studies ($I^2 = 37.9\%$, $p = .18$ and $I^2 = 62.7\%$, $p = .03$, for cardiovascular and all-cause mortality, respectively). Across studies with cardiac troponin I, heterogeneity for cardiovascular mortality decreased ($I^2 = .0\%$, $p = .40$), but remained high for all-cause mortality ($I^2 = 83.4\%$, $p < .01$).

Egger's regression analysis was not indicative for publication bias ($p = .50$ and $p = .68$, for cardiovascular and all-cause mortality, respectively) (Supplemental Figure 1 and 2).

Figure 2. Association between one standard deviation increase in basal cardiac troponin concentrations and hazard ratio for cardiovascular mortality in the general population. The boxes and lines in the forest plots indicate the hazard ratio for cardiovascular mortality per standard deviation increase (HR per 1-SD) in cardiac troponin concentrations for individual studies and a pooled estimate (random-effects model) for all studies (panel A), studies on cardiac troponin T (panel B), and studies on cardiac troponin I (panel C).

Figure 3. Association between one standard deviation increase in basal cardiac troponin concentrations and hazard ratio for all-cause mortality in the general population. The boxes and lines in the forest plots indicate the hazard ratio for all-cause mortality per standard deviation increase (HR per 1-SD) in cardiac troponin concentrations for individual studies and a pooled estimate (random-effects model) for all studies (panel A), studies on cardiac troponin T (panel B), and studies on cardiac troponin I (panel C).

Sensitivity analysis

For all-cause mortality two studies were excluded because they did not correct for at least the six conventional cardiovascular risk factors. To verify that pooled hazard ratios were robust and independent, a sensitivity analysis including those two studies was performed. Random-effects pooling after the addition of two additional studies^{30,31} revealed similar results regarding all-cause mortality and reduced heterogeneity for cardiac troponin T (HR per 1-SD 1.30, 95% confidence interval 1.16 to 1.47; $I^2=52.9\%$ ($p=.060$)), cardiac troponin I (1.14, 1.07 to 1.22; $I^2 = 79.3\%$ ($p<.01$)) and the combined analysis (1.19, 1.12 to 1.26; $I^2 = 71.3\%$ ($p<.01$)). The meta-regression analysis that was performed after the inclusion of two additional studies^{30,31} showed a significant effect of assay-type (cardiac troponin T versus I) on the observed heterogeneity ($p=.05$).

Translation to clinical practice

Together, the pooled data show that elevated basal cardiac troponin levels in the general population are associated with increased mortality during follow-up, and suggest that a slightly stronger association for cardiac troponin T than for cardiac troponin I. To relate the pooled summary measures per standard deviation, to a clinically comprehensible risk estimation tool, we transformed HR per 1-SD to absolute cardiac troponin concentrations and corresponding hazard ratios for all high-sensitive troponin assays that were included in the meta-analysis. Figure 4 depicts the relation between basal cardiac troponin T and I concentrations and mortality during follow-up.

The majority of included studies performed discrimination and reclassification statistics (Supplemental Table 2)^{15,17,21,22,24,25,27,29}. The results from these analyses suggest small, but significant improvements in (established) prediction models based on established risk factors after the inclusion of high-sensitive cardiac troponin T or I.

Figure 4. Cardiac troponin levels and corresponding hazard ratios. HR per 1-SD increase was transformed to absolute cardiac troponin concentrations and corresponding hazard ratios for all high-sensitive troponin assays that were included in the meta-analysis.

Discussion

This meta-analysis with data on 65019 participants from 11 prospective cohorts shows that basal elevated cardiac troponin concentrations, also below the 99th percentile, in the general population are associated with an increased risk for cardiovascular and all-cause mortality during follow-up. The pooled data analyses corroborates and extends the findings of previous studies^{7-9,32,33}. Besides, our results suggest that the quantitative relationship between cardiac troponin concentrations is stronger for cardiovascular than for all-cause mortality, and may be slightly stronger for cardiac troponin T than for cardiac troponin I.

The observed association between elevated cardiac troponin concentrations and an increased risk for cardiovascular mortality and, to a lesser extent, for all-cause mortality, are reflected in the discrimination and reclassification statistics of the included studies. These analyses indicate that the addition of cardiac troponins may cause a small, but significant improvement in risk prediction models^{15,17,21,22,24,25,27,29}. Some recent studies, but not all, suggest that the addition of cardiac troponin I to existing risk prediction models may be particularly useful among a subpopulation, especially in older and female subjects^{24,25,29,33}. Before cardiac troponins can be actually implemented in clinical practice, it is necessary to identify the suitable target population and the best combination of biomarkers for risk prediction, also from a cost-effectiveness point of view.

The observed differences in the quantitative relationships of high-sensitive cardiac troponin T and I are clinically and scholarly interesting and contrast with demonstrated equivalent performance of both assays in the acute setting, i.e. for diagnosing acute myocardial infarction³⁴. We can only speculate about the factors that might underlie these apparent prognostic differences. A first factor concerns the analytical aspects of the distinct cardiac troponin assays. It is conceivable that intrinsic assay differences, including the use of different antibodies³⁵, may affect distinctive power of troponin assays with direct consequences for prognostic performance. A second factor involves the release of cardiac troponins. The mechanism of chronic cardiac troponin release is not elucidated, but recent observations suggest differences in release patterns of cardiac troponin T and I³⁶. Third, post-

release modification like fragmentation, complex formation, and elimination of cardiac troponins may differ between both cardiac troponins^{37,38}.

This meta-analysis has several strengths, including its large patient populations, standardization of the outcome measure and the comparison of cardiac troponin T and I. Limitations of our study merit consideration: First, despite the consistent adjustment for at least six established cardiovascular risk factors and the use of a standardized outcome measure (HR per 1-SD), heterogeneity was substantial, which persisted in assay-stratified analyses, in particular across all-cause mortality studies. Meta-regression analyses did not identify other suggestive factors that contributed to heterogeneity. A likely, remaining source of heterogeneity is the diversity in populations, caused by variable in- and exclusion criteria of the individual studies. Nonetheless, despite the detected heterogeneity, all studies revealed the same direction of effect, with increased mortality upon higher levels of circulating cardiac troponin. Second, due to the fact that we did not have access to the individual, data and that the percentage of included women showed minimal variation, we were unable to examine the effect of gender. Therefore we cannot confirm that gender affects the relation between cardiac troponin concentrations and the risk on cardiovascular and all-cause mortality^{24,30,33}. With respect to age, we could only examine the effect of the mean age across the included studies. In line with previous observations³³, we found a borderline significant association between increasing age and a stronger correlation between troponin I and all-cause mortality. We cannot exclude that the variation in in- and exclusion criteria and adjusting factors might have masked a more explicit effect of age. Third, HR per 1-SD from stratified data has been mathematically derived. However, validation of this method in two studies revealed minimal discrepancies, and this approach to standardize risk-estimates enabled us to pool rather heterogeneously presented data, and to perform a meta-analysis with substantial statistical power and long-term follow-up. Fourth, the measurement and definition of mortality outcomes differs across studies. The definition of cardiovascular mortality varied slightly across studies, but in general it concerned the ICD-10 diagnosis I00-I99.

Conclusion

Elevated, basal cardiac troponin T and I levels are significantly associated with increased risk of cardiovascular and all-cause mortality during follow-up in the general population. The results of this meta-analysis suggest this association is stronger for cardiovascular than for all-cause mortality. The observation that the quantitative relationship between cardiac troponin concentration and mortality risk may be stronger for cardiac troponin T than for cardiac troponin I is an interesting finding that requires further research. In addition, future studies should focus on further establishment and validation of cardiac troponins for the prediction of long-term outcomes.

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Supplemental information

Supplemental Figures

Supplemental Figure S1. Funnel plot cardiovascular mortality

Supplemental Figure S2. Funnel plot all-cause mortality

Supplemental Tables

Supplemental table 1a. Quality assessment (EPHPP quality assessment tool)

	Selection bias	Study design	Confounders	Blinding	Data collect. and method	Withdrawals and dropouts	Global rating
Oluleye, Ann Epidemiol 2013	+/-	+/-	+	+/-	+	-	+/-
Hussein, J Am Coll Cardiol 2013	+/-	+/-	+	+/-	+	-	+/-
deFilippi, JAMA 2010	+/-	+/-	+	+/-	+	-	+/-
De Lemos, JAMA 2010	+/-	+/-	+	+/-	+	-	+/-
Masson, J Intern Med 2014	-	+/-	+	+/-	+	-	-
Eggers, Am Heart J 2013	+	+/-	+	+/-	+	-	+/-
Neumann, PLoS One 2014	+/-	+/-	+	+/-	+	+	+
Wang, Circulation 2012	+/-	+/-	+	+/-	+	-	+/-
Omland, Clin Chem 2015	+/-	+/-	+	+/-	+	-	+/-
Eggers, J Am Coll Cardiol 2013	-	+/-	+	+/-	+	-	-
Thorsteinsdottir, Clin Chem 2016	+/-	+/-	+	+/-	+	-	+/-
Zeller, Eur Heart J 2014	+/-	+/-	+	+/-	+	-	+/-

+ indicates strong, +/- moderate and - weak.

Supplemental Table 1b. Variables in cox regression model.

Article	Variables in multivariate cox regression analysis
Oluleye, Ann Epidemiol 2013	Age, gender, race, BMI, total cholesterol, HDL cholesterol, SBP, diet, sport index, FEV ₁ , eGFR, smoking, drinking, hormone use, antihypertensive medication, DM, history of cancer, CVD, stroke, heart failure, and respiratory disease, hs-CRP and NT-proBNP
Hussein, J Am Coll Cardiol 2013	Age, race, gender, smoking, SBP, serum glucose, total cholesterol, HDL cholesterol, eGFR, physical activity, history of heart failure, coronary disease, history of myocardial infarction, stroke, transient ischemic attack, ventricular conduction delay, Q and QS abnormalities, prolonged QT interval, LVEF, LV mass, CRP, NT-proBNP, use of aspirin, antihypertensives, antiarrhythmics, and digoxin
deFilippi, JAMA 2010	Age, gender, race, SBP, DBP, use of antihypertensives, diabetes, CHD, smoking, HDL cholesterol, LDL cholesterol, NT-proBNP and CRP
De Lemos, JAMA 2010	Age, gender, race/ethnicity, DM, hypertension, hypercholesterolemia, HDL cholesterol, smoking, hs-CRP, CKD categories, and NT-proBNP
Masson, J Intern Med 2014	Age, gender, dyslipidemia, DM, SBP, smoking, blood sample taken in the winter season, eGFR, COPD, alcohol consumption, atrial fibrillation, heart failure, history of ischemic heart disease, LVEF, LV mass/BSA, NT-proBNP and vitamin D
Eggers, Am Heart J 2013	Age, smoking, BMI, SBP, antihypertensive treatment, total cholesterol, HDL cholesterol, lipid-lowering treatment, DM, and previous or prevalent cancer, NT-proBNP, CRP, cystatin C
Neumann, PLoS One 2014	Age, gender, total cholesterol, HDL cholesterol, SBP, hypertension medication, DM, smoking, region of Finland, CRP, and NT-proBNP
Wang, Circulation 2012	Age, gender, BMI, SBP, hypertension therapy, DM, smoking, total cholesterol, HDL cholesterol, atrial fibrillation, major cardiovascular disease, ECG left ventricular disease, heart murmur, sST2, GDF-15, BNP and hsCRP
Omland, Clin Chem 2015	Age, gender, total and HDL cholesterol, SBP, treatment of hypertension, smoking, diabetes, eGFR, history of myocardial infarction, CRP
Eggers, J Am Coll Cardiol 2013	Gender, hypertension, DM, HDL cholesterol, LDL cholesterol, smoking, BMI, eGFR, CRP, NT-proBNP and previous cardiovascular disease
Thorsteinsdottir, Clin Chem 2016	Age, gender, diabetes, BMI, hypertension medication, systolic blood pressure, smoking, log CRP, log creatinine, cholesterol, HDL cholesterol, log triglycerides, statin medication
Zeller, Eur Heart J 2014	Age, sex, cohort, family history of CHD/stroke, diabetes, rheumatoid arthritis, use of antihypertensives, smoking, BMI, SBP, total cholesterol, HDL cholesterol

Supplemental Table 2. C-statistic and reclassification for the addition of hs-cTn*

All-cause mortality							
Study	C-statistic without hs-cTn	C-statistic with hs-cTn	p-value	NRI	p-value	IDI	p-value
hs-cTnT Roche							
CHS ²¹	NA	NA	NA	NA	NA	NA	NA
DHS ¹⁷	Model 1:	Model 1:	Model 1:	NA	NA	Model 1:	Model 1:
	0.793	0.818	<0.001			0.044	<0.001
	Model 2:	Model 2:	Model 2:			Model 2:	Model 2:
	0.793	0.818	<0.001			0.044	<0.001
	Model 3:	Model 3:	Model 3:			Model 3:	Model 3:
	0.806	0.823	<0.01			0.024	<0.001
	Model 4:	Model 4:	Model 4:			Model 4:	Model 4:
	0.821	0.827	0.02			0.010	0.01
ULSAM ²²	0.63	0.65	0.07	NA	NA	0.02	<0.001
Hs-TnI Abbott (Architect)							
AGES-R ²⁹	0.739	0.748	<0.001	0.26	NA	NA	NA
FINRISK ²⁷	0.8174	0.8173	ns	-0.0040	ns	-0.0002	ns
HUNT ²⁴	Women:	Women:	Women:	NA	NA	NA	NA
	0.934	0.937	0.011				
	Men:	Men:	Men:				
	Not reported	Not reported	ns				
PIVUS ¹⁵	Not reported	Model 1:	Model 1:	Model 1:	Model 1:	Model 1:	Model 1:
		+ 0.003	0.51	0.231	0.02	0.014	0.04
		Model 2:	Model 2:	Model 2:	Model 2:	Model 2:	Model 2:
		+0.006	0.60	0.140	0.17	0.120	0.07
		Model 3:	Model 3:	Model 3:	Model 3:	Model 3:	Model 3:
		+0.040	0.17	0.288	0.004	0.017	0.01
SSHEC ²⁵	NA	NA	NA	NA	NA	NA	NA
Cardiovascular mortality							
Study	C-statistic without hs-cTn	C-statistic with hs-cTn	p-value	NRI	p-value	IDI	p-value
hs-cTnT Roche							
CHS ²¹	0.779	0.792	<0.05	0.040	<0.05	0.021	<0.05
DHS ¹⁷	Model 1:	Model 1:	Model 1:	NA	NA	Model 1:	Model 1:
	0.832	0.868	<0.001			0.027	<0.001
	Model 2:	Model 2:	Model 2:			Model 2:	Model 2:
	0.833	0.869	<0.001			0.029	<0.001
	Model 3:	Model 3:	Model 3:			Model 3:	Model 3:
	0.865	0.882	0.02			0.010	0.10
	Model 4:	Model 4:	Model 4:			Model 4:	Model 4:
	0.889	0.893	0.37			0.005	0.24
ULSAM ²²	0.66	0.70	0.01	NA	NA	0.02	0.001
Hs-TnI Abbott (Architect)							
AGES-R ²⁹	NA	NA	NA	NA	NA	NA	NA
FINRISK ²⁷	NA	NA	NA	NA	NA	NA	NA
HUNT ²⁴	Women:	Women:	Women:	NA	NA	NA	NA
	0.875	0.878	0.003				
	Men:	Men:	Men:				
	Not reported	Not reported	ns				
PIVUS ¹⁵	NA	NA	NA	NA	NA	NA	NA
SSHEC ²⁵	0.8362	0.8439	<0.0001	0.0451	0.0119	0.0053	0.0150

*Addition of hs-cTn to models with established risk factors:

CHS: model includes age, gender, race, SBP, DBP, use of antihypertensives, diabetes, CHD, smoking, HDL cholesterol, LDL cholesterol, NT-proBNP and CRP

DHS: model 1 includes age, race/ethnicity, gender, diabetes mellitus, hypertension, hypercholesterolemia, LDH cholesterol, and current smoking, model 2 includes all factors in model 1 and CRP, model 3 includes all factors in model 2 and chronic kidney disease, and model 4 includes all factors in model 3 and NT-proBNP

ULSAM: model includes age, current smoking, BMI, systolic blood pressure, antihypertensive treatment, total cholesterol, HDL cholesterol, lipid-lowering treatment, diabetes treatment and previous or prevalent cancer

AGES-R: model includes age, diabetes mellitus, BMI, hypertension medication, systolic blood pressure, smoking, CRP, creatinine, total cholesterol, HDL cholesterol, triglycerides and statin medication

FINRISK: model includes total cholesterol, HDL cholesterol, systolic blood pressure, hypertension medication, diabetes mellitus, current smoking and region of Finland

HUNT: model includes gender, age, total cholesterol, HDL cholesterol, systolic blood pressure, treatment of hypertension, smoking, diabetes mellitus, eGFR, history of myocardial infarction and CRP

PIVUS: model 1 includes gender, hypertension, diabetes mellitus, HDL cholesterol, LDL cholesterol, current smoking, BMI, eGFR, CRP, and previous cardiovascular disease, model 2 includes all factors in model 1 and NT-proBNP, and model 3 includes the Framingham risk score

SSHEC: model includes age, gender, area, family history, diabetes mellitus, blood pressure, total cholesterol and HDL cholesterol

General discussion

The diagnosis of acute myocardial infarction has undergone a noticeable transformation since the introduction of the high-sensitivity cardiac troponin assays^{1,2}. The increased sensitivity of the assays empowered the detection of low levels and small changes in cardiac troponins and was therefore at the basis of the development of more accurate and faster diagnostic algorithms³⁻⁶. However, at the same time, the detection of previously unnoticed, slightly elevated cardiac troponin levels in various populations without acute cardiac injury complicated the interpretation of cardiac troponin concentrations⁷.

This thesis highlights four aspects associated with the complexity of interpretation of cardiac troponins in the high-sensitivity era (discussed in the following chapters): First, the variation in cardiac troponin concentrations over the day (*chapters 3 and 4*). Second, the effect of impaired renal function on cardiac troponin levels (*chapters 5 and 6*). Third, the differences between cTnT and cTnI (*chapters 7 and 8*). Fourth, persistently elevated basal cardiac troponin concentrations and their clinical significance (*chapters 9, 10 and 11*).

In this discussion section, we shine light on the four major findings that are described in this thesis and provide directions towards further research.

Cardiac troponin T, but not cardiac troponin I, exhibits a diurnal rhythm

The first major finding is the circadian rhythm in basal cTnT, but not in cTnI, concentrations (*chapters 2, 3, 4, and 5*)^{8,9}. This rhythm is characterized by gradually decreasing concentrations throughout daytime and rising concentrations during night-time¹⁰.

The presence of a diurnal rhythm implies that both the absolute concentrations and the changes in serially measured cTnT are of different magnitude at different time points. Since the current guidelines presume random variation in cardiac troponin levels and apply the same cut-off values during the whole day^{2,11}, the diurnal rhythm may affect the diagnosis of acute myocardial infarction. Our results from *chapter 3*, in which we examined the diagnostic accuracy during the high- (early morning) and low-level phase (evening), show that the diagnostic accuracy is not time-dependent, and indicate that the circadian rhythm in cTnT does not affect diagnosis of acute myocardial infarction. Nevertheless, the current approach

used to analyze the effect of the diurnal rhythm on the diagnosis of acute myocardial infarction by examining diagnostic accuracy, does not allow us to draw any conclusions on whether the optimal cut-off values are identical throughout the day. Future, extended or pooled, cohort studies establishing the sensitivity and specificity of different cut-off values at different time-intervals, may help us to determine whether cut-off values for cTnT should be time-dependent.

Decreased renal elimination is not the main driver

In addition to the possible clinical implications of the diurnal variation, we employed it as a model to examine the effect of impaired renal clearance on cTnT concentrations (*chapter 5*). At the basis of this study was the hypothesis that if impaired renal elimination and subsequent accumulation, and not increased cardiac release, are the main drivers behind elevated cardiac troponin release, this would affect the diurnal rhythm in cTnT concentrations: A prolongation of the terminal half-life of cTnT will fade the circadian rhythm. This hypothesis was supported by a mathematical model¹². In contrast, we demonstrated that the circadian rhythm remains similar in subjects with severe chronic kidney disease, and therefore we showed that impaired renal elimination is not the main driver behind elevated cTnT concentrations in subjects with impaired renal function.

Our conclusion is in line with the results of several cross-sectional studies indicating the multi-etiology of cardiac troponin elevations, including factors that are associated with myocardial injury^{13,14}. However, stronger support for our findings is provided by longitudinal cohort studies that show a robust association between basal elevated cardiac troponin concentrations and premature mortality and morbidity. The fact that these associations are almost unaffected by adjustment for renal function indicate that cardiac troponin levels rather reflect an underlying risk profile or (subclinical) disease, rather than a mere consequence of impaired renal elimination¹⁵⁻¹⁸.

In conclusion, we should take elevated cardiac troponin concentrations seriously as an indicator for underlying cardiovascular disease and future risk. Our results therefore advocate for an extensive diagnostic work-up, not limited to the rule-out of acute myocardial

infarction (based on (adjusted) diagnostic algorithms¹⁹), in all patients with elevated cardiac troponin concentrations irrespective of their estimated glomerular filtration rate¹³.

We cannot deny that the conclusion that decreased renal elimination is not the main driver behind persistently elevated cTnT levels in subjects with impaired renal function might feel contra intuitive considering the successful identification of cTnT fragments in the urine of patients suffering acute myocardial injury (*chapter 6*). However, it is important to consider that both studies examine two distinct settings. The presence of cardiac troponin fragments in urine in the acute setting has only been detected in patients with concomitant proteinuria²⁰. Furthermore, it is important to bear in mind that the finding that impaired renal clearance is not the main driver behind elevated cTnT levels, does not exclude that cardiac troponin (fragments) may, in part, be removed from the human body by renal clearance²¹⁻²³.

Combining cardiac troponin T and cardiac troponin I increases early rule-out

The third major finding involves the early diagnosis of acute myocardial infarction using a combination of cTnT and cTnI concentrations. In *chapter 8*, we demonstrated how combining cardiac troponin levels, both mathematical combinations and a combination algorithm, leads to a three-to-six-fold increase in the number of patients in whom acute myocardial infarction can be ruled-out after a single blood draw at presentation compared to the current algorithm (based on either cTnT or cTnI)². This may have major impact on clinical practice since it allows more efficient patient care and resource allocation²⁴⁻²⁹.

The addition of a second biomarker to cardiac troponin for the rapid rule-in and rule-out of acute myocardial infarction may increase the specificity of a single measurement at presentation and can be considered as a next step towards increasingly accurate and fast clinical decision making^{25,30-35}. Nevertheless, we should be aware of the fact that dual testing can also complicate interpretation and may be associated with logistic challenges. Since none of the current diagnostic platforms offers high-sensitivity cTnT and cTnI assays, a direct, broad and prompt implementation of this combination strategy is not (yet) achievable.

A second point that merits attention, is the performance of the diagnostic algorithms in the external validation cohort (*chapter 8*): Despite the high negative predictive value (99.4 – 99.6%), the number of false-negative findings (0.8-1.5%) is increased in comparison with the internal cohort. It is a common phenomenon that algorithms show different performances in distinct cohorts, and it is for example also the case for the 0-hour/1-hour algorithm³⁶. Nevertheless, it challenges us to reflect on the underlying mechanisms (different patient characteristics, distinct allocation strategies, analytical variation in the lower range and the outlier-driven approach of determining cut-off values³⁷⁻³⁹), and to consider future research strategies like recalibration of cut-off values in even bigger datasets and specific subgroup analyses³⁹⁻⁴¹.

Basal cardiac troponin levels predict premature mortality

Fourth and final major finding is the prognostic value of basal cardiac troponin values in the general population. We performed a meta-analysis, with data on 65019 participants from 11 cohort studies, in which we showed a significant relationship between basal cardiac troponin levels, also below the 99th percentile, and all-cause and cardiovascular mortality during follow-up (*chapter 11*)⁴². Our data corroborate and extend the findings of previous studies^{43,44}.

The finding that cardiac troponin levels correlate to prognosis in asymptomatic individuals, raises the question whether and how baseline cardiac troponin concentrations are of additional value in cardiovascular risk management¹⁴. *Chapter 11* shows that the addition of cardiac troponins leads to a significant, but numerically small improvement of existing prediction models⁴². Nevertheless, remaining questions merit attention: First, do changes in cardiac troponin concentrations translate directly into risk prediction? Second, may interventions affect both the outcome and the basal concentration of cardiac troponins? Recent studies indicate that changes in cardiac troponin levels over time are reflected in the prognosis⁴⁵⁻⁴⁹. Furthermore, whereas exercise training interventions did not affect basal cardiac troponin levels (*chapters 9 and 10*)^{50,51}, other interventions like statin therapy (modestly) reduced both cTnI concentrations and associated risk^{52,53}. Notwithstanding,

additional studies towards the effect of possible risk stratification strategies – including the target population, biomarker panel and timing – on health related and economic outcomes seem to be necessary prior to implementation^{45,49,54-57}.

From state of the art to the future

This thesis paid attention to multiple aspects of cardiac troponins in acute and chronic conditions. Based on our main findings, we propose a number of future directions. Besides, the ultimate challenge for the upcoming years seems to be finding a solution for the limited specificity of cardiac troponin elevations for the diagnosis of acute myocardial infarction.

Three future strategies may help us to increase the specificity of cardiac troponin testing for acute myocardial infarction: The first strategy involves the optimization of interpretation of cardiac troponin concentrations measured with the current assays. By determining adjusted, optimal cut-off values for factors like time of presentation, gender, age or previous concentrations^{58,59}, cardiac troponin testing may become more specific to detect acute myocardial infarction. The second strategy relates to *chapter 8* of this thesis: The specificity of cardiac troponin testing may increase by addition of a second cardiac or ischemic biomarker to the diagnostic algorithm^{25,30-34}. The third, and most challenging strategy is the development of new, high-specificity (cardiac troponin) assays for the diagnosis of acute myocardial infarction. Over the last decades, our research group has partially solved the puzzle on release, degradation, and elimination by unraveling the fragmentation of cardiac troponins⁶⁰⁻⁶². A next step may involve the identification of cardiac troponin forms that are specific for acute myocardial infarction, and can be targeted by new assays.

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Summary

Cardiac troponins, cardiac troponin T (cTnT) and cardiac troponin I (cTnI), are the biochemical cornerstones in the diagnosis of acute myocardial infarction without characteristic changes on the electrocardiogram (ECG). The recent introduction of the high-sensitivity cardiac troponin assays was at the basis of the increasingly accurate and rapid clinical decision making in patients with acute chest pain. Nevertheless, the increased sensitivity of the recent assays has not only expedited diagnosis of acute myocardial infarction, but also resulted in the detection of, previously undetectable, low cardiac troponin concentrations in subjects without acute myocardial infarction. The aim of this thesis is to increase our understanding of basal cardiac troponin levels in non-acute conditions, their impact on the diagnostics of acute myocardial infarction, and how these aspects can be used to improve the interpretation of cardiac troponins levels.

Chapter 1 provides a general introduction on cardiac troponins and the diagnosis of acute myocardial infarction. Furthermore, it describes the prevalence and prognostic value of persistently elevated cardiac troponin concentrations.

Chapter 2 illustrates the impact of chronically elevated cardiac troponin concentrations with a case. This case highlights four aspects that are discussed in this thesis (discussed in the following chapters): First, the variation in cardiac troponin concentrations over the day (*chapters 3 and 4*). Second, the effect of impaired renal function on cardiac troponin levels (*chapters 5 and 6*). Third, the differences between cTnT and cTnI (*chapters 7 and 8*). Fourth, persistently elevated basal cardiac troponin concentrations and their clinical significance (*chapters 9, 10 and 11*).

Since increased cardiac troponin concentrations are not specific for the presence of an acute myocardial infarction, serial measurement of cardiac troponins is of profound importance in diagnostics. An important assumption for the interpretation of these serial cardiac troponin concentrations is the random variation of concentrations in clinically stable conditions. In *chapters 3 and 4*, we demonstrate that this assumption holds true for cTnI, but not for cTnT. Specifically, in *chapter 3*, which contains the results of a biological variation study with hourly sampling during day and night in 24 patients without chronic kidney disease, we show an

omnipresent diurnal rhythm in cTnT. This rhythm is characterized by gradually decreasing concentrations during the day and increasing concentrations of cTnT during night-time. Since the diurnal pattern in cTnT concentrations may cause increases or decreases in serial measurements to be more likely in certain timeframes, we additionally evaluated the effect of time on the diagnostic accuracy in an international multicenter diagnostic trial (2782 patients with acute chest pain). This evaluation shows a very high and time-frame independent diagnostic accuracy of cTnT for the diagnosis of acute myocardial infarction. Nevertheless, in early presenters (≤ 3 h after the onset of chest pain) the effect of the timeframe seems to be more pronounced and leads to a borderline significant decrease in diagnostic accuracy for cTnT concentrations at one hour after presentation and for the combination of cTnT concentration at presentation combined with the absolute change within the first hour. Therefore, the diurnal rhythm may provide an explanation in cases where the clinical assessment is discordant with the biochemical kinetics. In addition, this chapter shows random variation in cTnI levels over the day without an effect on the diagnostic accuracy of cTnI. Moreover, *chapter 4* focuses on the variation in cTnI levels during day and night. In addition to the population described in *chapter 3*, we included 20 subjects with chronic kidney disease. Their baseline cTnI levels were slightly higher and accompanied by a significant lower analytical coefficient of variation compared to the subjects without chronic kidney disease. Nevertheless, we observed a similar random variation in cTnI levels over the day in both groups.

Persistently elevated cardiac troponin concentrations are highly prevalent among subjects with impaired renal function. A better understanding and interpretation of these elevations, requires more insight in the underlying mechanism: Is it the heart, by increased release, or the kidneys, by decreased renal elimination? *Chapters 5 and 6* consider the role of the kidneys in the terminal elimination of cTnT from the circulation. *Chapter 5* builds upon the previous chapters and employs the diurnal oscillation pattern of cTnT as a model to assess the contribution of impaired renal elimination to persistently elevated cTnT levels in patients with chronic kidney disease. Mathematically modeling shows that impaired elimination of cTnT should lead to a faded circadian rhythm characterized by a substantial

decrease in amplitude. Nevertheless, the pattern and the amplitude of the circadian rhythm in cTnT is similar in subjects with or without chronic kidney disease. These observations strongly suggest that other factors, such as increased release of cardiac troponins from the heart due to (subclinical) myocardial injury, are responsible for persistently elevated cardiac troponin levels in subjects with decreased renal function. Whereas the previous chapter focuses on the elimination of cTnT in a chronic setting, *chapter 6* examines the elimination of cTnT in patients suffering from acute myocardial infarction. We are the first to develop and validate a targeted mass spectrometry assay for the detection of cTnT (fragments) in urine. Subsequently, we show the presence of cTnT fragments in a subset of patients. Nevertheless, whether the presence of cTnT in urine reflects a physiological or rather a pathophysiological process needs to be elucidated.

cTnT and cTnI are both proteins from the same complex inside the cardiomyocytes and are equally recommended for the diagnosis of acute myocardial infarction. Although there are some clear differences between both cardiac troponins, which are further highlighted in *chapters 7 and 8*. In *chapter 7*, we show a substantial variation in cTnT concentrations in both serum and plasma of different individuals after the addition of a standardized amount of cardiac troponin. This evident variation is not present for other cardiac biomarkers (cTnI, creatine kinase MB isoenzyme and myoglobine) and disappears when the experiment is replicated in deproteinated serum. These findings indicate the presence of one or more factors that have specific interaction with cTnT and not with cTnI. Additional studies have to elucidate whether this effect can also be found *in vivo* and what its exact clinical implications are. *Chapter 8* examines the clinical impact of the use of the different cardiac troponins for the diagnosis of acute myocardial infarction and evaluates whether combining them might overcome individual limitations. When applying the current 0-hour/1-hour algorithm for cTnT or for cTnI in an international multicenter center diagnostic trial (2225 patients with acute chest pain), the agreement on the early diagnosis of acute myocardial infarction is moderate and the rule-out of patients without an acute myocardial infarction at presentation is limited. The combination of cTnT and cTnI for the rule-out of acute myocardial infarction leads to a significant increase in the number of safe rule-outs after a single blood draw. These

results are supported by the validation of combination strategies in an external cohort (2539 patients). Nevertheless, some limitations of this strategies merit attention: First, the 3-to-6-fold increase in rule-outs at presentation due to combining cTnT and cTnI seems to lead to a very small, but inevitable increase in the absolute number of false-negative results. Second, the clinical implementation of this dual-marker approach would currently be associated with substantial logistic obstacles.

The final part of this thesis reflects on persistently elevated cardiac troponin concentrations that are common in various subpopulations and associated with an unfavorable prognosis. *Chapters 9 and 10* explore the effects of exercise training, a preventive lifestyle intervention, on the course of basal cardiac troponin levels. In *chapter 9*, we analyze the effects of a 24-week resistance-type exercise training program on the cTnT levels of (pre)frail elderly. There is no significant difference in the course of cTnT levels between the intervention (25 subjects) and the control group (27 subjects). A limitation of this study might be the limited (absolute) training load due to the frailty of the subjects. Furthermore, the effect on cTnI concentrations is unclear. To overcome these limitations, *chapter 10* presents two additional exercise training studies (including 13 and 39 subjects) and an additional analysis of cardiac cTnI in the previous study. Despite the beneficial changes in metabolic profile and physical performance, there is also no effect on circulating cardiac troponins levels in these studies. To quantify the relationship between basal cardiac troponin levels and prognosis in the general population, we perform a meta-analysis in *Chapter 11*. This meta-analysis with data on 65019 participants from 11 cohort studies shows a robust association of basal cardiac troponin levels with all-cause and cardiovascular mortality during follow-up in the general population.

Lastly, *chapter 12*, contains a general discussion of the work presented in this thesis and provides directions for future research.

Samenvatting

Cardiale troponines, cardiaal troponine T (cTnT) en cardiaal troponine I (cTnI), zijn de biochemische pijlers in de diagnostiek van een acuut myocardinfarct in afwezigheid van kenmerkende veranderingen op het elektrocardiogram (ECG). Door de recent geïntroduceerde hoog-sensitieve cardiale troponinetesten, kunnen infarcten steeds accurater en sneller worden gediagnosticeerd bij patiënten die zich melden met pijn op de borst. De hoge sensitiviteit zorgt er echter ook voor dat er regelmatig (licht) verhoogde concentraties worden gevonden bij personen die geen acuut myocardinfarct hebben. Het doel van dit proefschrift is om deze niet-acute verhogingen verder in kaart te brengen, te bestuderen hoe zij kunnen interfereren met de diagnostiek en uiteindelijk te onderzoeken of deze kennis ons kan helpen om cardiale troponineconcentraties beter te interpreteren.

Hoofdstuk 1 vormt de introductie van dit proefschrift. Het geeft een korte beschrijving van de diagnostiek van het acute myocardinfarct en de rol die cardiale troponines hierin spelen. Daarnaast worden in dit hoofdstuk chronische troponineverhogingen en hun prognostische implicaties besproken.

Hoofdstuk 2 illustreert de impact van chronisch verhoogde cardiale troponines aan de hand van een casus. In deze casus komen vier aspecten aan de orde die verder in dit proefschrift aan bod komen: Ten eerste, de schommelingen in cardiale troponineconcentraties over de dag (*hoofdstuk 3 en 4*). Ten tweede, de mogelijke effecten van de nieren op cardiale troponines (*hoofdstuk 5 en 6*). Ten derde, de verschillen tussen cTnT en cTnI (*hoofdstuk 7 en 8*). Ten vierde, de prognostische betekenis van chronische verhoogde cardiale troponineconcentraties (*hoofdstuk 9, 10 en 11*).

Omdat verhoogde cardiale troponineconcentraties niet specifiek zijn voor de aanwezigheid van een acuut myocardinfarct, is het serieel meten van licht verhoogde cardiale troponineconcentraties van belang bij de diagnostiek. Een belangrijke aanname bij de interpretatie van seriële waarden is dat de concentraties onder normale omstandigheden (dus in de afwezigheid van een infarct) willekeurig schommelen over de dag. In *hoofdstuk 3 en 4* laten we zien dat deze aanname klopt voor cTnI, maar niet voor cTnT. *Hoofdstuk 3* rapporteert de resultaten van een biologische variatie studie waarbij gedurende een etmaal bij 24 proefpersonen ieder uur bloed is afgenomen. Hierbij vertonen alle proefpersonen een

duidelijk dag-nacht ritme in cTnT: Over de dag neemt de concentratie cTnT in het bloed langzaam af en gedurende de nacht neemt deze weer toe. Het dag-nacht ritme zou ertoe kunnen leiden dat (grote) verschillen in serieel gemeten waarden op sommige tijdstippen waarschijnlijker zijn dan op andere tijdstippen en dat hiermee het vermogen om onderscheid te maken tussen wel of geen infarct tijdsafhankelijk is. Daarom hebben we in dit hoofdstuk tevens gekeken of het diagnostische vermogen van cardiale troponines afhankelijk is van het moment van de dag. Dit hebben we gedaan in een internationale diagnostische multicenter studie (2782 patiënten met acute pijn op de borst). Uit deze analyse blijkt dat het vermogen van cTnT om patiënten met en zonder een infarct te onderscheiden hoog is, en niet lijkt te worden gestoord door het dag-nacht ritme. Echter, in de groep die zich vroeg presenteert (≤ 3 uur na het ontstaan van pijn op de borst) zien we dat het diagnostisch vermogen van cTnT beter lijkt in de avonduren (dit verschil is significant wanneer alleen gekeken wordt naar de concentratie na een uur of naar het verschil in seriële metingen en bijna significant als dit wordt gecombineerd met de meting bij presentatie). Dit zou mogelijk kunnen verklaren waarom bij sommige patiënten de klinische presentatie niet overeenkomt met het beeld in de laboratoriumuitslag. Daarnaast laat dit hoofdstuk zien dat cTnI concentraties wél random fluctueren gedurende de dag en het moment waarop de patiënt zich presenteert geen effect heeft op het diagnostisch vermogen van de test. In aanvulling hierop focust *hoofdstuk 4* zich volledig op de variatie in cTnI concentraties gedurende de dag en de nacht. Hiertoe zijn 20 proefpersonen met een chronische nierziekte toegevoegd aan de populatie zoals deze beschreven is in *hoofdstuk 3*. De beginconcentraties van cTnI waren significant hoger in deze tweede groep, hetgeen gepaard ging met een lagere analytische variatie. Ondanks dit verschil zien we in beide groepen een even grote, random spreiding van cTnI levels over de dag.

Individen met een verminderde nierfunctie hebben relatief vaak chronisch verhoogde cardiale troponineconcentraties in hun bloed. Het onderliggende mechanisme voor deze verhogingen is echter nog onopgehelderd. De centrale vraag hierbij is: Komt het door de nieren (doordat zij minder cardiale troponines verwijderen uit het bloed) of komt het door het hart (doordat het meer cardiale troponines afgeeft aan het bloed)? In *hoofdstuk 5 en 6* hebben we gekeken naar de rol die de nieren spelen bij het verwijderen van cardiale

troponines. *Hoofdstuk 5* is een vervolg op de voorgaande hoofdstukken en gebruikt het ritme in cTnT om de mate waarin de nieren verantwoordelijk zijn voor de basaal verhoogde troponineconcentraties te onderzoeken. We hebben hierbij allereerst aan de hand van een mathematisch model laten zien dat als er minder cTnT uit het bloed wordt verwijderd bij patiënten met een verminderde nierfunctie, het ritme in cTnT over de dag zal afnemen. Als we echter naar de ritmes in cTnT in de individuen met en in de individuen zonder nierfalen kijken, dan zien we dat het ritme hetzelfde is (ook qua sterkte). Deze bevindingen suggereren dat niet een verminderde uitscheiding door de nieren, maar andere factoren, zoals een verhoogd vrijkomen van cardiale troponines als het resultaat subklinische hartschade, de drijvende krachten zijn achter de troponine verhogingen in individuen met een verminderde nierfunctie. In tegenstelling tot het vorige hoofdstuk richt *hoofdstuk 6* zich op de rol van de nieren in de acute setting. In dit hoofdstuk hebben we een methodiek ontworpen en gevalideerd om cTnT middels massaspectrometrie te meten in urine. Vervolgens hebben we hiermee de aanwezigheid van cTnT fragmenten in de urine van een aantal patiënten met een acuut myocardinfarct aangetoond. Het is echter niet duidelijk of de aanwezigheid van troponine in urine een 'normaal', fysiologisch proces is (dat door de lage concentraties niet in ieder monster gemeten kan worden) of dat dit het gevolg is van overloopproteïnurie en enkel bij heel hoge troponineconcentraties in het bloed kan worden gemeten.

cTnT en cTnI zijn twee eiwitten die afkomstig zijn vanuit een gemeenschappelijk eiwitcomplex in de hartspiercel en worden gezien als diagnostische equivalenten (dit wil zeggen; in de huidige richtlijnen worden zij als gelijkwaardige biomarkers gezien). Er zijn echter ook verschillen tussen beide troponines. In *hoofdstuk 7 en 8* wordt hier verder bij stilgestaan. In *hoofdstuk 7* laten we grote verschillen in de gemeten concentraties cTnT zien als aan het serum of plasma van verschillende individuen dezelfde hoeveelheid cardiaal troponine wordt toegevoegd. Dit is niet het geval voor de andere cardiale biomarkers (cTnI, creatine kinase MB en myoglobine) of als we het serum eerst eiwit-vrij maken. Het lijkt erop dat er één of meerdere factoren in min of meerdere mate aanwezig zijn in het serum en plasma van verschillende individuen en dat deze factoren wel een effect hebben op cTnT, maar niet op cTnI. Vervolgstudies zullen moeten aantonen of dit effect ook in vivo aanwezig is en wat hiervan de klinische implicaties zijn. *Hoofdstuk 8* bestudeert welke impact de keuze

voor het testen van cTnT of cTnI kan hebben en bekijkt of het wellicht beter is om deze testen te combineren. In een internationale multicenter cohortstudie (2225 patiënten met acute pijn op de borst) is de overeenkomst in vroege diagnostiek van het acute myocardinfarct matig als cTnT en cTnI worden vergeleken. Hierbij valt eveneens op dat slechts bij een klein deel van alle patiënten dat geen infarct heeft, een infarct direct na één bloedafname op het moment van presentatie kan worden uitgesloten. Door het combineren van de beide cardiale troponinetesten neemt het aantal patiënten waarbij direct na één bloedafname veilig een infarct kan worden uitgesloten significant toe. Hetzelfde beeld zien we terug als deze combinatiestrategie wordt toegepast in een extern validatie cohort (2539 patiënten met acute pijn op de borst). Kanttekeningen bij deze combinatiestrategieën zijn: 1) de kleine toename in het absolute aantal vals-negatieven (patiënten die voor rule-out in aanmerking komen, maar toch een acuut myocardinfarct blijken te hebben) ten gevolge van een 3-tot-6-voudige toename in het aantal vroege rule-outs, en 2) het feit dat het meten van twee verschillende cardiale troponines op dit moment in veel ziekenhuizen nog niet mogelijk is vanwege de logistiek.

Het laatste deel van dit proefschrift richt zich op prognostische betekenis van chronische cardiale troponine verhogingen. *Hoofdstuk 9 en 10* onderzoeken het effect van levensstijlinterventies, in de vorm van trainingsprogramma's, op cardiale troponineconcentraties over de tijd. In *hoofdstuk 9* hebben we gekeken naar het effect van een 24-weeks krachttrainingsprogramma op cTnT concentraties in (pre)fragiele ouderen. Hierbij hebben wij geen verschil kunnen aantonen tussen groep die wel (25 deelnemers) en de groep die geen trainingsprogramma heeft gevolgd (27 deelnemers). Een mogelijke limitatie van deze studie was de beperkte belastbaarheid van de (pre)fragiele deelnemers. Daarnaast laat deze studie in het midden of er mogelijke effecten op cTnI zijn. In *hoofdstuk 10* hebben we, rekening houdend met de beperkingen van de vorige studie, de studie uit *hoofdstuk 9* verder uitgebreid. Hiertoe hebben we twee extra trainingsstudies toegevoegd (met respectievelijk 13 en 39 deelnemers) en hebben we in alle studies ook cTnI bekeken. Ondanks duidelijke verbeteringen op metabole en fysieke parameters, zagen we ook in deze studie geen effect op cardiale troponineconcentraties. Ten slotte, hebben we in *hoofdstuk 11* middels een meta-analyse de relatie tussen basale cardiale troponine levels en het risico

op vroegtijdige overlijden van mensen in de algemene populatie gekwantificeerd. De uitgevoerde meta-analyse bevat data van 11 cohort studies (in totaal 65019 deelnemers) en laat zien dat verhoogde basale cardiale troponineconcentraties samenhangen met een grotere kans op vroegtijdig overlijden.

Tot slot bevat *hoofdstuk 12* een algemene discussie over het in dit proefschrift beschreven werk en worden suggesties voor vervolgonderzoek aangedragen.

This chapter describes the (future) valorization of the research described in this thesis. Valorization is thereby defined as the social and economic relevance of research, the target populations of the findings, the related concrete products, services, processes and activities, their innovativeness and future directions¹.

Relevance

According to the World Health Organization (WHO), cardiovascular disease, of which acute myocardial infarction is considered as an important entity², is the world's number one killer, responsible for approximately 31% of all deaths worldwide³. Along, cardiovascular disease substantially contributes to disability and decreased quality of life^{4,5}. A recent economic evaluation showed that cardiovascular disease accounts for approximately 12% of the total European health care expenditures^{6,7}. However, this may be even an underestimation since the total economic impact on society is bigger due to productivity losses and informal care as result of chronic disease and disability⁷. Urbanization and associated changes in lifestyle at one hand, and longevity at the other hand, will further increase the prevalence of cardiovascular disease in the coming years^{8,9}.

This thesis focuses on two ways in which the accurate interpretation of cardiac troponin concentrations may contribute to lower the burden of cardiovascular disease: The distinction of patients with and without myocardial infarction in the acute setting, and the identification of subjects at increased risk for future events based on basal cardiac troponin concentrations.

Target population

Since this thesis focuses on the optimization of interpretation of cardiac troponin concentrations in both the acute (diagnostic) and chronic (prognostic) setting, its findings are relevant for a large population. For example, whereas the novel insights on combining cardiac troponin T and cardiac troponin I for the early rule-out of acute myocardial infarction refer to emergency department patients, the results on the prognostic value of cardiac troponins are applicable to the general population.

Products

As already described in the discussion chapter of this thesis, many of our findings trigger further research. An example of a concept that requires additional research is the application of cardiac troponins for prognosis and prevention. In contrast, a more concrete and innovative concept is the combination of cardiac troponin T and cardiac troponin I for the diagnosis of acute myocardial infarction.

Implementation of combination testing in clinical practice

We showed that combining cardiac troponin T and cardiac troponin I in the diagnostic setting is highly promising to overcome the limited specificity of the separate assays. It results in a significant increase in patients in which acute myocardial infarction could be safely ruled-out at presentation (from approximately 10% to more than 40%). Therefore, the implementation of this combination strategy may have profound consequences for clinical practice since it may help to overcome unnecessary resource use, overcrowding of the emergency department and anxiety among patients¹⁰. The fact that high-sensitivity cardiac troponin T and high-sensitivity cardiac troponin I assays do currently not run on the same platform and most laboratories have only one platform running 24/7 for routine measurements is an important obstacle for the introduction of this strategy in clinical practice. The development of (point of care) devices suitable for combined measurement – a project involving diagnostic companies, laboratories and physicians – is therefore crucial. Parallel, we should evaluate the optimal place of combination testing in the diagnostic chain – directly at presentation in the hospital or maybe even earlier at the general practice or in the ambulance setting –, and define optimal algorithms with optimal cut-off values, taking into account the effects of combination testing and the increased number of rule-outs at presentation downstream the diagnostic chain.

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Dankwoord

Curriculum Vitae

List of publications

Dankwoord

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Curriculum Vitae

Noreen van der Linden was born on October 20th, 1987 in Breda, the Netherlands. After completing her secondary school with the distinction cum laude at Stedelijk Gymnasium Breda (Breda, the Netherlands) in 2006, she studied Molecular Life Sciences at Maastricht University (Maastricht, the Netherlands). After obtaining her Bachelor's degree, she enrolled in the 4-year Physician-Clinical Investigator Research Master at the same university. During her medical training, she was

awarded the top 3% student award. In 2013 she graduated with the distinction cum laude. After her graduation, Noreen started her PhD-program under supervision of prof. dr. Marja van Dieijen-Visser, prof. dr. Luc van Loon and dr. Steven Meex at the department of Clinical Chemistry of the Central Diagnostic Laboratory of the Maastricht University Medical Center+ (MUMC+) (Maastricht, the Netherlands). As part of her PhD-program, she visited the Cardiovascular Research Institute Basel (CRIB) at Basel University Hospital (Basel, Switzerland) (supervised by prof. dr. Christian Mueller).

During the first two years of her PhD-program, Noreen was a board member of the AAV-MUMC+. In 2015, she interrupted her PhD-program for four months to take part in the National ThinkTank. In a team of twenty-four (PhD-)students, she examined the educational system in the Netherlands and presented an advice on future improvement. Afterwards, she was involved in various ThinkTank projects on topics like e-Health and intergenerational processes. During the academic year 2016-2017, she joined the KNAW Academy Honors Program for Young Artists and Scientists.

The scientific work in this thesis was published in several peer-reviewed journals and presented on national and international conferences. In addition, Noreen won the Dutch Society of Clinical Chemistry Abstract Price, was nominated for the Pélerin Award and received a travel grant from the European Society of Cardiology.

Noreen will pursue her career in healthcare strategy consulting at Gupta Strategists.

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