Mass Spectrometric Identification of Cardiac Troponin T in Urine of Patients Suffering from Acute Myocardial Infarction

Mass Spectrometric Identification of Cardiac Troponin T in Urine of Patients Suffering from Acute Myocardial Infarction

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Background: Because of its high cardiospecificity, cardiac troponin T (cTnT) is one of the first-choice biomarkers to diagnose acute myocardial infarction (AMI). cTnT is extensively fragmented in serum of patients suffering from AMI. However, it is currently unknown whether all cTnT is completely degraded in the body or whether some cTnT fragments can leave the body via 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 20 patients were precipitated using a cTnT-specific immunoprecipitation technique and a nonspecific acetonitrile protein precipitation. After in-solution digestion of the precipitated proteins, the resulting peptides were separated and analyzed using HPLC and mass spectrometry with a targeted selected ion monitoring assay with data-dependent tandem mass spectrometry (t-SIM/dd-MS2).

Results: The t-SIM/dd-MS2 assay was validated using a synthetic peptide standard containing 10 specific cTnT peptides of interest and with purified human intact cTnT spiked in urine from healthy individuals. Using this assay, 6 different cTnT-specific peptides were identified in urine samples from 3 different patients, all suffering from AMI.

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

IMPACT STATEMENT

This article provides novel evidence that cardiac troponin T (cTnT) can be detected with LC-MS/MS in urine from patients with acute myocardial infarction. This information is of interest to researchers studying release, degradation, and elimination pathways of cTnT. The article also provides directions for future research and debates the cause of the cTnT presence in urine. Is cTnT in urine a physiological process involving renal clearance of cTnT or a pathophysiological process related to aspecific proteinuria?

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[3] **Nonstandard abbreviations:** cTnT, cardiac troponin T; AMI, acute myocardial infarction; ACN, acetonitrile; TFA, trifluoroacetic acid; AMBIC, ammonium bicarbonate; t-SIM/dd-MS2, targeted-selected ion monitoring with data dependent tandem mass spectrometry.

Cardiac troponin T (cTnT)[3] is one of the preferred biomarkers to establish the diagnosis of acute myocardial infarction (AMI), along with the electrocardiogram and physical examination (*1*–*3*). Nevertheless, the interpretation of cardiac troponin concentrations can be challenging, especially in individuals with persistently increased levels (*4*, *5*), like those found in chronic kidney disease patients. The high prevalence of persistently increased cardiac troponin concentrations among individuals with impaired renal function has raised the question if, and if so how, the kidneys are involved in the terminal elimination of cardiac troponins (*6*–*8*). A better understanding of the final elimination of cardiac troponins might help us better interpret 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 to 14 days (*10*–*12*). In addition, it has been shown that cTnT is highly degraded in the 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 filtration (*15*) and should normally be degraded and reabsorbed by the proximal tubule (*16*, *17*). These filtered cTnT fragments may also be excreted via the urine because of, for example, dysfunction or overload of the tubuli (*18*).

Recently, we have developed a gel-based targeted mass spectrometry assay for the identification of cTnT and its fragments in serum (*19*–*21*). In the present study, we have modified and validated this assay for the analysis of urine samples. We explored 2 different sample workup methods and applied them to urine samples from patients with acute myocardial injury.

MATERIALS AND METHODS

Reagents, synthetic peptide standard, and positive control

UPLC-grade water, acetonitrile (ACN), formic acid, and trifluoroacetic acid (TFA) for sample preparation and mass spectrometry were obtained from Biosolve. Ammonium bicarbonate (AMBIC), ≥99.5%, was from Sigma-Aldrich. Trypsin Gold and ProteaseMAX™ surfactant were from Promega.

Ten tryptic cTnT peptides of interest were synthesized by Pepscan with an average purity >95%. These peptides were dissolved in 20% ACN and subsequently pooled in 2.0% ACN and 0.01% TFA at a concentration of 40 nmol/L to produce a synthetic peptide standard. This standard was used as a quality control sample throughout the experiment and was also used to schedule the targeted mass spectrometry experiments.

Validation experiments were performed with a positive control consisting of purified human intact cTnT (catalog no. 8T13, HyTest LTD) spiked in various concentrations in both 50-mmol/L AMBIC and fresh urine from a healthy volunteer.

Urine samples

Twenty urine samples were collected from the following acute myocardial injury patient groups: ST-elevation myocardial infarction (n = 9), non–ST-elevation myocardial infarction (n = 4), myocarditis (n = 5), cardiac arrest (n = 1), and miscellaneous (n = 1). These samples were submitted for routine diagnostic analysis, and all had cTnT serum measurements that were >1000 ng/L. See Table 1 in the Data Supplement that accompanies the online version of this article (http://www.jalm.org/content/vol2/issue6) for the relevant patient characteristics. Urine samples were typically collected

within 2 or 3 days after the increased serum cTnT measurement; they 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 2000*g* for 10 min and subsequently treated as described below. Patient samples were randomized and treated according to the code of proper secondary use of human tissue in the Netherlands (www.fmwv.nl). As controls, 19 urine samples were collected from presumably healthy volunteers who neither reported symptoms related to AMI nor had a medical history of cardiovascular disease.

Sample workup: protein concentration and digestion

In this study, all samples were analyzed 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 µL of urine was performed by the addition of 50 µL of M-270 Streptavidin Dynabeads (Invitrogen) coupled to the biotinylated anti-cTnT antibody M11.7 (Roche Diagnostics) (*22*). After incubation for 1 h at room temperature, immunoprecipitated proteins were eluted in 50-mmol/L 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 µL of ice-cold (−20 °C) ACN to 400 µL of urine, followed by 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 of ice-cold ACN, air dried, and redissolved in 50 µL of AMBIC (50 mmol/L, pH 8). The digestion of all samples was performed with 1 µg of Trypsin Gold in the presence of 0.03% ProteaseMAX surfactant for 3 h 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 UPLC Dionex Ultimate 3000 (ThermoFisher Scientific). Solvent A consisted of 0.1% formic acid in 100% water; solvent B consisted of 0.08% formic acid, 20% water, and 80% ACN. Peptides were first trapped on an Acclaim PepMap 100, 100 µm × 2 cm, C18, 5 µm, 100 A trap column in 0.1% TFA, 2% ACN, and 98% water. Next, peptides were separated on an Acclaim PepMap RSLC, 75 µm × 15 cm, C18, 2 µm, 100 A analytical column by a 30-min gradient of 4% to 55% solvent B. Washout was performed by a 1-min gradient of 55% to 90% solvent B and a constant flow of 90% solvent B for 4 min at 300 nL/min.

Selected ion monitoring scans were acquired in the Orbitrap with a width of 2.0 Th at a resolution of 70 000 full width at half maximum at 200 *m/z*, automated gain control of 10^5, and a maximum injection time of 250 ms. Selected ion monitoring 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 selected ion monitoring scan were selected for higher-energy collisional dissociation with an isolation window of 1.0 Th and a normalized collision energy that was optimized for each peptide. Product ions were detected in the range of 250 to 1500 *m/z* at a resolution of 17 500 full width at half maximum at 200 *m/z*, automated gain control target of $2{\cdot}10^5$, maximum injection time of 200 ms, and a dynamic exclusion window of 30 s.

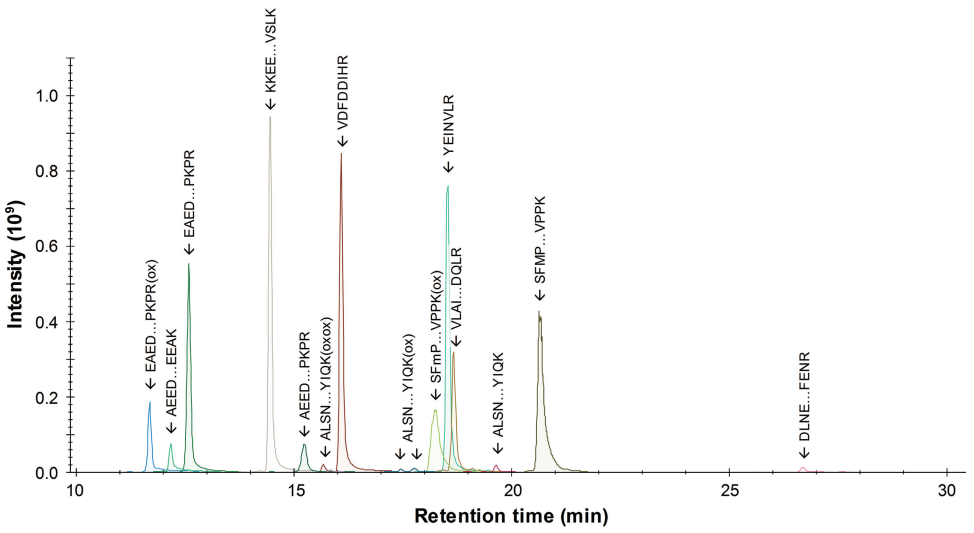

Fig. 1. Selected ion chromatogram of the precursor ions of all peptides present in the synthetic peptide standard.
The synthetic peptide standard consisted of 10 synthesized peptides spiked in equimolar amounts. All labelled peaks were identified with tandem mass spectrometry. Methionine residues contained within some peptides can be oxidized, designated with "(ox)".

cTnT immunoreactivity determination

cTnT-specific immunoreactivity within the collected urine samples was determined with the fifth-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% at >30 ng/L (*23*).

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

Data analysis

Product ion spectra were searched against the Uniprot Human database dated November 13, 2013 (39690 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 semitryptic with a maximum of 2 missed cleavages allowed. A fixed-value peptide-spectral match (PSM) validator was used with default Xcorr cutoffs. All accepted spectra were manually evaluated.

Statistical significance of cTnT concentrations in urine as measured with the fifth-generation Roche assay was assessed with a 2-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 synthesized peptides specific to cTnT, was first analyzed using a full scan with data-dependent tandem mass spectrometry (dd-MS2). Fig. 1 shows

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

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

[a] Purified human intact cTnT was dissolved in urine and serially diluted. Both dilution series were then subjected to either 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).

a representative precursor ion chromatogram of this measurement. Every peptide present in the synthetic standard was positively identified using dd-MS2. Although all peptides were pooled in equimolar amounts, it can be observed that the measured abundance of each peptide varied (e.g., because of ionization differences). This analysis was repeated regularly to assess the mass accuracy of the instrument and retention time drift between experiments.

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 tandem mass spectrometry in both dilution experiments. The listed cTnT concentrations were calculated based on the added amount of cTnT and the dilution factors used in both experiments. As shown, both protocols could identify cTnT spiked in low concentrations in urine. However, some peptides are identified in lower concentrations than others, whereas others may not have been identified at all. In addition, some of the peptides (like ^{108}DLNE...FENR^{123} and ^{228}VLAI...DQLR^{240}) are not detected using ACN precipitation but can be identified in very low concentrations using immunoprecipitation. In general, it has been shown that the limit of identification is lower when using immunoprecipitation than when using ACN precipitation.

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 2 of the analyzed samples. In one sample, four peptides could be identified (Fig. 2A). In the other sample, only one peptide was identified (Fig. 2B). When ACN precipitation was used, cTnT peptides were identified in a single additional sample (Fig. 2C). The identification of cTnT in these 3 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 ΔCn = 0 for all peptides; Table 2).

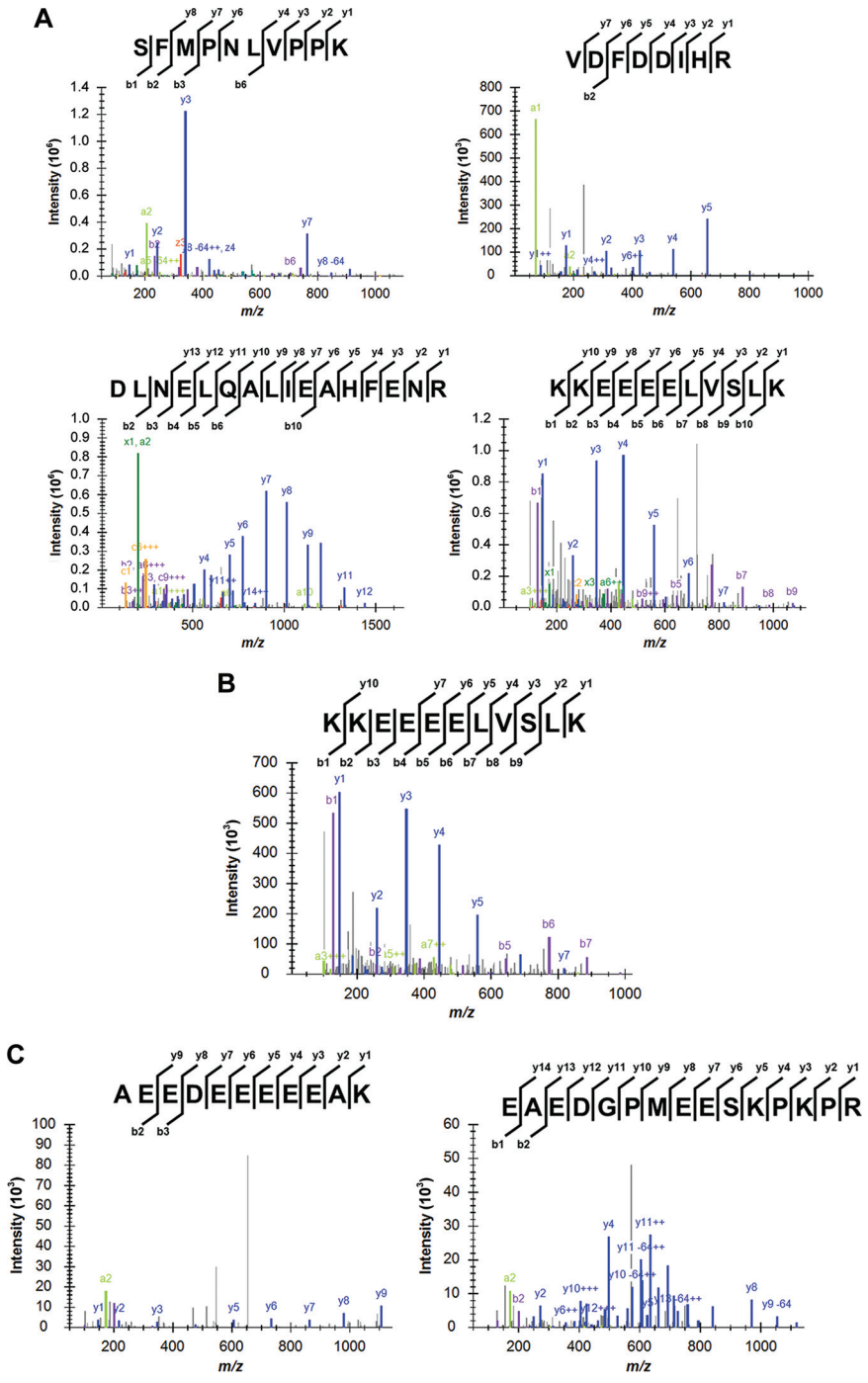

Fig. 2. Product ion spectra of cTnT peptides present in patient urine samples.
Four peptides, located at the center region of cTnT, were identified in Patient (a) using immunoprecipitation (A). One peptide, located at the center region of cTnT, was identified in Patient (b) using immunoprecipitation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

Table 2. Cross-correlation (Xcorr) and delta correlation (ΔCn) values of identified cTnT peptides in patient urine.[a]

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}EAED. . .PKPR^{78}	3.48	0

[a] Xcorr and ΔCn scores are listed for all the spectra in Fig. 3. Xcorr cutoff value was set to 1.9; maximum ΔCn score allowed was 0.05.

In addition to the LC-MS/MS identifications, cTnT immunoreactivity was measured in all urine samples using the fifth-generation Roche assay. Fig. 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. Fig. 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, data not shown). Table 1 in the online Data Supplement shows the relevant laboratory measurements of each patient included in the study.

DISCUSSION

In this study, a targeted LC-MS/MS assay and 2 different proteomic workflows were used to detect the presence of cTnT in urine. Using this method, we are the first to provide conclusive evidence that

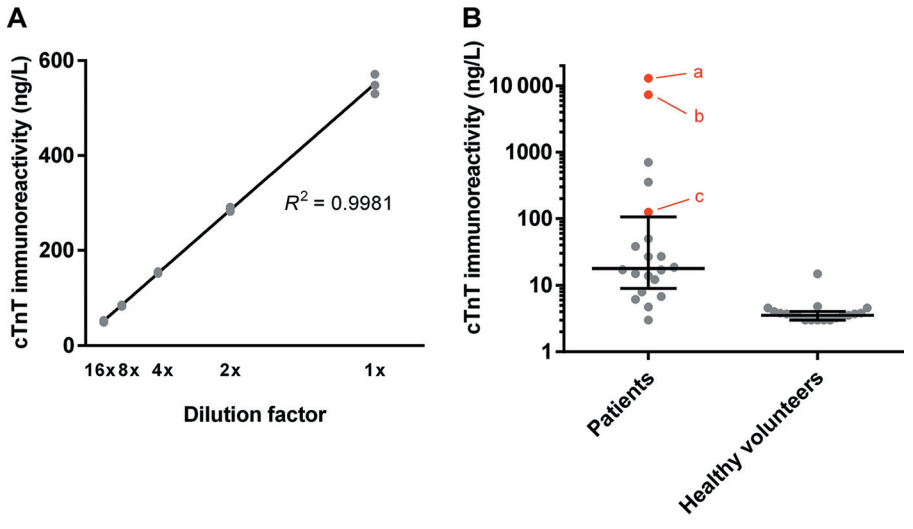

Fig. 3. Immunoreactivity in urine samples as measured with the fifth-generation clinical cTnT immunoassay by Roche Diagnostics.
Serial dilution series of the positive control spiked in urine of a healthy individual. R^2 = 0.9981, n = 3 (A). Twenty urine samples of patients suffering from acute myocardial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

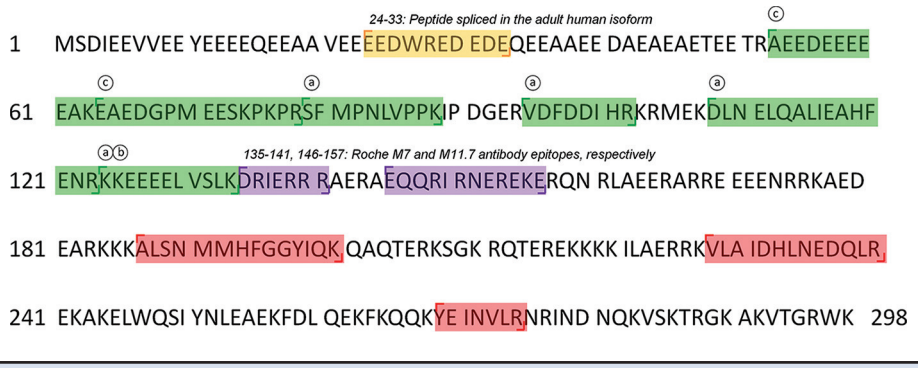

Fig. 4. Annotated sequence of the canonical isoforms of cardiac troponin T. Green highlights indicate peptides that were successfully identified using mass spectrometry in Patients (a), (b), and (c), as depicted. Red highlights indicate targeted peptides that were not identified in any of the samples. 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.

cTnT can be present in the urine of patients with high serum concentrations of cTnT. We could show the presence of cTnT-specific peptides in urine in 3 of 20 acute myocardial injury patients. All 3 of these patients suffered from AMI, with concomitant proteinuria.

Although urine is a biological matrix that can be conveniently and noninvasively 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 concentrations (*24*). Because 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 selective in its binding to the central region of cTnT. The peptides identified with this workflow (Fig. 2, A and B) indeed do originate from this central region (Fig. 4). Because 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 nonselective workflow based on the precipitation of proteins using an organic solvent (*24*, *26–29*). This resulted in the identification of 2 peptides originating from the N terminus, which are cleaved in the first step in the degradation of cTnT (*12*, *21*) in an additional sample (Figs. 2C and 4). The peak serum concentration of cTnT in this particular patient was high (31 199 ng/L; see Table 1 in the online Data Supplement), which may explain why we identified cTnT peptides in this patient's urine, even though the measured cTnT immunoreactivity in his urine was much lower (125 ng/L). The other 2 patients in which cTnT peptides were identified had a high cTnT-specific immunoreactivity in urine (13 000 and 7365 ng/L; red dots in Fig. 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 with serum. When the arginine- and lysine-rich midregion is the only part of cTnT that remains in urine, it may become completely degraded during tryptic digestion and will be missed with the t-SIM/

dd-MS2 assay, but possibly not with the immunoassay, in which digestion is not performed. Because we used leftover samples from patients routinely analyzed in our laboratory, no protease inhibitors or other stabilizers were added to the urine upon collection. Therefore, it is possible that the 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 points into account.

It is important to note that we were able to identify cTnT in only 3 samples, and that in 1 of these 3 samples only a single cTnT peptide was identified, violating commonly used guidelines for the identification of proteins (*30*). However, these guidelines are often focused on intact proteins, making the requirement of identifying >1 peptide more feasible than for this specific case, in which we are most likely dealing with protein fragments. Considering the high confidence of this match, the quality of the tandem mass spectrometry spectrum, and the fact that an abundant cTnT-specific immunoreactivity was also determined, we believe this match is convincing evidence. Although 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 occurs only 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 3 patients in whom cTnT was identified with LC-MS/MS additionally suffered from proteinuria, possibly causing filtered cTnT fragments to end up in the urine because of protein overload of the proximal tubule.

Previously, using the clinical cTnT immunoassay, Ziebig et al. also detected cTnT in urine of patients suffering from AMI or undergoing heart surgery (*31*). Patients included in this work also suffered from mild to severe proteinuria; however, Ziebig et al.'s methodology was not intended to be used in urine and was not properly validated for that matrix. This makes drawing firm conclusions difficult, as positive results may have been caused by matrix effects or other disturbances. After all, although immunoassays may have relatively low limits of detection, their limit of identification may be more questionable. Immunoassays will require multiple epitopes to reach the same level of identification certainty as tandem mass spectrometry. In this study, we could confirm the findings by Ziebig et al. using the fifth-generation cTnT immunoassay (whereas Ziebig used the second-generation immunoassay, both by Roche Diagnostics). The measured cTnT-specific immunoreactivity was considerable in most patient samples and differed significantly from those measured in healthy controls (Fig. 3B). However, because of the urinary matrix, the identity of this immunoreactivity is uncertain. Therefore, it is important to interpret Fig. 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 increased cardiac troponin concentrations (*8*). Although the results presented here may seem to be at odds with that conclusion, it is important to consider that the current study demonstrated that cardiac troponin in urine is limited to AMI patients 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 troponin elevations in the absence of myocardial damage.

In conclusion, we showed that with the use of a targeted mass spectrometry assay, the presence of the highly cardiospecific biomarker cTnT can be

demonstrated in urine of patients with AMI. Further study examining the extent and implications of these observations is required, as well as determining whether this is a nonspecific effect caused by concomitant proteinuria or a more general elimination pathway of cTnT.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

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