Multi-Site Coronary Vein Sampling Study on Cardiac Troponin T Degradation in Non–ST-Segment–Elevation Myocardial Infarction: Toward a More Specific Cardiac Troponin T Assay

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Background—Cardiac troponin T (cTnT) is seen in many other conditions besides myocardial infarction, and recent studies demonstrated distinct forms of cTnT. At present, the in vivo formation of these different cTnT forms is incompletely understood. We therefore performed a study on the composition of cTnT during the course of myocardial infarction, including coronary venous system sampling, close to its site of release.

Methods and Results—Baseline samples were obtained from multiple coronary venous system locations, and a peripheral artery and vein in 71 non–ST-segment–elevation myocardial infarction patients. Additionally, peripheral blood was drawn at 6- and 12-hours postcatheterization. cTnT concentrations were measured using the high-sensitivity-cTnT immunoassay. The cTnT composition was determined via gel filtration chromatography and Western blotting in an early and late presenting patient. High-sensitivity -cTnT concentrations were 28% higher in the coronary venous system than peripherally (n=71, $P<0.001$). Coronary venous system samples demonstrated cTn T-I-C complex, free intact cTnT, and 29 kDa and 15 to 18 kDa cTnT fragments, all in higher concentrations than in simultaneously obtained peripheral samples. While cTn T-I-C complex proportionally decreased, and disappeared over time, 15 to 18 kDa cTnT fragments increased. Moreover, cTn T-I-C complex was more prominent in the early than in the late presenting patient.

Conclusions—This explorative study in non–ST-segment–elevation myocardial infarction shows that cTnT is released from cardiomyocytes as a combination of cTn T-I-C complex, free intact cTnT, and multiple cTnT fragments indicating intracellular cTnT degradation. Over time, the cTn T-I-C complex disappeared because of in vivo degradation. These insights might serve as a stepping stone toward a high-sensitivity-cTnT immunoassay more specific for myocardial infarction. (*J Am Heart Assoc.* 2019;8: e012602. DOI: 10.1161/JAHA.119.012602.)

The introduction of high-sensitivity cardiac troponin (hs-cTn) immunoassays to diagnose myocardial infarction (MI) has led to frequent reporting of elevated cTn levels in conditions other than MI.[1–5] This limitation in specificity complicates clinical decision making on a daily basis, and new strategies to overcome this issue are highly warranted.[6]

For cardiac troponin T (cTnT), degradation of its intact form into multiple cTnT fragments was observed in patients who have acute MI.[7–9] Interestingly, in other conditions associated with cTnT elevations (eg, end-stage renal disease), only the smallest cTnT forms were present.[10,11] The current clinical high-sensitivity (hs)-cTnT immunoassay detects all these circulating cTnT forms. As suggested by

Clinical Perspective

What Is New?

- Cardiac troponin T (cTnT) is released from the injured myocardium in multiple forms (ie, cardiac troponin T-I-C complex, free intact cTnT subunit, and several cTnT fragments).
- cTnT is subject to in vivo degradation, which may already take place within the cardiomyocyte.
- The cTnT composition is dependent on the interval that passed since the onset of symptoms.

What Are the Clinical Implications?

- A high-sensitivity cTnT immunoassay solely targeting cTnT forms ≥29 kDa may discriminate cTnT release in the setting of a myocardial infarction from cTnT release in other (patho)physiologies.
- The proportion of ternary cardiac troponin T-I-C complex might hint toward the age of the myocardial infarction.

others, a novel assay that would specifically target distinct cTnT forms might increase diagnostic specificity for MI.[12–14] Before this will become feasible, however, more basic knowledge on the origin and formation of troponin forms seems indispensable.

Until now, the majority of studies on cTnT degradation in MI patients were conducted on serum samples.[7,8,11,15–17] This is of particular importance because thrombin is generated during serum collection and thrombin has repeatedly been shown to cause cTnT degradation.[18,19] Some have therefore postulated that cTnT degradation is merely a pre-analytical effect instead of an in vivo degradation pathway.[18] Alternatively, as cTnT degradation in MI patients follows a time-dependent pattern, it has been suggested that in vivo processes, at least in part, also contribute to cTnT degradation.[7,15,16,20] In addition, in vitro studies have shown that intracellular proteases are also involved in the degradation of cTnT, but supportive in vivo evidence is lacking.[21–24]

In view of the above, it remains to be determined at which site(s) cTnT degradation actually occurs. The goal of this study was to investigate the cTnT composition(s) close to the site of release and during the course of MI. This was studied through multisite sampling both in the coronary venous system (CVS) as well as in the peripheral circulation in patients with non–ST-segment–elevation myocardial infraction (NSTEMI) using gel filtration chromatography (GFC) and Western blotting (WB). To assess the impact of pre-analytical degradation, all analyses were performed in both lithium-heparinized (LH) plasma and serum samples. These insights might serve as a stepping stone for the development of a novel hs-cTnT assay more specific for MI.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Patient Population

The TRAMICI study (Transcardiac Assessment of Myocardial Injury and Coronary Inflammation; see online supplemental Data S1 for description of the inclusion and exclusion criteria, and study procedures) was used to select eligible patients for the present study. The goal of the TRAMICI study was central, peripheral, and transcardiac assessment of cardiac biomarkers and inflammatory parameters in non–ST-segment–elevation acute coronary syndromes. The TRAMICI cohort included 71 patients with NSTEMI undergoing clinically indicated cardiac catheterization. Patients were treated according to the acute coronary syndrome guidelines, including treatment with aspirin and a P2Y12-inhibitor. During coronary angiography, every patient was anticoagulated with heparin.

With regard to patient selection for cTnT composition analyses, the following criteria were specified: (1) definite culprit lesion in the left anterior descending coronary artery (coronary artery that delivers blood to the anterior wall); (2) availability of all samples in accordance with the TRAMICI protocol; and (3) a minimum hs-cTnT concentration of 400 ng/L (sensitivity threshold of our laboratory setup, see below) in all CVS and peripheral samples. Of the patients who met these criteria, we selected the patient with the shortest and longest interval between symptom onset and study procedures. The TRAMICI protocol was approved by the local ethical committee (2004-186) of the Radboud University Medical Center (Nijmegen, The Netherlands). After obtaining oral informed consent before the procedure, participants provided written informed consent. Study procedures were in accordance with the Declaration of Helsinki.

Sample Collection

Our extensive blood sampling protocol is described in the supplemental material. In short, access to the CVS was gained after identification of a culprit artery by the attending interventional cardiologist. Blood samples were obtained from the great cardiac vein, the coronary sinus at the site of the most proximal posterolateral vein, and at the ostium of the middle cardiac vein (Figure S1). Hereafter, samples were obtained from the peripheral venous and arterial sheaths. Subsequently, peripheral venous samples were obtained by venipuncture from the antecubital vein 6 and 12 hours after cardiac catheterization. All blood samples were collected in LH plasma and serum tubes, centrifuged, divided over multiple aliquots preventing freeze–thaw cycles, and stored at −80°C until further analysis.

Laboratory Techniques

Details on the different laboratory techniques used are presented in the online supplement (Data S1) and described in brief as follows.

Biochemical assessment

hs-cTnT concentrations were measured with the hs-cTnT immunoassay (Roche Diagnostics, Basel, Switzerland). This assay utilizes 2 monoclonal antibodies: M11.7 and M7. These epitopes are present in all cTnT forms (ie, cTn T-I-C complex, free intact 40 kDa cTnT, 29 kDa cTnT, and 15 to 18 kDa cTnT fragments). In addition, creatinine levels were assessed (CREP2; Roche Diagnostics). Assay characteristics were as provided by the manufacturer and measurements were performed on the e601 module of the COBAS 6000 analyzer series and c702 module of the COBAS 8000 analyzer series (Roche Diagnostics). The estimated glomerular filtration rate was calculated according to the Chronic Kidney Disease Epidemiology Collaboration Formula (CKD-EPI).[25]

Gel filtration chromatography

GFC was used to separate cTn T-I-C complex and free cTnT forms based on size exclusion. Our GFC laboratory setup is currently the most sensitive technology available to study the cTnT composition and has already been validated for cTn T-I-C complex and cTnT form separation in serum.[10] A schematic illustration of a GFC elution profile and the expected corresponding cTnT forms are depicted in Figure S2. To validate potential blood matrix effects, purified human ternary cTn T-I-C complex (#8T62; HyTest, Turku, Finland) and free intact 40 kDa cTnT (#8T13; HyTest) standards were added to GFC running buffer and hs-cTnT-negative (<3 ng/L) LH plasma and serum from a pool of healthy individuals in a concentration comparable to the 2 selected NSTEMI patients for elaborate cTn T-I-C complex and cTnT composition analyses.

For each sample loaded on the GFC column (0.25 mL), either cTnT standard or patient sample, 83 fractions of 1 mL were collected and analyzed for hs-cTnT concentration with the hs-cTnT immunoassay.

Western blotting

To extend on the GFC data and further characterize the cTnT composition, immunoprecipitation on both LH plasma and serum samples was followed by Western blotting (WB) analysis. To increase the discriminatory value of the GFC technique with regard to cTnT form differentiation, WB analysis was used to determine the exact cTnT composition of the eluted GFC peaks.

The anti-cTnT monoclonal antibodies used for immunoprecipitation (M11.7) and WB (M7) were identical to those used in the commercially available hs-cTnT immunoassay (kindly provided by Roche Diagnostics). In contrast to GFC, it should be noted that the cTn T-I-C complex cannot be analyzed by WB because of the presence of the detergent sodium dodecyl sulfate, which disintegrates the cTn T-I-C complex into the different free intact cTn subunits. Therefore, GFC and WB are considered complementary techniques.

Statistical Analysis

Results are presented as median (interquartile range) or mean±SD depending on Gaussian distribution. The Wilcoxon signed-rank test was used to compare paired non-Gaussian distributed hs-cTnT concentrations of the 3 CVS samples (ie, great cardiac vein, posterolateral vein, and middle cardiac vein) with the baseline peripheral arterial sample. To account for multiple testing, the conservative Bonferroni correction was applied to reduce the chance of type 1 errors (αBonferroni=0.017). Hence, a $P<0.017$ was considered statistically significant. Comparison of the peripheral arterial and venous sample was performed with the Wilcoxon signed-rank test. Additionally, hs-cTnT concentration differences over time were evaluated with the Friedman test followed by post hoc Wilcoxon signed-rank test for pairwise comparisons. For these analyses, a $P<0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism (version 5.04; GraphPad Software, Inc., San Diego, CA).

For the GFC analysis, the hs-cTnT concentration of each fraction was plotted on the y-axis of the graph, and on the x-axis the 83 eluted fractions. To account for analytical noise, we determined the mean hs-cTnT concentration of the fractions between 0 and 15 mL and 64 to 83 mL, which are exterior to the GFC elution peaks representing the cTnT forms. This mean noise hs-cTnT concentration was subtracted from the hs-cTnT concentration measured in the respective fractions of each cTnT peak. After this noise correction, the surface of each individual peak was calculated and expressed relative (%) to the total surface of all observed cTnT peaks.

Results

Patient Characteristics

Baseline characteristics of the entire cohort of 71 NSTEMI patients are depicted in Table. Mean age was 65±12 years and 68% were male. Cardiac catheterization was successfully completed for all patients with a median (interquartile range) time from symptom onset to cardiac catheterization of 1559 (1183–2167) minutes. The time between symptom onset and cardiac catheterization was 461 and 1354 minutes for patients 1 and 2, respectively. Patient 1 had a 95% stenosis in segment 10 identified as the culprit lesion; for patient 2 the culprit lesion was an 80% stenosis in segment 7 (Figure S3).

Table. Baseline Characteristics of the Total Population (n=71) and the 2 Selected Patients for cTnT Composition Analysis

	Total Population (n=71)	Patient 1	Patient 2
Sex, male	68%	Male	Male
Age, y	65±12	61	53
BMI, kg/m^2	26.1 (24.2–29.4)	30.8	30.7
Medical history			
History of MI	23%	No	No
History of PCI/CABG	8%	No	No
History of stroke	6%	No	No
History of DM	11%	No	No
Blood pressure, mm Hg			
Systolic	144±31	135	115
Diastolic	78±18	89	65
Heart rate, per min	72±19	100	80
Hypercholesterolemia, %	37%	Yes	No
Current smoking, %	45%	No	No
Creatinine, μmol/L	83 (69–95)	69	88
eGFR per CKD-EPI$_{creat}$, mL/min per 1.73 m^2	82 (63–94)	98	87

BMI indicates body mass index; CABG, coronary artery bypass graft; CKD-EPI, chronic kidney disease–epidemiology collaboration equation; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; MI, myocardial infarction; PCI, percutaneous coronary intervention.

Highest hs-cTnT Concentrations in the CVS

In the entire cohort (n=71), the median hs-cTnT concentration in the CVS was 230 (111–547) ng/L and significantly higher as compared with the peripheral artery sample with a median hs-cTnT concentration of 180 (95–369) ng/L (28%, $P<0.001$) (Figure 1). Median peripheral venous hs-cTnT concentrations changed significantly over time (Friedman $P=0.002$), which was ascribed to an increase in concentration between baseline and 6 hours postprocedure ($P=0.004$, Figure 1).

Also for the 2 selected patients, the highest hs-cTnT concentrations were measured in the CVS. Between baseline and 6 hours postprocedure, patient 1 showed an increase in hs-cTnT from 474 to 1177 ng/L and patient 2 showed a decrease from 1498 to 1214 ng/L. In line with the analytical validation article, no hs-cTnT concentration differences between serum and LH plasma were detected (R=0.995, Figure S4).[5]

Validation of GFC Elution Profiles for cTnT Standards

GFC elution profiles of the validation experiments using cTn standards are depicted in Figure 2.

GFC buffer

The addition of cTn T-I-C complex and free intact 40 kDa cTnT standards to the GFC buffer resulted in the detection of cTnT peaks in the GFC elution profiles at 21 and 30 mL (Figure 2A) and 29 mL (Figure 2B), respectively. WB characterization revealed that cTnT in both standards consisted of intact 40 kDa cTnT (100%, Figure 3). Therefore, the peak at 21 mL in Figure 2A is assigned to cTn T-I-C complex, and the peak at 30 mL in Figure 2A and 29 mL in Figure 2B to free intact 40 kDa cTnT.

LH plasma

Addition of cTn T-I-C complex and free intact 40 kDa cTnT standards to LH plasma resulted in cTnT peaks in the GFC elution profiles at 21 and 32 mL (Figure 2C) and 29 mL (Figure 2D), respectively. WB analysis revealed that cTnT in both standards consisted of free intact 40 kDa cTnT (100%, Figure 3). Again, the 21 mL peak in Figure 2C corroborates with cTn T-I-C complex, and the 32 and 29 mL peak (Figures 2C and 2D) with free intact 40 kDa cTnT. In contrast to the 2 peaks observed in Figure 2A, the second peak in Figure 2C is larger than the first, suggesting more elaborate turnover of cTn T-I-C complex to free intact 40 kDa cTnT in LH plasma as compared with the GFC buffer.

Serum

Addition of cTn T-I-C complex and free intact 40 kDa cTnT standards to serum resulted in cTnT peaks in the GFC profiles at 30 and 44 mL (Figure 2E) and at 30 and 41 mL (Figure 2F), respectively. WB analysis of the cTn T-I-C complex standard illustrated presence of free intact 40 kDa cTnT (29%), 29 kDa cTnT (60%), and 15 to 18 kDa cTnT (11%) fragments (Figure 3). WB analysis of the free intact 40 kDa cTnT standard revealed exclusive presence of cTnT fragments; 29 kDa cTnT (49%) and 15 to 18 kDa cTnT (51%) fragments (Figure 3).

As expected, in the GFC elution profiles of hs-cTnT-negative LH plasma and serum, no cTnT peaks were identified (Figures 2G and 2H).

From these validation experiments the following could be concluded: (1) the 21 mL peak was assigned to cTn T-I-C complex; (2) the 29 to 32 mL peak was assigned to free intact 40 kDa cTnT and/or 29 kDa cTnT fragments; and (3) the 41 to 44 mL peak was assigned to 15 to 18 kDa cTnT fragments (Figure S2).

In Vitro Disintegration of cTn T-I-C Complex and Degradation of cTnT in Serum

To determine the effect of the blood matrix, we compared GFC profiles (Figure 4 and Figures S5 and S6) between simultaneously obtained LH plasma and serum samples of the 2 selected patients.

In the CVS and peripheral baseline (T0) samples of both patients, we observed 3 peaks in LH plasma, and only 2
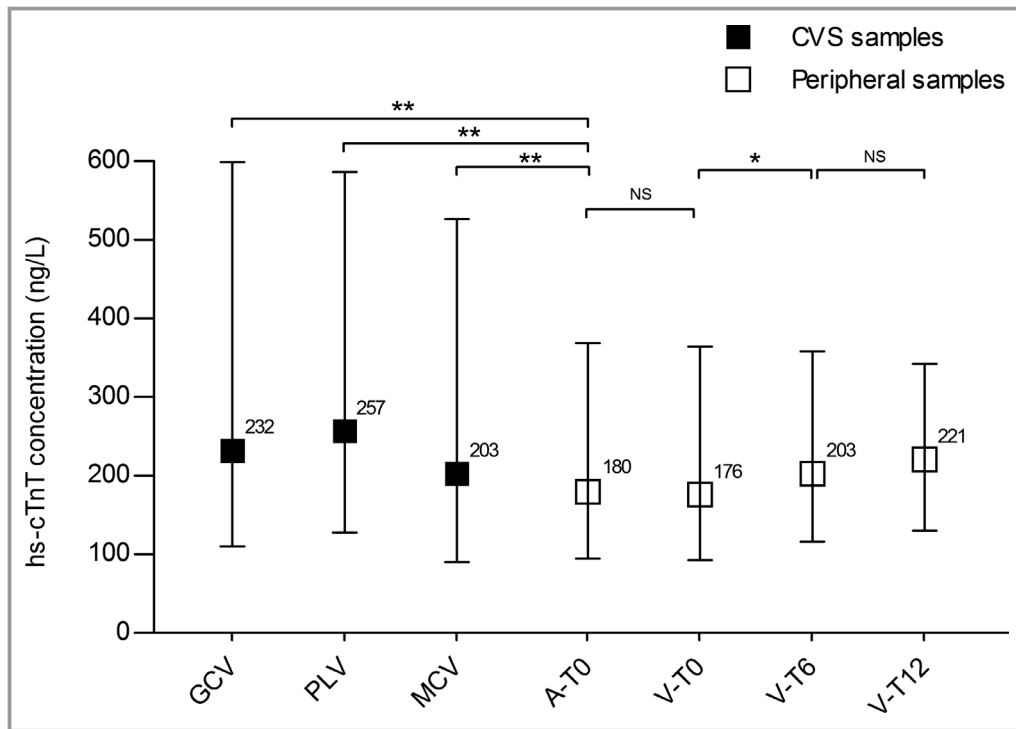
Figure 1. Median (box) and interquartile range (whiskers) hs-cTnT concentrations (ng/L) of the entire cohort at the different coronary venous system (CVS) and peripheral sampling sites. *P<0.05; **P<0.001; A-T0 indicates peripheral artery sample at baseline; GCV, great cardiac vein sample; hs-cTnT, high-sensitivity cardiac troponin T; MCV, coronary sinus sample at the ostium of the middle cardiac vein; NS, not significant; PLV, coronary sinus sample near the proximal posterolateral vein; V-T0, peripheral vein sample at baseline; V-T12, peripheral vein sample 12 hours postprocedure; V-T6, peripheral vein sample 6 hours postprocedure.

peaks in the corresponding serum samples (Figures S5 and S6). As compared with LH plasma, in serum there was no 20 to 22 mL peak, which indicated absence of cTn T-I-C complex. In addition, all observed 45 to 47 mL peaks were greater in serum than in LH plasma, indicating a higher proportion of 15 to 18 kDa cTnT fragments in serum (67−100%) as compared with LH plasma (41−75%) (Figure 4). Finally, GFC analyses of the V-T12 sample of both patients showed 2 peaks for the LH plasma sample at 31 and 45 to 46 mL, and only 1 peak for the corresponding serum sample at 46 mL (Figures S5G and S5N; S6G and S6M). These data indicate total absence of free intact 40 and 29 kDa cTnT fragments in serum.

WB analysis of the baseline (T0) LH plasma samples primarily revealed the presence of free intact 40 kDa cTnT, but 29 kDa and 15 to 18 kDa cTnT fragments were also present (Figure 5). In serum we exclusively observed 29 kDa and 15 to 18 kDa cTnT fragments (Figure 5). In addition to a clear presence of 15 to 18 kDa cTnT fragments on WB analysis of the V-T12 samples, there was also evidence of 29 kDa cTnT in both patients at T12 (Figure 5).

Hence, comparing simultaneously collected LH plasma and serum samples revealed in vitro induced disintegration of the cTn T-I-C complex and cTnT degradation in serum.

cTn T-I-C Complex Disintegration and cTnT Degradation Occurs In Vivo

In order to investigate potential in vivo processes contributing to cTn T-I-C complex disintegration and cTnT degradation, we evaluated the cTnT composition in LH plasma over time and between the early presenting patient 1 and late presenting patient 2. In addition, we compared the observed cTnT forms with the validation experiments.

GFC analyses showed that the proportion of cTn T-I-C complex decreased over time in peripheral venous LH plasma samples in patient 1 from 41% at V-T0 to 0% at V-T12 and patient 2 from 2% at V-T0 to 0% at V-T6 (Figure 4). Simultaneously, the proportion of 15 to 18 kDa cTnT fragments increased between V-T0 and V-T12 from 41% to 68% for patient 1 and from 56% to 75% for patient 2 (Figure 4). Additionally, cTnT composition comparison between patient 1 and 2 revealed higher proportions of the larger cTnT forms (cTn complex, free intact 40 and 29 kDa cTnT fragments) in patient 1 for all CVS and peripheral samples.

As depicted in Figure 5 using WB, detection of 29 kDa and 15 to 18 kDa cTnT fragments in LH plasma samples at T0 of both our patients provides evidence of in vivo degradation. As
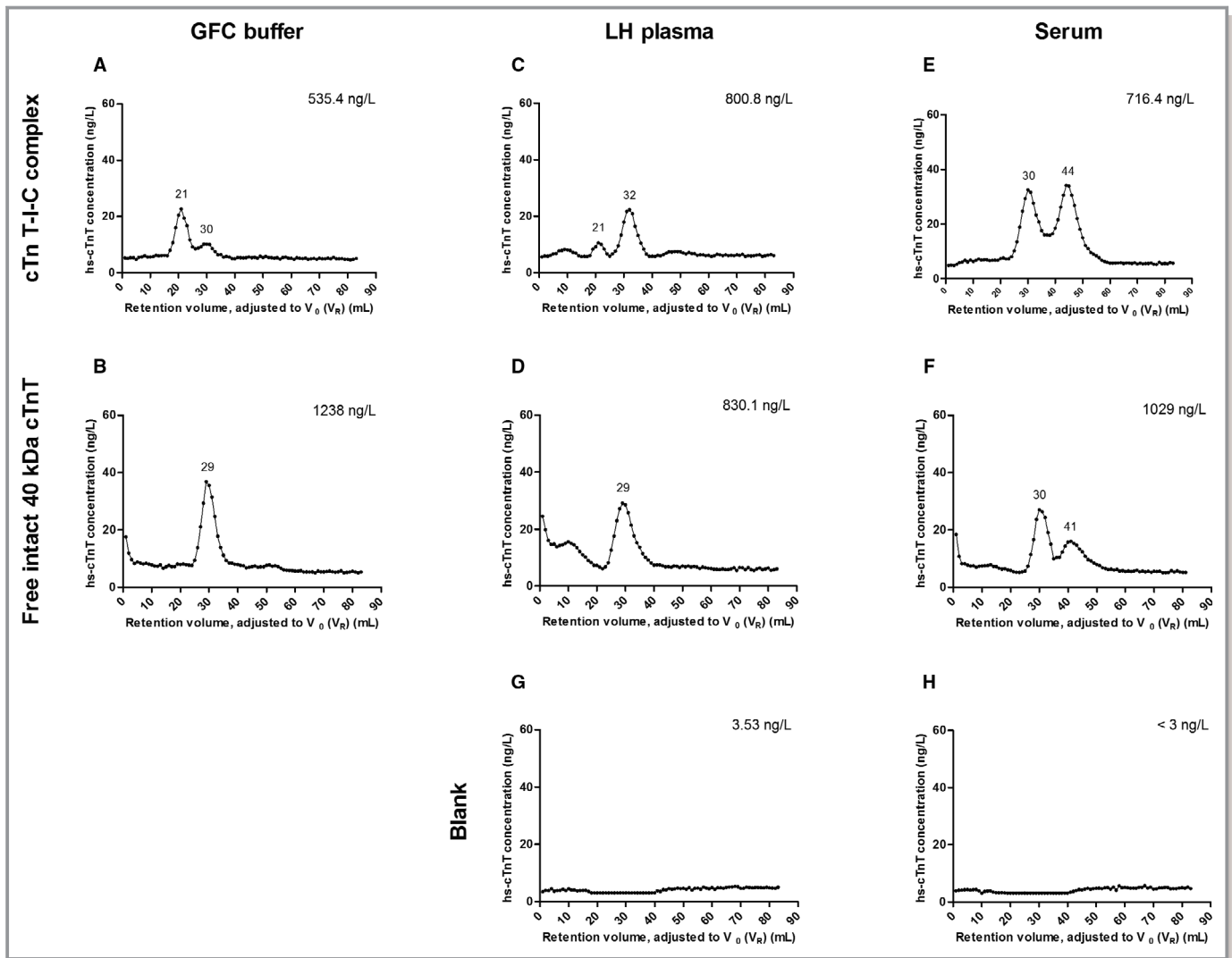
Figure 2. Gel filtration chromatography (GFC) analyses of cTnT standards (cTn T-I-C complex and free intact 40 kDa cTnT) in multiple matrices. cTn T-I-C complex in (**A**) GFC buffer, (**C**) lithium-heparinized (LH) plasma, and (**E**) serum. Free intact 40 kDa cTnT in (**B**) GFC buffer, (**D**) LH plasma, and (**F**) serum. cTnT-negative (**G**) LH plasma and (**H**) serum. The hs-cTnT concentration (ng/L) before GFC fractionation is displayed in the upper right corner of each panel. Also, the retention volume (mL) with highest hs-cTnT concentration per peak is displayed. hs-cTnT indicates high-sensitivity cardiac troponin T.

already shown in our validation experiments, in vitro degradation of free intact 40 kDa cTnT to smaller 29 kDa and 15 to 18 kDa cTnT fragments did not occur in LH plasma.

cTn T-I-C Complex Disintegration and cTnT Degradation Close to the Myocardium

To investigate potential in vivo cTn T-I-C complex disintegration and cTnT degradation within the heart, we compared the cTnT composition in samples obtained in close vicinity of the injured myocardium (ie, the CVS) with simultaneously obtained peripheral samples.

On GFC analyses, the absolute concentrations of all cTnT forms were higher within the CVS as compared with the peripheral arterial samples (Figure 4 and Figures S5A through S5D and S6A through S6D), which agrees with cardiac release of each cTnT form. The relative contribution of each separate cTnT peak to the total observed cTnT that eluted from the GFC column remained constant among the 3 CVS sampling sites (Figure 4 and Figures S5A through S5C and S6A through S6C). The maximum variation within the CVS between the 3 different peaks were 3%, 6%, and 8% for patient 1 and 3%, 2%, and 2% for patient 2 (Figure 4).

Thus, the identical cTnT composition in all CVS and baseline peripheral samples, but higher hs-cTnT concentrations measured in the GFC elution profiles of the CVS samples, indicates release of all cTnT forms (cTn T-I-C complex, free intact 40 kDa cTnT and cTnT fragments) from the myocardium.
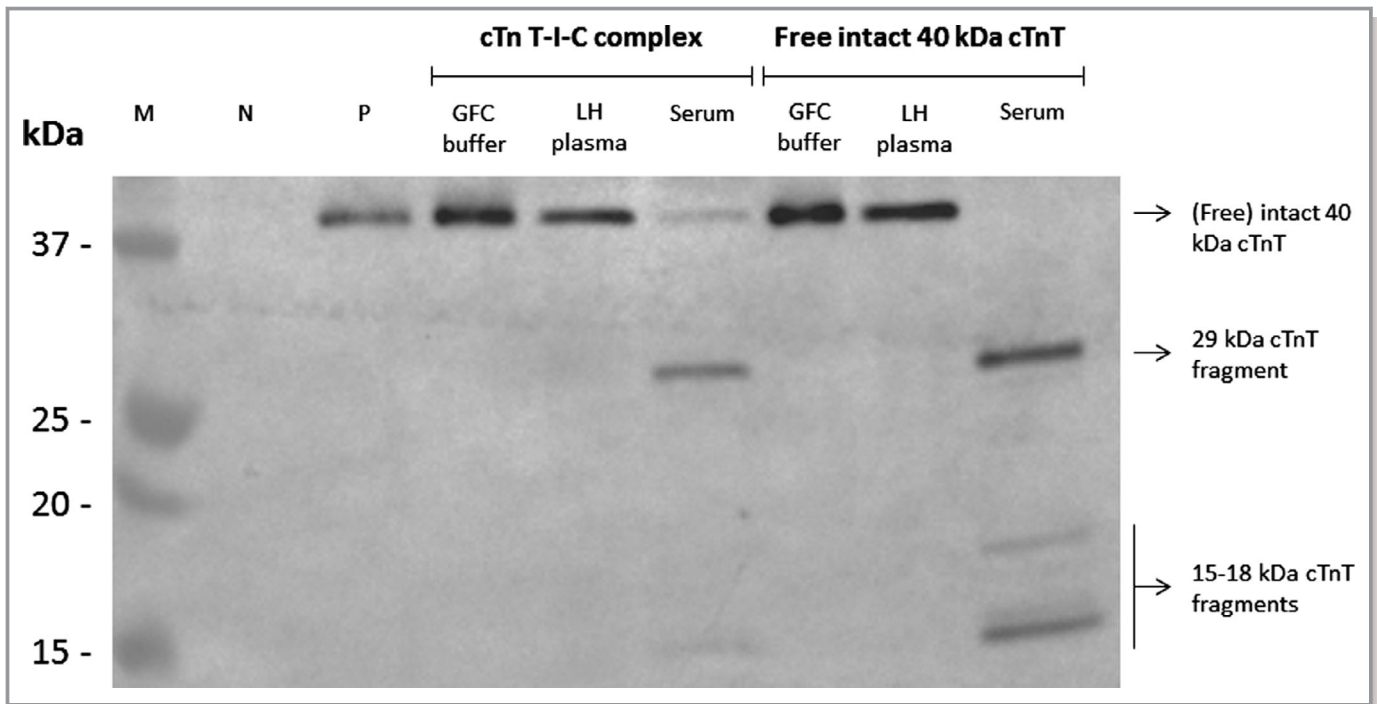
Figure 3. Immunoprecipitation followed by Western blot analysis of cTnT standards (cTn T-I-C complex and free intact 40 kDa cTnT) added to gel filtration chromatography (GFC) buffer, cTnT negative lithium-heparinized (LH) plasma and serum. hs-cTnT indicates high-sensitivity cardiac troponin T; M, protein standard marker; N, negative control; P, cTnT positive control.

Discussion

In search of improved cTnT assay specificity, detailed knowledge of the in vivo molecular appearance of cTnT in the setting of ischemic heart disease is fundamentally important. The unique design of the present study enabled us to determine the in vivo cTnT composition in close vicinity of the infarcted myocardium using multisite blood sampling in the CVS and compare it with the peripheral circulation in NSTEMI patients. We report 4 major findings:

First, to the best of our knowledge, this is the first study investigating hs-cTnT concentrations at multiple sites within the CVS. We showed ≈30% higher hs-cTnT concentrations in the CVS samples compared with baseline peripheral samples, which is in line with active cTnT release from the injured myocardial cells. This concentration gradient was also noticed by Turer et al, who demonstrated hs-cTnT elevation through coronary sinus sampling after pacing-induced stress in stable angina patients.[26]

Second, we found matrix-induced in vitro cTn T-I-C complex disintegration and more elaborate cTnT degradation in serum as compared with LH plasma. As illustrated by our GFC validation analyses, the cTn T-I-C complex was no longer demonstrable in serum, whereas in LH plasma little cTn T-I-C complex was observed. This difference between serum and LH plasma was also observed in the 2 patients, in whom cTn T-I-C complex was fully absent in serum. In addition, GFC data of the 2 patients

showed that cTnT in serum was primarily present as 15 to 18 kDa fragments in all CVS and peripheral samples with proportions greater than observed in LH plasma. These data are in alignment with Katrukha et al, who predominantly detected free intact 40 kDa cTnT in LH plasma of acute MI patients, while in simultaneously collected serum samples exclusively cTnT fragments were observed.[18] This pre-analytical effect has been allocated to abundantly generated thrombin during serum production,[18–20,27] and we conclude that the cTnT composition in LH plasma samples is a better representation of the in vivo situation in these patients.

Third, we also found evidence of in vivo processes that contributed to cTn T-I-C complex disintegration and cTnT degradation. As was observed in LH plasma samples of the 2 patients, there was a time-dependent degradation pattern. Over time a change in cTnT composition occurred with a decreasing magnitude of cTn T-I-C complex and increasing proportions of 15 to 18 kDa cTnT fragments. Moreover, the difference between the early and late presenting patient appeared to be in accordance with this. In the early presenting patient, the proportion of cTn T-I-C complex was still 25% (peripheral artery), whereas in the late presenting patient cTn T-I-C complex was almost absent (4%, peripheral artery). All these findings were observed using LH plasma as blood matrix. Importantly, the change in cTnT composition over time corroborates with previous observations in peripheral serum samples of MI patients.[7,17,28] Interestingly, our WB data were also confirmative
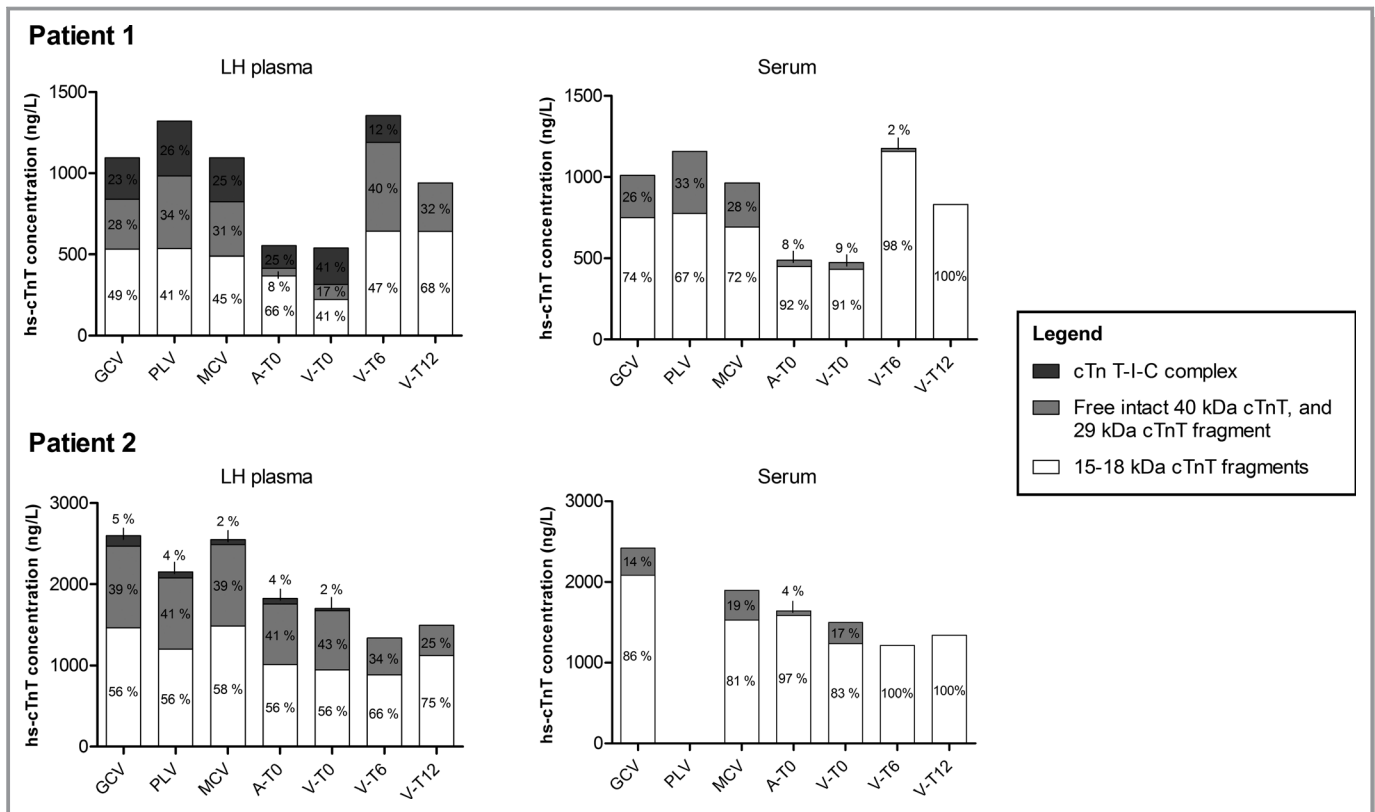
Figure 4. cTnT composition through gel filtration chromatography in lithium–heparinized (LH) plasma and serum samples of patients 1 and 2 at the different coronary venous system and peripheral sampling sites. A-T0 indicates peripheral artery sample at baseline; GCV, great cardiac vein sample; hs-cTnT, high-sensitivity cardiac troponin T; MCV, coronary sinus sample at the ostium of the middle cardiac vein; PLV, coronary sinus sample near the proximal posterolateral vein; V-T0, peripheral vein sample at baseline; V-T12, peripheral vein sample 12 hours postprocedure; V-T6, peripheral vein sample 6 hours postprocedure.

of in vivo degradation. As we showed in our LH plasma validation experiments, the cTnT that was present in the standard solutions was exclusively free intact 40 kDa cTnT. Therefore, the presence of the 29 kDa and 15 to 18 kDa cTnT fragments in the 2 patients is also proof of in vivo cTnT degradation.

Fourth, we provide evidence of each separate cTnT form (ie, cTn T-I-C complex, free intact 40 kDa cTnT, 29 kDa, and 15 to 18 kDa cTnT fragments), detected in the CVS, to be released from the injured myocardium. For each cTnT form, the absolute hs-cTnT concentration was higher in the CVS as compared with baseline peripheral measurements. This is an important first-time observation because our findings of in vivo cTnT degradation could be interpreted as the natural course of peripheral protein degradation. This evidence of release into the CVS suggests that degradation occurred in vivo inside the ischemic cardiomyocytes. In this regard, intracellular μ-calpain and caspase-3 were suggested as contributors to intracellular cTnT degradation and were shown to have proteolytic capacity on the N-terminal region of cTnT.[21–24] Alternatively, despite the observed transcardiac concentration gradient of each cTnT form, the possibility remains that degradation occurred after cTnT was released into the bloodstream. In this regard,

thrombin has been identified as an extracellular protease capable of cleaving cTnT at the N-terminal end.[18,19] Thrombin is abundantly generated in patients with MI, and therefore could have caused extensive cleavage especially inside the CVS, which is close to the infarcted myocardium.[29] Still, we consider it unlikely that circulating thrombin played a significant role because the relative contribution of each cTnT form remained stable throughout the coronary circulation. In view of the small chance of significant cTnT proteolysis in the trajectory between the area of injured myocardium and the blood collected closest to the injured area, it is assumed that the samples from the CVS reflect normal venous cardiac metabolism, which is also recognized by others.[30–32] Importantly, cleavage at the C-terminal end of cTnT is also required for the generation of the smallest cTnT fragments and thus other unidentified (intracellular) proteases need to be involved in cTnT degradation.

Implications

The data presented in this explorative study have important implications. Recently, it has been suggested that condition-specific cTnT degradation patterns might serve as a proxy for
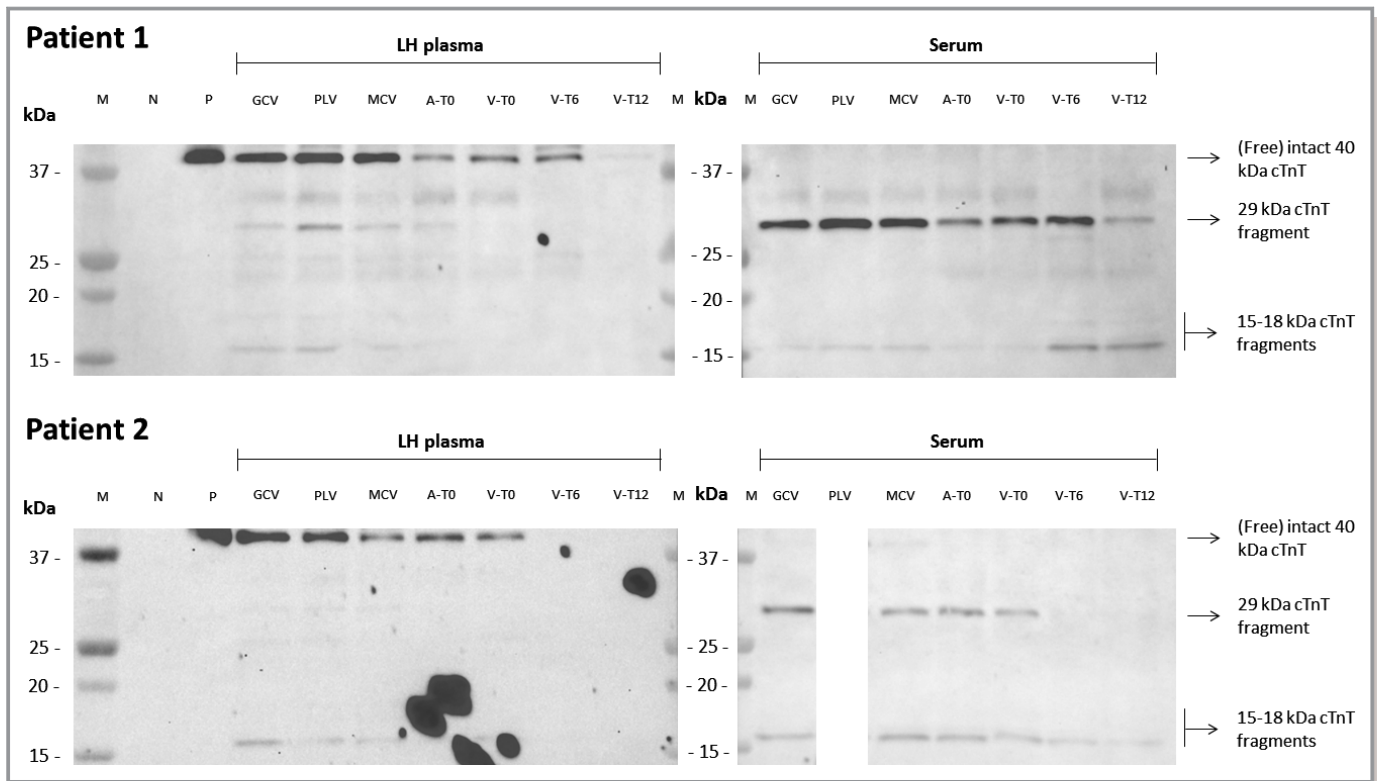
Figure 5. Western blot analysis of lithium-heparinized (LH) plasma and serum samples of patient 1 and patient 2 at the different coronary venous system and peripheral sampling sites. A-T0 indicates peripheral artery sample at baseline; cTnT, cardiac troponin T; GCV, great cardiac vein sample; M, protein standard marker; MCV, coronary sinus sample at the ostium of the middle cardiac vein; N, negative control; P, purified intact cTnT positive control; PLV, coronary sinus sample near the proximal posterolateral vein; V-T0, peripheral vein sample at baseline; V-T12, peripheral vein sample 12 hours postprocedure; V-T6, peripheral vein sample 6 hours postprocedure.

a more specific hs-cTnT immunoassay.[12–14] In patients with end-stage renal disease and in asymptomatic recreational runners, who finished a marathon, only the smallest 15 to 18 kDa cTnT fragments were observed.[10,11] In contrast, in our NSTEMI patients we observed release of larger cTnT forms (cTn T-I-C complex, free intact 40 kDa cTnT, and 29 kDa cTnT fragments). In addition, we showed that these forms were likely to be generated inside the heart. Therefore, they could be the result of intracellular disease-specific cTnT cleavage instead of normal in vivo protein breakdown, or pre-analytical proteolysis. Consequently, if the observed forms reflect a specific state of disease, a hs-cTnT immunoassay only targeting cTnT forms ≥29 kDa may discriminate cTnT release in the setting of an MI from cTnT release in other (patho)-physiologies. Noteworthy, given the observed pre-analytically induced cTn T-I-C complex disintegration and cTnT degradation in serum, LH plasma should be the preferred blood matrix for such a next-generation hs-cTnT immunoassay. Additional blood matrices are approved by the manufacturer (eg, EDTA plasma), but their potential pre-analytical effects on the cTnT composition remain to be elucidated.

Another interesting observation was the disappearance over time of cTn T-I-C complex in our NSTEMI patients. In the late presenting patient, a markedly lower proportion of cTn T-I-C complex was observed as compared with the early presenting patient. The presence of cTn T-I-C complex might therefore hint toward the "age of the infarction." This is of particular interest in patients in whom symptom onset is uncertain. Given the complete cTn T-I-C complex disintegration ≈20 hours after symptom onset, the presence of cTn T-I-C complex seems indicative of an "early" presentation, whereas the absence of cTn T-I-C complex may reflect a "late" presentation. Ultimately, the cTn T-I-C complex proportion could provide guidance in the selection of patients who will benefit the most from an invasive strategy with a percutaneous coronary intervention. Larger numbers of patients are needed to investigate the relation between the cTnT composition and time from infarction to blood draw. Also, it would be interesting to investigate the cTnT composition in case of troponin elevation in patients with conditions other than MI.

Limitations

This work is limited by the fact that only 2 patients were selected for elaborate cTnT composition analyses. Given the
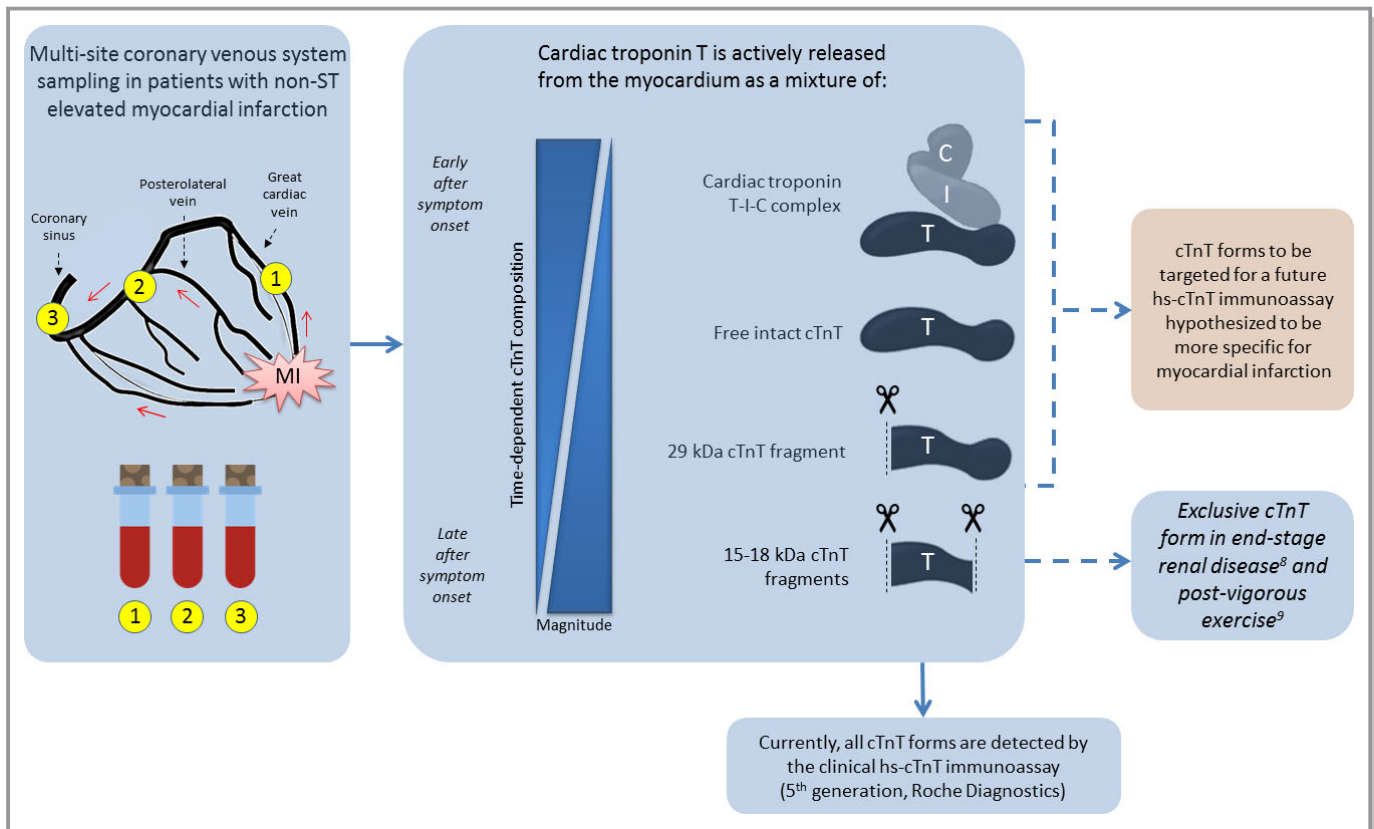
Figure 6. Through multisite sampling in both the coronary venous system (yellow dots 1, 2, and 3) and the peripheral circulation, we unraveled that cardiac troponin T (cTnT) is released from the myocardium as a mixture of cTn T-I-C complex, free intact cTnT, and multiple cTnT fragments in patients with non–ST-segment–elevation myocardial infarction (MI, star) supporting intracellular degradation. In addition, we observed in vivo degradation in a time-dependent manner. Based on descriptions of cTnT composition in conditions other than myocardial infarction (eg, end-stage renal disease, vigorous exercise) current findings may pave the way towards the development of a new generation hs (high-sensitivity)-cTnT immunoassay with improved specificity for myocardial infarction.

conceptual nature of our study, the analyses should be considered as a proof of concept to describe cTnT forms close to their origin of release, and as such fuel the search for condition-specific cTnT degradation patterns. Studying the cTnT composition with our GFC laboratory setup is a highly specialized and labor-intensive analysis requiring 1200 hs-cTnT concentration measurements per patient. The present GFC setup is currently the most sensitive technology to study the cTnT composition. Therefore, in our opinion, we provide one of the most detailed descriptions of cTnT composition in NSTEMI patients to date, including observations from samples taken near its site of release. Despite the age of the blood samples (patients consented in 2006), we previously evaluated that long-time storage at −80°C does not impact the cTnT composition. Additionally, fresh sample aliquots were used to prevent freeze–thaw cycles impacting the hs-cTnT concentration or cTnT composition. Unfortunately, there was too little posterolateral vein serum material available from patient 2 for GFC and WB analysis. In addition, our study did not include patients who presented very early after symptom onset (<3 hours). In theory, in such a population the cTnT

composition might have been different with cTn T-I-C complex and intact cTnT as prevailing forms. Finally, hs-cTnT concentrations were not sufficient to meet the analytical sensitivity requirements for mass-spectrometric evaluation. However, previous work from our group showed that the observed cTnT fragments on GFC analyses were cTnT-derived degradation products.[8]

Conclusion

In conclusion, in this explorative multisite coronary and peripheral sampling study we demonstrated that the cTnT composition is dependent on the interval that passed since the onset of symptoms. This indicates that cTnT is subject to in vivo degradation. More importantly, we demonstrated that cTnT is released from the injured myocardium in multiple forms (cTn T-I-C complex, free intact 40 kDa cTnT, 29 kDa, and 15 to 18 kDa cTnT fragments), which is the first in vivo proof of cTnT degradation within the cardiomyocyte. These new insights ultimately may provide guidance in the development of a next-generation hs-cTnT assay with improved specificity for the acute phase of MI (Figure 6).

Sources of Funding

Disclosures

None.

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SUPPLEMENTAL MATERIAL

Data S1.

TRAMICI IN- AND EXCLUSION CRITERIA

Inclusion criteria:

Patients (≥ 18 years) from the Radboud University Medical Center (Radboud UMC) and three referring centers were eligible if they presented early after symptom onset, were diagnosed with a NSTEMI and had evidence of elevated (i.e. above the 99th percentile upper reference limit from a healthy population) and rising cardiac troponin levels based on conventional troponin assays used in the respective hospitals. Subsequently, patients were referred for a clinically indicated coronary angiography (CAG) in the Radboud UMC. Upon identification of the culprit coronary artery by the interventional cardiologist, patients were included and study procedures were started. After obtaining oral informed consent prior to the procedure, participants provided written informed consent.

Exclusion criteria:

1) Prior anginal complaints or myocardial infarction in the 3 weeks preceding the qualifying episode of chest discomfort in the case of a positive admission troponin concentration;

2) Indication for treatment with fibrinolysis or urgent PCI at index presentation;

3) Prior CABG, PCI within last 3 months;

4) Evidence of other acute or severe cardiac disease (Killip class III or IV) requiring intubation or an intra-aortic balloon pump;

5) Other suspected, potentially life-threatening, non-cardiac disease including pulmonary embolism or aortic dissection;

6) Occurrence of recurrent ischemia at rest after the positive symptom-limited bicycle test and before the planned coronary angiography;

7)   Presence of a pacemaker;

8)   Presence of catheters for hemodynamic monitoring;

9)   Any situation that precludes using the brachial vein as a puncture site, i.e. after surgery of the brachial or axillary region;

10)   Severe peripheral arterial disease (Fontaine III and IV);

11)   Severe main stem stenosis (> 50%);

12)   Known congenital heart disease or anomalous coronary anatomy;

13)   Known renal insufficiency (serum creatinine > 150 μmol/L);

14)   Pregnancy;

15)   Systemic infection, hematologic disorder or treatment with an immune suppressive agent; treatment with NSAID; treatment with antibiotics.

16)   Any other condition which, in the opinion of the investigator, may pose a significant hazard to the subject if he or she is enrolled in the study.

17)   Presence of collateral coronary artery filling at angiography was considered a relative exclusion criterion.

BLOOD SAMPLING PROCEDURES

After identification of a culprit lesion by the interventional cardiologist and local team of investigators, the coronary venous anatomy was recorded during coronary angiography by filming the complete washout of contrast dye. Access to the coronary sinus (CS) was gained by means of a right-sided catheterization procedure. For cannulation and blood sampling of the coronary venous system (CVS) a Terumo wire (Terumo Europe NV, Leuven, Belgium) and CHAMP multipurpose catheter (Medtronic, Santa Rosa, CA, USA) were used, respectively.

Coronary venous blood samples: after cannulation of the CS, the CHAMP catheter was advanced into the great cardiac vein (GCV). This vein primarily drains blood from the anteroseptal walls. Consequently, a selective blood sample from this site allows measurement of biomarker concentrations in blood that just passed injured (culprit lesion in the left anterior descending (LAD) artery) myocardium. Before blood sampling, the position of the catheter was confirmed using contrast dye, and then flushed. After sampling, the catheter was pulled back towards the posterolateral vein (PLV), were blood from the posterolateral wall enters the CS. Again, position was confirmed with contrast dye, and the catheter flushed before sampling. Finally, the catheter was pulled back in the CS at the point where the middle cardiac vein merges with the CS. This location can be seen as the gathering point of all the blood that ran through the myocardium. Again, position of the catheter was confirmed with contrast dye, and the catheter flushed before blood sampling. For all blood sample collections, a 30 second waiting period was incorporated between flushing and obtaining the sample to give ample time for the washout of the flushing fluid. In addition, because some flushing fluid could remain in the sampling catheter, we discarded the first blood sample syringe.

Systemic blood samples: after removal of the catheter from the CVS, systemic blood samples were obtained from the femoral venous and arterial sheaths.

Follow-up blood samples: at 6 and 12 hours post-procedure, additional peripheral venous samples were obtained from the antecubital vein.

LABORATORY TECHNIQUES

Biochemical analyses: cTnT concentrations were measured by utilizing the high-sensitivity (hs-)cTnT immunoassay (Roche Diagnostics, Basel, Switzerland) with a limit of blank (LoB) at 3 ng/L, limit of detection (LoD) at 5 ng/L, 10% coefficient of variation (CV) cutoff at 13 ng/L and a determined 99th percentile from a healthy reference population at 14 ng/L. Creatinine concentrations (Roche Diagnostics) were determined with a LoD at 5 µmol/L and a 10% CV cutoff at 80 µmol/L.

Gel filtration chromatography (GFC): Separation of the different cTnT forms was performed by GFC on the AKTA Prime Plus (GE Healthcare, Chicago, Illinois, United States) equipped with the HiPrep™ 16/60 Sephacryl™ S-300 HR column (#17-1167-01, GE Healthcare). The column was equilibrated with GFC running buffer consisted of 0.26 mol/L NaCl, 2.5 mmol/L CaCl$_2$·2 H$_2$O, 0.02 mol/L Tris, 6 mmol/L NaN$_3$ and 1 g/L BSA buffer, pH 7.4 and operated at a flow of 0.5 mL/min. Two columns of identical lot numbers were used for the fractionation of all samples. The void volume (V$_0$) of each column was assessed using blue dextran and determined to be 42 mL. To investigate circulating cTnT forms in the two selected patients 0.25 mL LH plasma or serum was loaded on the GFC column. For each sample loaded, 83 fractions of 1 mL were collected and kept on ice until hs-cTnT concentration determination (<1 h), and subsequently stored at -80 °C.

From previous experience, utilizing the identical system and GFC column, retention volumes (V$_R$; corrected for V$_0$) for the cTn T-I-C complex, free intact 40 kDa cTnT and its 29 kDa fragment, and 15-18 kDa cTnT fragments were expected at approximately 21, 30 and 45 mL, respectively. Separation of free intact 40 kDa cTnT and its 29 kDa fragment was not achieved with GFC (both ± 30 mL), but obtained by Western blotting.

cTnT purification and Western blotting: Purification of cTnT in all patient samples was achieved by immunoprecipitation. Ten µg biotinylated M11.7 monoclonal antibody (as kindly provided by Roche Diagnostics) was cross-linked to 1 mL M-270 Streptavidin DynaBeads (#65306, Thermo Fisher

Scientific, Waltham, Massachusetts, United States). Subsequently, 50 µL beads were incubated with 200 µL LH plasma or serum for 1 hour at 4 °C. After washing the beads 4 times in phosphate buffered saline with 0.1 % (v/v) Tween-20 (#P1379, Merck, Darmstadt, Germany), captured cTnT was eluted in 25 µL citric acid at pH = 2.5 for 15 min at 56 °C. After adding 6 µL XT Sample Buffer 4x (#1610791, Bio-Rad, Hercules, California, United States) to 18 µL eluate, samples were loaded on 12 % criterion XT (#345-0118, Bio-Rad) precast gels, together with the Precision Plus Protein™ molecular weight marker (#1610373, Bio-Rad). After stacking the samples for 15 min at 100 V, cTnT proteoforms were separated for 45 min at 200 V. The gels were subsequently blotted on 0.45 µm nitrocellulose membranes (#1620115, Bio-Rad) for 1 hour at 100 V. Antibody application to the nitrocellulose membrane was performed using the SNAP i.d.® 2.0 Protein Detection System (Merck). The primary detector antibody used was the anti-cTnT M7 monoclonal antibody (as kindly provided by Roche Diagnostics) and horseradish peroxidase-labeled goat anti-mouse polyclonal antibody served as the secondary detector antibody. Finally, blots were incubated with in SuperSignal West Femto Maximum Sensitivity Substrate (#34096, Thermo Scientific) and bands were detected using the ChemiDoc XRS system (Bio-Rad) and Quantity One software (Bio-Rad, version 4.6.6.).

Figure S1. CORONARY VENOUS SYSTEM BLOOD SAMPLE COLLECTION.
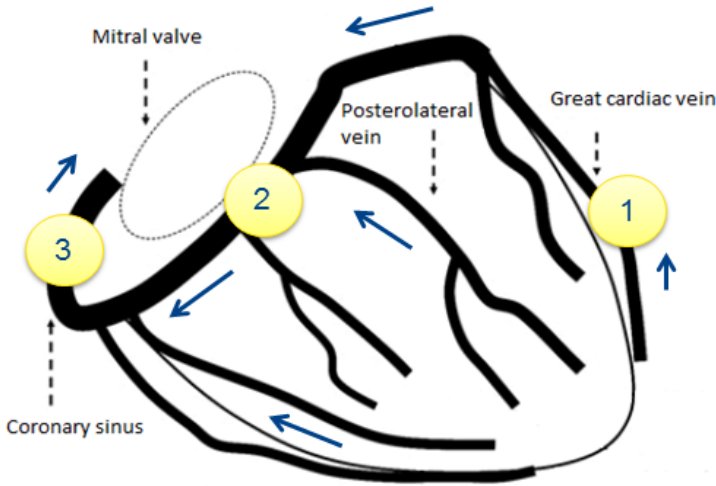
Schematic view of the blood samples collected from the coronary venous system. 1 = great cardiac vein sample; 2 = coronary sinus sample at the ostium of the most proximal posterolateral vein; 3 = coronary sinus sample at the ostium of the middle cardiac vein; arrows = blood flow. Adapted from Khan et al[1] with permission. Copyright ©2009, Oxford University Press.
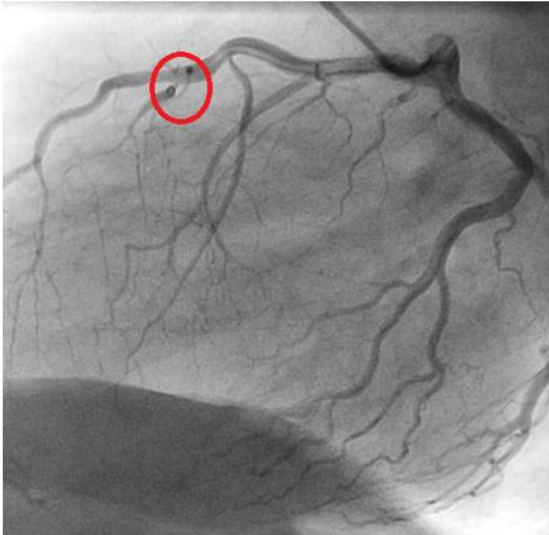
Figure S2. GFC ELUTION PROFILE.
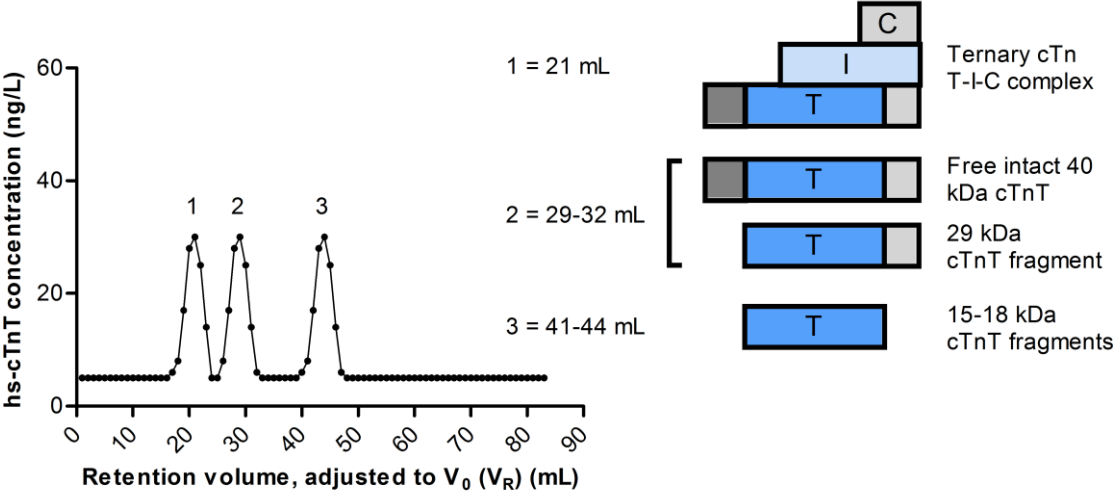
Schematic view of the different cTnT forms and their expected location on the GFC elution profile.

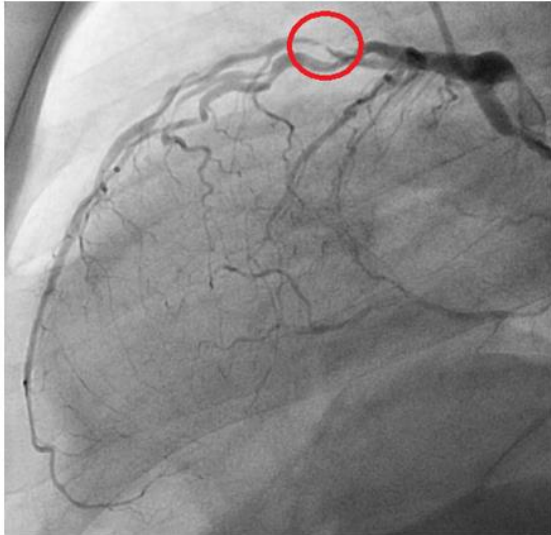The following forms of cTnT are reported in this manuscript:

- *Peak at ± 21 mL*: ternary cardiac troponin T-I-C complex

- *Peak at ± 29-32 mL*: free intact 40 kDa cTnT and/or 29 kDa cTnT fragments

- *Peak at ± 41-44 mL*: 15-18 kDa cTnT fragments
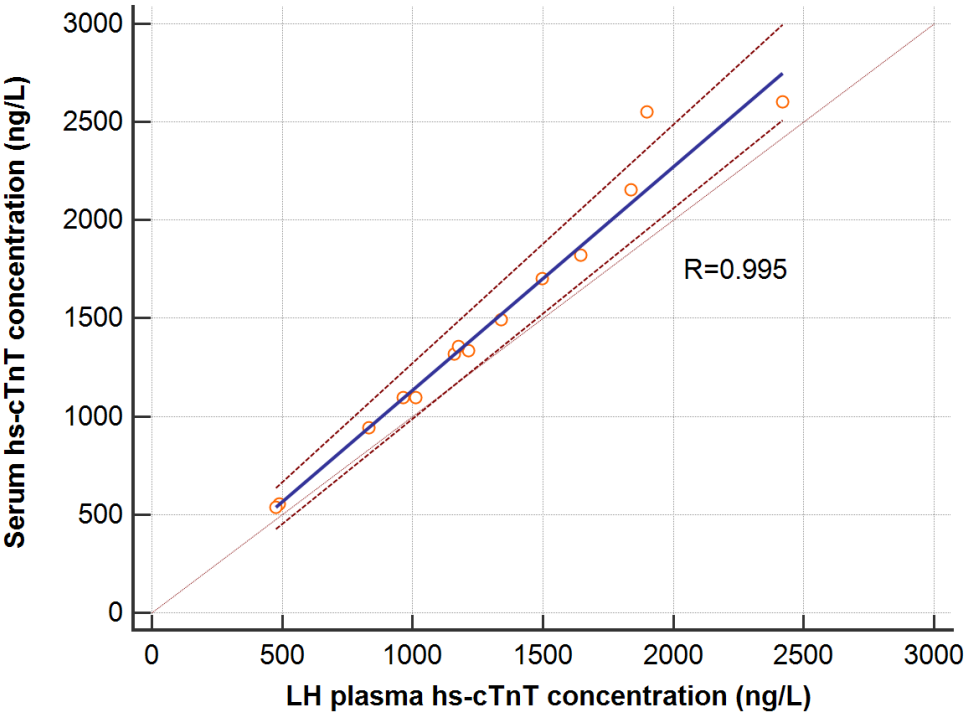
Figure S3. ANGIOGRAMS PATIENT 1 & 2.

Diagnostic coronary angiogram of patient 1 and patient 2. Red circle = location of the stenosis.

**Figure S4. HS-CTNT CONCENTRATION REGRESSION ANALYSIS BETWEEN LITHIUM-HEPARINIZED PLASMA AND SERUM.**

Passing-Bablok regression analysis comparing lithium-heparinized (LH) plasma and serum hs-cTnT concentrations (ng/L) measured in duplicate in all coronary venous system and peripheral samples of the two patients selected for cTnT composition analysis (n=14).

Figure S5. GFC ELUTION PROFILE PATIENT 1.
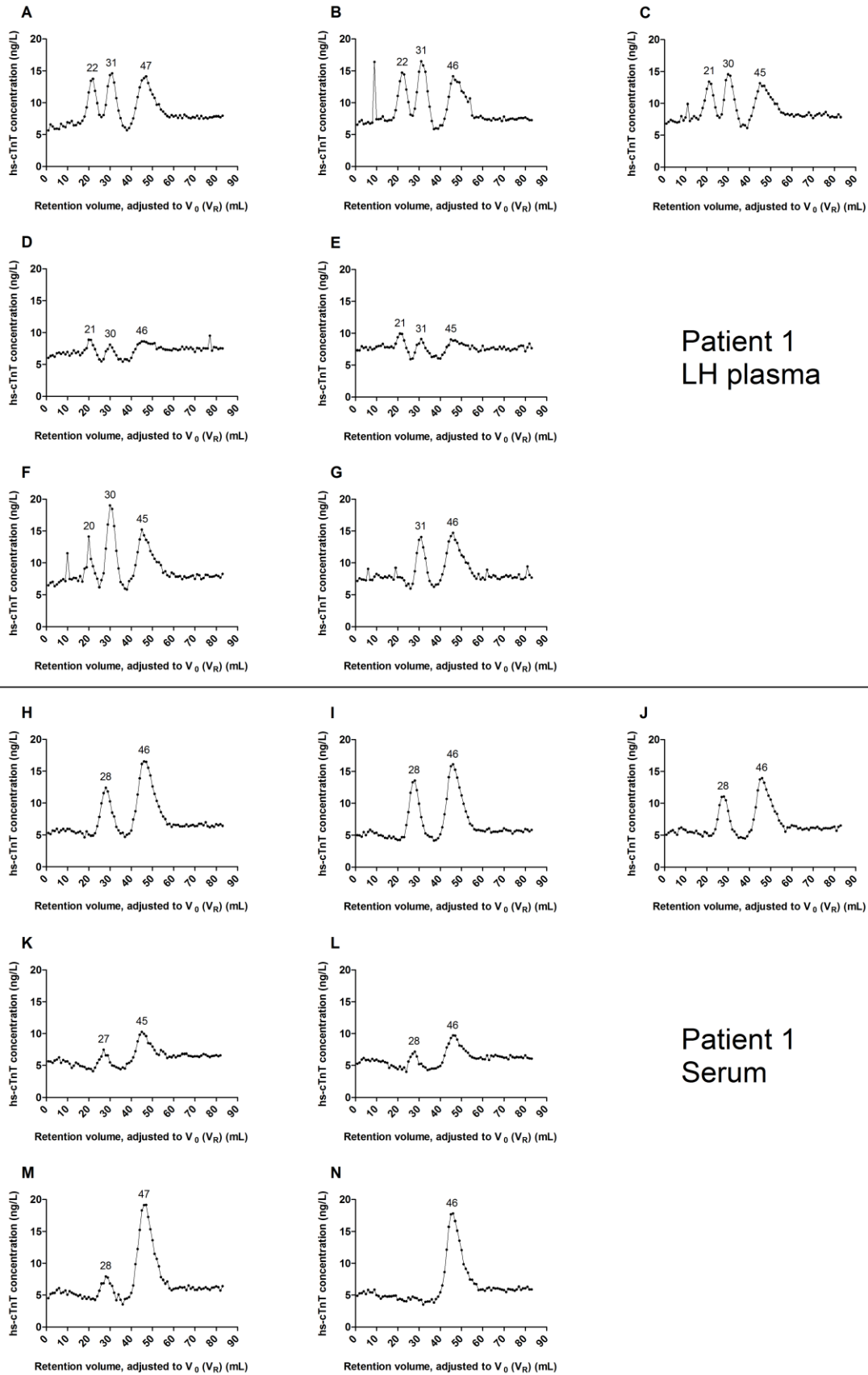
Patient 1
LH plasma

Patient 1
Serum

Gel filtration chromatography elution profiles of all lithium-heparinized (LH) plasma (A-G) and serum (H-N) samples from patient 1. A = great cardiac vein LH plasma sample; B = coronary sinus LH plasma sample near the proximal posterolateral vein; C = coronary sinus LH plasma sample at the ostium of the middle cardiac vein; D = peripheral artery LH plasma sample at baseline; E = peripheral vein LH plasma sample at baseline; F = peripheral vein LH plasma sample 6 hours post-procedure; G = peripheral vein LH plasma sample 12 hours post-procedure; H = great cardiac vein serum sample; I = coronary sinus serum sample near the proximal posterolateral vein; J = coronary sinus serum sample at the ostium of the middle cardiac vein; K = peripheral artery serum sample at baseline; L = peripheral vein serum sample at baseline; M = peripheral vein serum sample 6 hours post-procedure; N = peripheral vein serum sample 12 hours post-procedure.

Figure S6. GFC ELUTION PROFILE PATIENT 2.
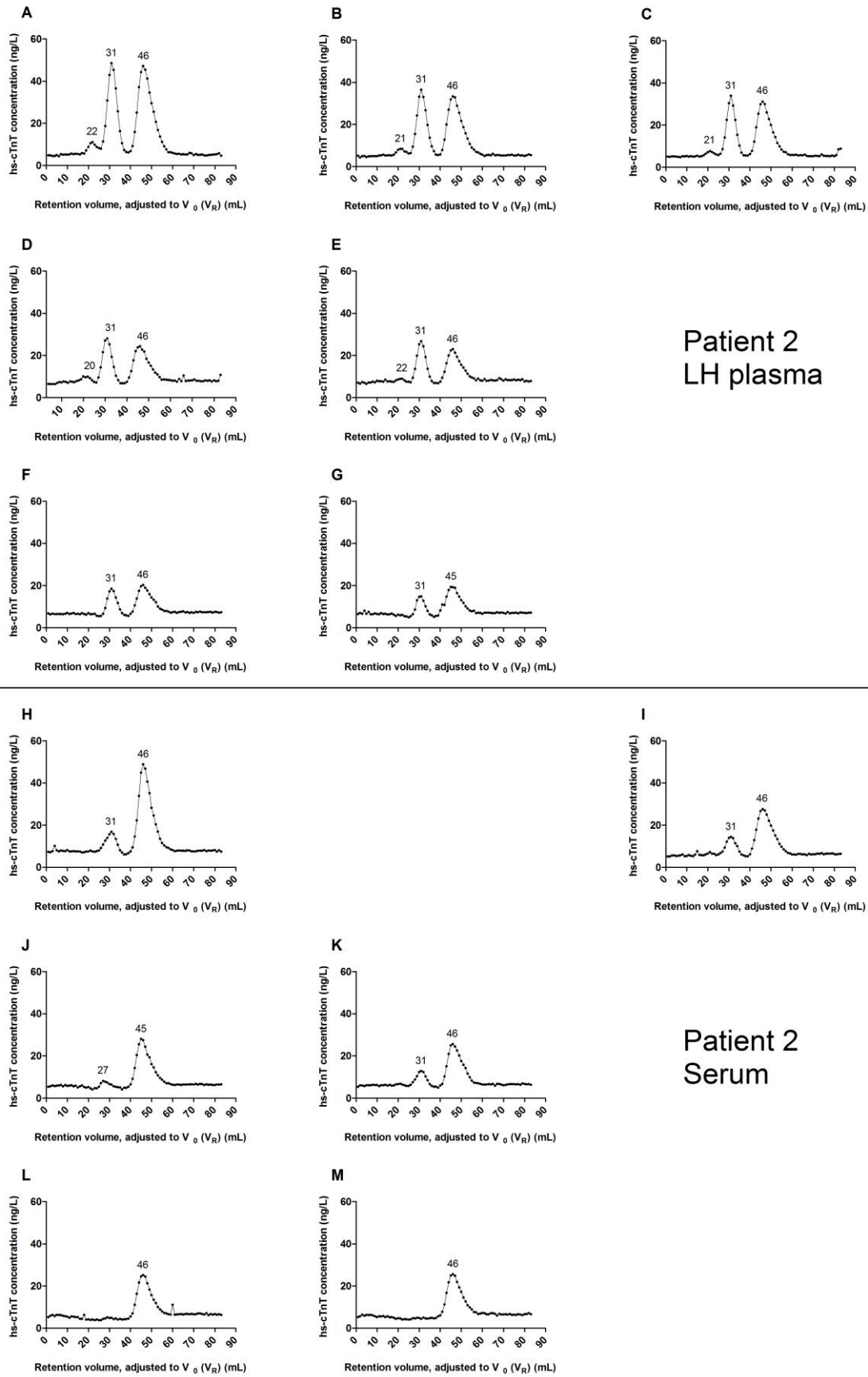
Patient 2
LH plasma

Patient 2
Serum

Gel filtration chromatography elution profiles of all lithium-heparinized (LH) plasma (A-G) and serum (H-M) samples from patient 2. A = great cardiac vein LH plasma sample; B = coronary sinus LH plasma sample near the proximal posterolateral vein; C = coronary sinus LH plasma sample at the ostium of the middle cardiac vein; D = peripheral artery LH plasma sample at baseline; E = peripheral vein LH plasma sample at baseline; F = peripheral vein LH plasma sample 6 hours post-procedure; G = peripheral vein LH plasma sample 12 hours post-procedure; H = great cardiac vein serum sample; I = coronary sinus serum sample at the ostium of the middle cardiac vein; J = peripheral artery serum sample at baseline; K = peripheral vein serum sample at baseline; L = peripheral vein serum sample 6 hours post-procedure; M = peripheral vein serum sample 12 hours post-procedure.

Supplemental Reference:

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