

Cardiac Troponin T

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Cardiac Troponin T: Smaller Molecules in Patients with End-Stage Renal Disease than after Onset of Acute Myocardial Infarction

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BACKGROUND: We have found previously that in acute myocardial infarction (AMI), cardiac troponin T (cTnT) is degraded in a time-dependent pattern. We investigated whether cTnT forms differed in patients with chronic cTnT increases, as seen with renal dysfunction, from those in the acute phase of myocardial infarction.

METHODS: We separated cTnT forms by gel filtration chromatography (GFC) in end-stage renal disease (ESRD) patients: prehemodialysis (pre-HD) and post-HD (n = 10) and 2 months follow-up (n = 6). Purified (cTnT) standards, quality control materials of the clinical cTnT immunoassay (Roche), and AMI patients' sera also were analyzed. Immunoprecipitation and Western blotting were performed with the original cTnT antibodies from the clinical assay and antibodies against the N- and C-terminal end of cTnT.

RESULTS: GFC analysis revealed the retention of purified cTnT at 27.5 mL, identical to that for cTnT in quality controls. For all ESRD patients, one cTnT peak was found at 45 mL, pre- and post-HD, and stable over time. Western blotting illustrated that this peak corresponded to cTnT fragments <18 kDa missing the N- and C-terminal ends. AMI patients' sera revealed cTnT peaks at 27.5 and 45 mL, respectively, corresponding to N-terminal truncated cTnT of 29 kDa and N- and C-terminal truncated fragments of <18 kDa, respectively.

CONCLUSIONS: We found that cTnT forms in ESRD patients are small (<18 kDa) and different from forms seen in AMI patients. These insights may prove useful for development of a more specific cTnT immunoassay, es-

pecially for the acute and diagnostic phase of myocardial infarction.

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Release of cardiac troponins (cTnT and cTnI)⁵ in the patient's circulation is considered to be a hallmark for myocardial damage owing to the unique cardiospecificity of these biomarkers. The diagnosis of an acute myocardial infarction (AMI), therefore, relies on a rise or fall in cardiac troponin concentrations, preferably performed with high-sensitivity cardiac troponin assays (1, 2). Previously, we have demonstrated in serial serum samples from AMI patients that cTnT is degraded in a time-dependent pattern (3). Using gel filtration chromatography (GFC) and Western blotting, we illustrated that during the first hours after presentation at the emergency department, intact cTnT and predominantly cTnT fragments of 29 kDa (primary fragment) were present, while later (>24 h after presentation), only fragments of <18 kDa (secondary fragments) were detected (3). The specific amino acid sequence of these cTnT fragments has been determined very recently using mass spectrometry (4).

However, chronically increased cTnT concentrations are also common in patients suffering from renal dysfunction (5–7). The presence of such increases has been associated with abnormal left ventricular morphology and function (8), myocardial stunning (9), and a higher risk of future cardiovascular events and mortality (10, 11).

Significant associations between cTnT and renal clearance have indeed been demonstrated in chronic kid-

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⁵ Nonstandard abbreviations: cTnT, cardiac troponin T; cTnI, cardiac troponin I; AMI, acute myocardial infarction; GFC, gel filtration chromatography; ESRD, end-stage renal disease; HD, hemodialysis; CVD, cardiovascular diseases; hs, high-sensitivity; NT-proBNP, N-terminal fragment of the prohormone B-type natriuretic peptide; IQR, interquartile range; AUC, area under the curve.

ney disease patients apart from cardiac explanations (12). It has been shown that chronically increased cTn concentrations reflect the accumulation of cTnT fragments, as we previously illustrated for end-stage renal disease (ESRD) patients using Western blotting (13). However, since we employed different antibodies than those of the clinical cTnT immunoassay, these findings have been challenged by others who found only intact cTnT when analyzing the serum with GFC (14). To date, it still is not fully known which cTnT forms circulate in the serum of ESRD patients and which are detected by the clinical cTnT assay. Gaining insight into the molecular forms of cTnT following ESRD will further improve our understanding of the nature of acute vs chronic cTnT elevations.

In the current study, we hypothesize that the molecular cTnT forms in patients with chronically increased concentrations are different from those seen in patients in the acute phase of myocardial infarction. We therefore investigated cTnT forms that are present in the circulation of patients on hemodialysis (HD) using a similar approach to the one that we previously performed in AMI patients (3).

Materials and Methods

STUDY POPULATIONS

Molecular forms of circulating cTnT were assessed in serum samples of 2 ESRD populations, both on chronic HD therapy (conventional HD). Out of an existing cohort of 47 ESRD patients (15), 10 patients were selected based on availability of two serum samples of approximately 2 mL: immediately before the onset of HD (pre-HD) and immediately after the HD session (post-HD). Samples were obtained via vascular access (arteriovenous fistula or central venous catheter). From another longitudinal ESRD cohort (5), predialysis serum samples from 6 patients were analyzed at baseline and also 2 months later. History of cardiovascular diseases (CVD) was considered for all patients. Finally, for comparison with our previous results in AMI patients (3), serum samples of 10 AMI patients from our previously described cohort were also analyzed see Table 1 in the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol63/issue3>. On collection, all samples were immediately stored at -80°C until analysis by GFC and/or Western blotting. This study was conducted according to the principles of the Declaration of Helsinki and approved by the Ethical Committee (METC 12-4-062 and METC 06-2-035) and the Hospital Board of the Maastricht University Medical Center. Written informed consent was obtained from the patients before participation.

BIOCHEMICAL TESTING

cTnT concentrations were determined using the high-sensitivity (hs)-cTnT assay (Roche Diagnostics) with a limit of detection at 5 ng/L and 10% CV cutoff at 13 ng/L. Moreover, duplicate hs-cTnT measurements in GFC eluates ranging between 10–20 ng/L yielded a mean (SD) CV of 2% (1%). N-terminal fragment of the prohormone B-type natriuretic peptide (NT-proBNP) was measured (proBNP II STAT, Roche Diagnostics) with a limit of detection at 5.08 ng/L (0.6 pmol/L) and 20% CV cutoff at 50 ng/L (5.9 pmol/L). Albumin was analyzed using the microalbumin assay with a measuring range of 2.0–970 mg/L. All assay characteristics were as given by the manufacturer, and all the measurements were performed on the COBAS 6000 or 8000 analyzer (Roche Diagnostics).

STANDARDS AND QC MATERIALS

The Gel Filtration Calibration kit (GE Healthcare) contained blue dextran (MW = 2000 kDa), conalbumin (MW = 75.0 kDa), ovalbumin (MW = 43.0 kDa), carbonic anhydrase (MW = 29.0 kDa) and ribonuclease (MW = 13.7 kDa), which were dissolved in GFC running buffer as described below.

Purified human cTn T-I-C complex (MW 77 kDa, Hytest) and purified intact, free cTnT (MW 37 kDa, Hytest) were dissolved as prescribed by the manufacturer and were further diluted in a serum pool of healthy volunteers (cTnT <14 ng/L).

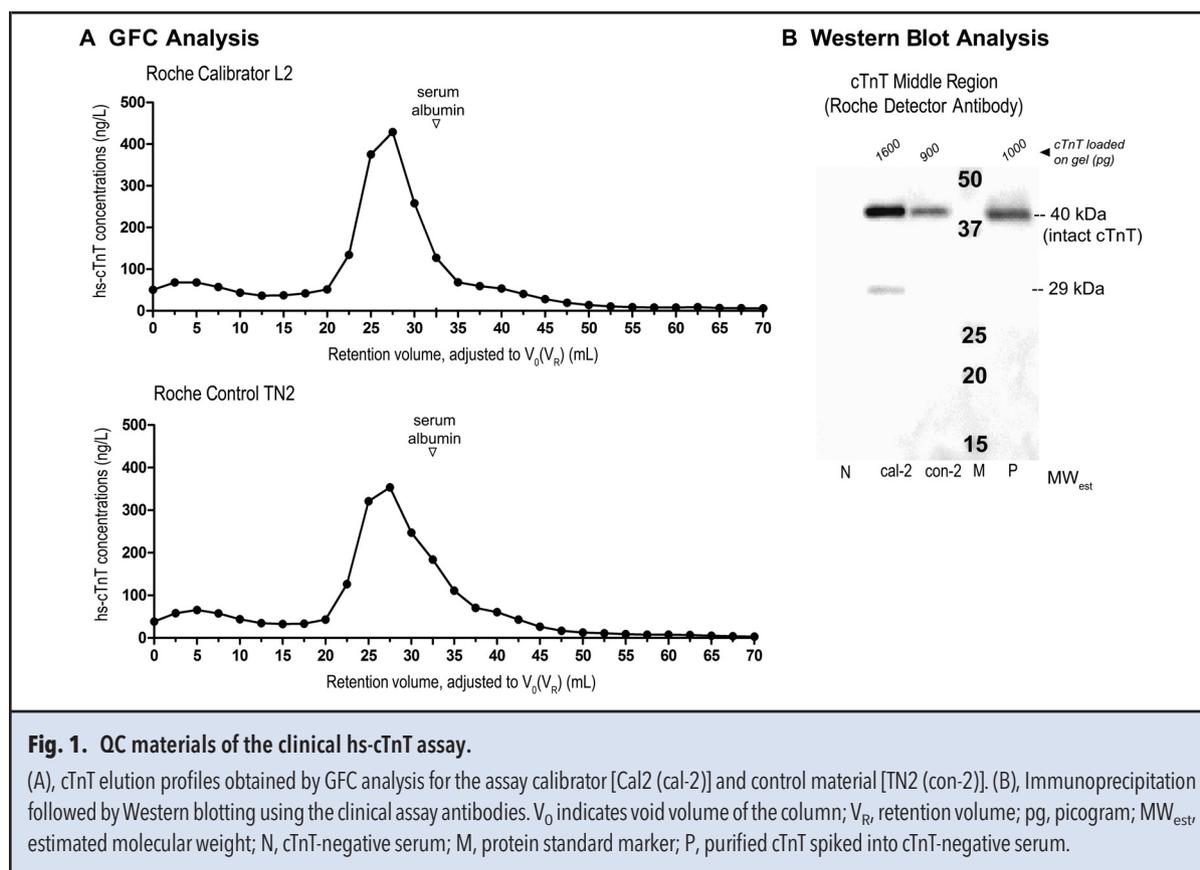
Calibration (Cal2) and control (TN2) materials of the clinical hs-cTnT immunoassay were dissolved as prescribed by the manufacturer.

FRACTIONATION BY GFC

GFC was performed on the AKTA Prime Plus (GE Healthcare) equipped with a 1.6×60 cm Sephacryl-S300 column (GE Healthcare). The equipment and column were different from our previous setup using a Sephacryl-S100 column (3), since we optimized the separation of the larger cTnT forms. The column was again equilibrated with 0.26 mol/L NaCl, 2.5 mmol/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.02 mol/L Tris, 6 mmol/L NaN_3 , and 1 g/L BSA buffer, pH 7.4 (3, 16) and operated at 0.5 mL/min.

The void volume (V_0) determination and calibration were performed as previously (3) using blue dextran (1 g/L) and globular protein standards (3–4 g/L) in running buffer; 0.5 mL was loaded on the column; everything was performed in duplicate. V_0 was determined to be 40 mL. The standard calibration curve $K_{av} = V_R/(V_c - V_0)$ was plotted against the logarithm of the MW of globular proteins following the equation $y = -0.34x + 1.04$. V_R signifies the retention volume adjusted to V_0 , while V_c indicates column volume and corresponds to 120 mL.

For serum samples, 1.0–2.0 mL was loaded on the GFC column. Serum albumin (67 kDa) and serum NT-



proBNP were used as internal retention markers in the patients' sera. For each sample loaded, fractions of 1.25 mL were collected, kept on ice until immunoassay measurements (<1 h), and subsequently stored at -80°C .

PURIFICATION AND WESTERN BLOT DETECTION OF cTnT

Purification and characterization of cTnT was performed as described previously (3) using the antibodies from the Roche cTnT assay [as kindly provided by Roche Diagnostics (www.roche.com)]. In short, the capture cTnT antibody (M11.7, biotinylated, epitope amino acid residues (a.a.r.) 136–147, according to package insert) was used for immunoprecipitation using streptavidin-coated Dynabeads (Invitrogen), serum was precipitated for 1 h at 4°C and eluted with 1 mol/L glycine (18 μL), pH 3 for 15 min at 56°C .

As negative controls for immunoblotting, either running buffer or a cTnT-negative serum pool of healthy volunteers was used. As positive controls, purified cTnT was spiked into running buffer and cTnT-negative pooled serum, respectively. Only GFC fractions containing sufficiently high cTnT concentrations (>500 ng/L) could be visualized on the immunoblot.

Immunoprecipitates were diluted with XT Sample Buffer (6 μL) (Bio-Rad), reduced at 56°C , separated on

a 12% Criterion XT SDS-PAGE gel (Bio-Rad), together with the Precision Plus Protein Standard (Bio-Rad), transferred to nitrocellulose membrane (Bio-Rad, 0.45 μm) and treated with the vacuum SNAP i.d. protein detection system (Millipore). Primary antibodies were Roche M7 anti-cTnT (a.a.r. 125–131, according to package insert), anti-cTnT against the middle region (Hytest, 1C11, epitope a.a.r. 171–190), the N-terminal end of cTnT (Hytest, 9G6, epitope a.a.r. 1–60), and the C-terminal end of cTnT (custom-created rabbit polyclonal antibody produced by Médimabs, epitope a.a.r. 275–286, although the antibody's specificity was not independently confirmed). Secondary antibodies were goat antimouse or goat antirabbit peroxidase (both Dako, 0.4 $\mu\text{g}/\text{mL}$), dependent on the primary antibody. Finally, membranes were incubated in Super Signal West Femto Substrate (Thermo Scientific) and bands were detected using the ChemiDoc XRS scanner (Bio-Rad) and Quantity One Software (Bio-Rad, version 4.6.5.).

STATISTICAL ANALYSIS

Results are presented as mean (SD) or, when not normally distributed, as median [interquartile range (IQR)]. The Wilcoxon signed-rank test was applied to investigate pre- vs post-HD concentrations. For each GFC analysis,

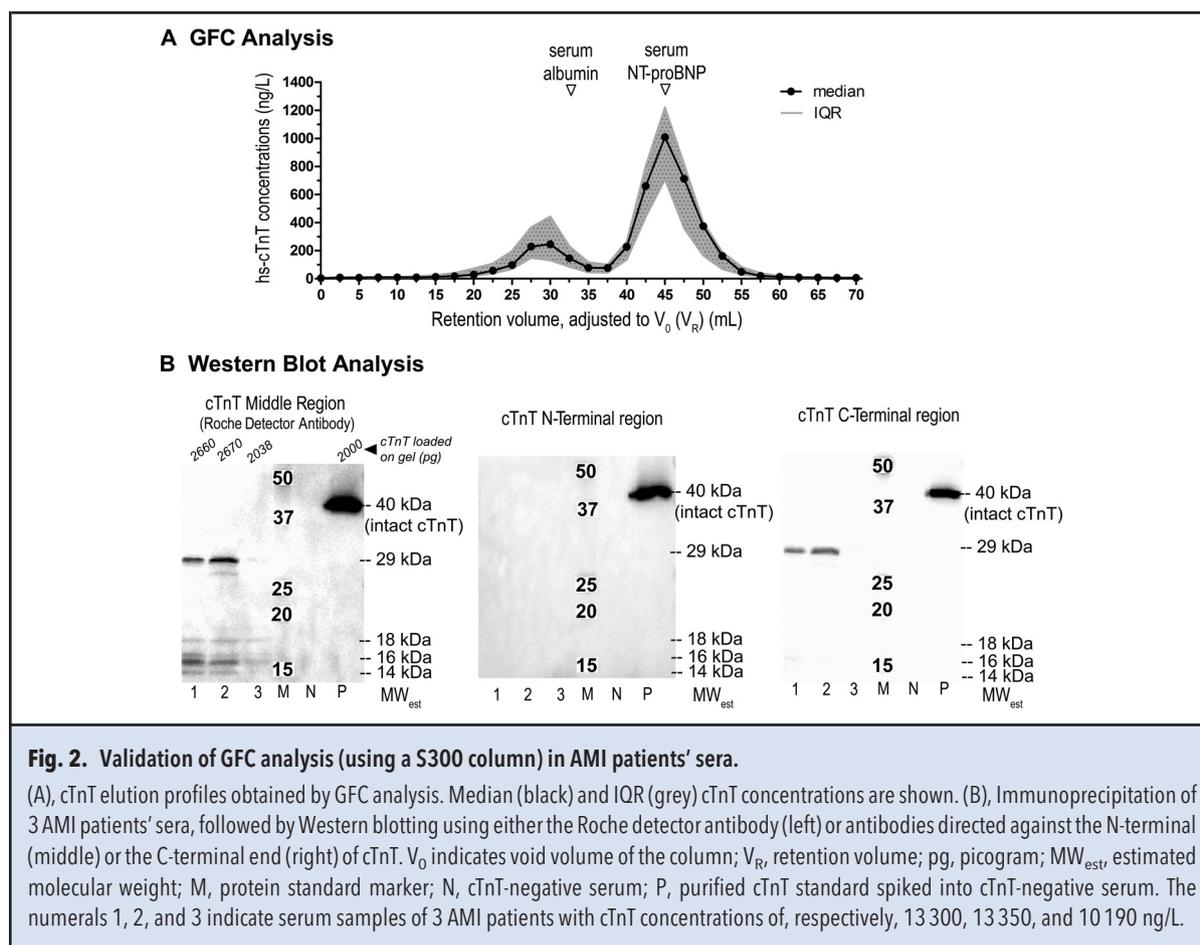


Fig. 2. Validation of GFC analysis (using a S300 column) in AMI patients' sera.

(A), cTnT elution profiles obtained by GFC analysis. Median (black) and IQR (grey) cTnT concentrations are shown. (B), Immunoprecipitation of 3 AMI patients' sera, followed by Western blotting using either the Roche detector antibody (left) or antibodies directed against the N-terminal (middle) or the C-terminal end (right) of cTnT. V_0 indicates void volume of the column; V_R , retention volume; pg, picogram; MW_{est}, estimated molecular weight; M, protein standard marker; N, cTnT-negative serum; P, purified cTnT standard spiked into cTnT-negative serum. The numerals 1, 2, and 3 indicate serum samples of 3 AMI patients with cTnT concentrations of, respectively, 13 300, 13 350, and 10 190 ng/L.

recovery (%) was calculated and was based on the initial amount of cTnT that was loaded on the column (hs-cTnT concentration and the injected volume), the hs-cTnT concentrations that were measured in the eluates, and the eluate volumes. Area under the curve (AUC) was calculated for each GFC elution profile and was based on the hs-cTnT concentrations that were measured in the eluates and the volumes of the eluates. For recoveries and AUC, hs-cTnT concentrations, <5 ng/L were considered zero. All statistical analyses were performed using SPSS (version 23; IBM). A P value <0.05 was regarded as statistically significant.

Results

VALIDATION OF GFC ELUTION PROFILES FOR cTnT STANDARDS

By using the S300 GFC column, elution profiles of purified cTn standards (cTn T-I-C complex and intact cTnT) revealed retention volumes for intact cTnT at $V_R = 20$ and 27.5 mL, respectively (see online Supplemental Fig. 1). Both cTnT standards deviated from the elution profiles of globular protein standards. Moreover,

when spiked into cTnT-negative serum, cTnT standards eluted before the internal retention markers albumin ($V_R = 32.5$ mL) and NT-proBNP ($V_R = 45$ mL). Western blotting of the GFC fractions confirmed that the cTnT peaks consisted of intact cTnT forms (estimated MW, MW_{est} of 40 kDa), as shown in the inserts of online Supplemental Fig. 1.

GFC PEAK ALLOCATION FOR hs-cTnT ASSAY QUALITY CONTROL MATERIALS

GFC elution profiles of the calibrator (level 2; cTnT = 5586 ng/L) and control material (TN2; cTnT = 1921 ng/L) of the clinical hs-cTnT assay both displayed a cTnT peak at 27.5 mL (Fig. 1A). Western blotting employing the original clinical antibodies showed that mainly intact cTnT and only minor 29-kDa cTnT fragments were present in these quality control materials (Fig. 1B).

GFC PEAK ALLOCATION FOR AMI PATIENTS

GFC elution profiles of AMI serum samples revealed 2 cTnT peaks, with the S300 column similar to the results obtained with the S100 column (3). Retention volumes for these peaks were established at 27.5 and 45 mL, re-

Table 1. Baseline characteristics of ESRD patients analyzed using GFC pre- and post-HD.

No	Age, years	Sex	History of CVD	hs-cTnT concentrations, ng/L			GFC peaks: AUC _{Post-HD} /AUC _{Pre-HD}
				Pre-HD	Post-HD	hs-cTnT _{Post-HD} /hs-cTnT _{Pre-HD}	
1	73	Male	No	40.3	31.9	79%	68%
2	57	Male	No	71.5	80.4	113%	106%
3	62	Female	No	50.7	49.9	98%	93%
4	88	Male	Pacemaker	335.5	312.5	93%	107%
5	76	Male	Brady and tachycardia	65.0	71.6	110%	90%
6	79	Male	Myocardial infarction	77.7	73.7	95%	68%
7	81	Female	Aortic valve stenosis	56.8	41.3	73%	63%
8	87	Female	Atrial fibrillation	61.3	51.4	84%	80%
9	80	Male	Atrial fibrillation	47.0	31.6	67%	74%
10	63	Male	Coronary artery stenosis	128.1	108.5	85%	82%
				63.2 (49.8-90.3) ^a	61.5 (38.9-87.4) ^a	89% (78%-101%) ^a	81% (70%-92%) ^a

^a Median (IQR).

spectively (Fig. 2A), eluting before and after the internal retention marker albumin ($V_R = 32.5$ mL), respectively.

Immunoprecipitation followed by Western blotting, either of AMI patients' sera directly (Fig. 2B) or after GFC fractionation (see online Supplemental Fig. 2), assigned the first AMI cTnT peak ($V_R = 27.5$ mL) to mainly a 29-kDa degradation product of cTnT. This 29-kDa fragment demonstrated immunoreactivity using the antibodies directed against the middle region (both the Roche detector antibody and 1C11 antibody) and the C-terminal end of cTnT. The second AMI cTnT peak ($V_R = 45$ mL) was allocated to smaller cTnT degradation products ranging in size between 14 and 18 kDa (Fig. 2B and online Supplemental Fig. 2). None of these smaller fragments were recognized by antibodies directed against the N- and C-terminal ends of cTnT.

PRESENCE AND STABILITY OF N- AND C-TERMINAL TRUNCATED cTnT FRAGMENTS IN ESRD PATIENTS

Baseline characteristics of the two ESRD populations are presented in Tables 1 and 2. In the first ESRD cohort ($n = 10$), serum samples from pre- and post-HD demonstrated median hs-cTnT concentrations of 63.2 and 61.5 ng/L, respectively (Table 1). Following the HD, only a minor and marginal reduction in hs-cTnT was observed ($P = 0.05$).

GFC elution profiles of these serum samples indicated one cTnT peak at $V_R = 45$ mL for all patients, either before or after HD (Fig. 3). The AUC of the GFC profiles demonstrated no significant reduction in cTnT following HD. Additionally, post-/pre-hs-cTnT ratios for serum concentrations were comparable to the ratios that were based on the AUC of the GFC profiles (Table 1).

In the second ESRD population ($n = 6$, Table 2), predialysis samples at baseline and 2 months later yielded median hs-cTnT concentrations of 151.8 and 131.8 ng/L, respectively ($P = 0.917$). GFC profiles at both time points could distinguish one cTnT peak at 45 mL for all patients (Fig. 4A). Subjecting serum samples of these patients directly to immunoblotting using an antibody against the middle region of cTnT illustrated cTnT fragments of <18 kDa (Fig. 4B).

Moreover, in both populations, the same GFC profiles were observed in patients with and without a previous history of CVD.

Discussion

In the present study, we demonstrate that chronic cTnT increases in ESRD patients' sera reflect N- and C-terminal truncated cTnT forms only. These cTnT fragments were observed pre- and post-HD and were found to be stable over time.

By means of GFC analysis, we repeatedly distinguished one cTnT peak at 45 mL in all ESRD patients. This peak was equivalent to the second cTnT peak found in AMI patients, either when characterized with an S100 GFC column (3) or with an S300 GFC column as shown in the present study. By using Western blot analysis with several anti-cTnT antibodies, as well as the unique capture and detector antibodies of the commercial Roche cTnT immunoassay, this peak was allocated to small N- and C-terminal truncated cTnT forms of <18 kDa.

These results indicate that cTnT increases merely reflect completely degraded cTnT fragments in cases of ESRD, as previously suggested (13), and not intact cTnT,

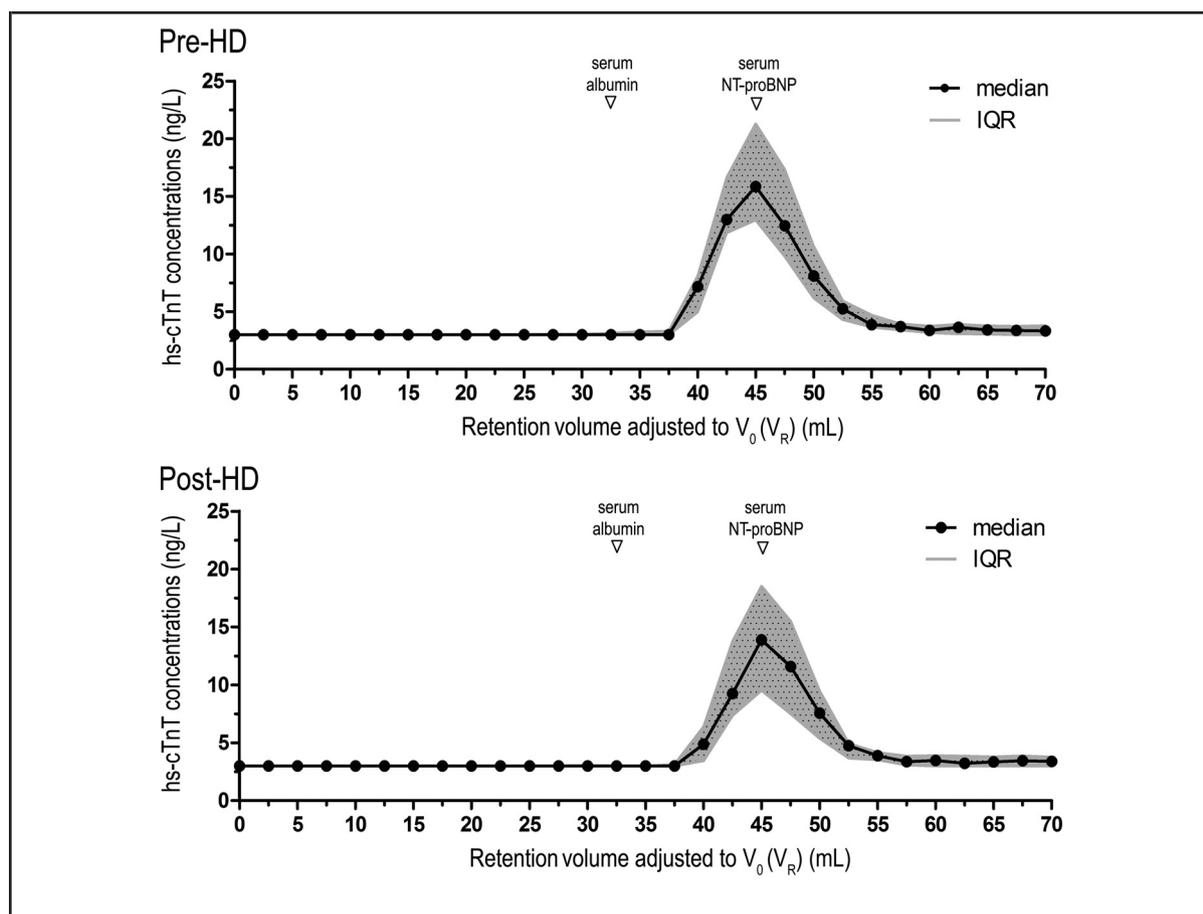


Fig. 3. Circulating cTnT forms in ESRD patients pre- and post-HD.

cTnT elution profiles obtained by GFC analysis. Median (black) and IQR (grey) cTnT concentrations are shown. Serum albumin and NT-proBNP were used as internal retention markers. V_0 indicates void volume of the column; V_R , retention volume.

as assumed by others (14). The truncation of the N-terminal end of cTnT has been described to occur in the intracellular environment of cardiomyocytes (17–20). However, whether observed cTnT fragments presented in this study are formed intracellularly or extracellularly goes beyond the purpose of this study and merits further investigation.

Furthermore, using GFC analysis, we found similar cTnT forms pre-HD and immediately post-HD. A minor to nonsignificant decrease in the cTnT peak was distinguished following HD, in line with another cohort and also in line with HD results obtained for hs-cTnI (21). By using extensive hemodiafiltration, however, significant reductions were found for hs-cTnT and hs-cTnI (21) and confirm that cTnT (fragments) are cleared during specific forms of renal replacement therapy.

Similar cTnT fragments were found in serum samples drawn 2 months apart. Unlike the time-dependent cTnT degradation pattern following AMI (3, 22), cTnT

fragments in ESRD patients' sera seem more stable over time. Moreover, the composition of cTnT forms clearly differs between AMI and ESRD patients, mainly during the time frame of clinical diagnosis of AMI (<12 h after onset of symptoms) (3). Consequently, it would be interesting to assess whether or not future cTnT immunoassays can be developed that can only detect intact cTnT and its 29 kDa fragment through a redesign of the capture and detector antibodies. Such assays might increase the diagnostic performance of troponin assays in predicting acute coronary syndromes in patients with ESRD. In current daily practice, increases in cTn concentrations occurring during the acute phase of AMI are primarily differentiated from chronic increases by the use of serial hs-cTn measurements; however, clinicians are often still challenged (23, 24).

Monitoring the calibration and precision of the assay is necessary to ensure quality hs-cTnT measurements. As shown in the present study, the quality control materials used for the hs-cTnT assay mainly contain intact

Table 2. Baseline characteristics of ESRD patients analyzed using GFC at baseline and after 2 months of follow-up.

Patient no.	Age, years	Sex	History of CVD	hs-cTnT concentrations, ng/L	
				Baseline	2 months
1	67	Male	No	149.3	166.6
2	68	Female	No	97.6	84.3
3	69	Male	No	154.3	110.8
4	59	Male	Myocardial infarction	161.3	130.4
5	70	Male	Myocardial infarction	129.8	133.1
6	75	Male	Myocardial infarction	213.8	274.6
				151.8 (121.7–174.4) ^a	131.8 (104.2–193.6) ^a

^a Median (IQR).

cTnT molecules. Therefore, these quality control materials are clearly not identical to the cTnT forms that are present in ESRD and AMI patients' sera. Despite this, hs-cTnT measurements unquestionably have very important diagnostic and prognostic value in ESRD (10, 11), as well as other populations (25–27). It is un-

known whether changes in immunoreactivity for different cTnT forms carry implications for clinical usage of the assay (24, 28). It should be noted that since only one (hs-)cTnT assay is available on the market, cTnT assay harmonization is not as much of concern as it has been for the several cTnI assays (28).

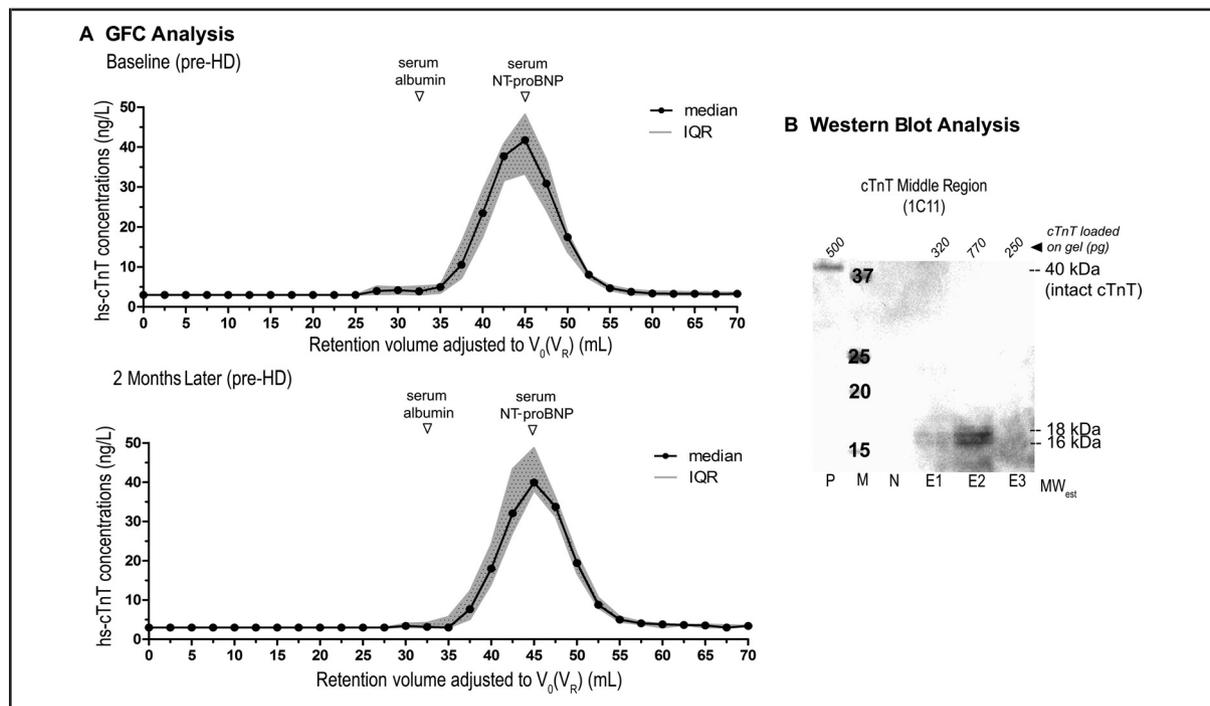


Fig. 4. Circulating cTnT forms in ESRD patients at baseline and after 2 months follow-up. (A), cTnT elution profiles obtained by GFC analysis. Median (black) and IQR (grey) cTnT concentrations are shown. Serum albumin and NT-proBNP were used as internal retention markers. (B), Immunoprecipitation followed by Western blotting using an antibody directed against the middle region of cTnT. V₀ indicates void volume of the column; V_R, retention volume; pg, picograms; MW_{est}, estimated molecular weight; P, purified cTnT spiked into serum; M, protein standard marker; N, cTnT-negative serum. E1, E2, and E3 indicate serum samples of 3 ESRD patients with cTnT concentrations of, respectively, 230, 453, and 200 ng/L.

The present study is limited by the sensitivity of the Western blotting technique, and we cannot exclude the presence of other cTnT bands at lower concentrations. Moreover, only serum samples were available from our ESRD and AMI study populations, so extrapolation to lithium-heparin plasma or EDTA-plasma remains uncertain.

In conclusion, this is the first study to conclusively reveal that only small cTnT fragments (<18 kDa) are present in the serum of ESRD patients. Importantly, these fragments are different from cTnT forms that are recognized by the clinical assay in the acute phase of AMI and in the quality control materials of the clinical cTnT immunoassay. These insights might help in the development of a better cTnT immunoassay that is more specific for detecting the onset of a myocardial infarction.

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