

openheart Cardiac troponin I but not cardiac troponin T adheres to polysulfone dialyser membranes in an in vitro haemodialysis model: explanation for lower serum cTnI concentrations following dialysis

David C Gaze, Paul O Collinson

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ABSTRACT

Background: Elevated serum cardiac troponin T (cTnT) and I (cTnI) can occur in patients with chronic kidney disease. Differences in cTn concentrations between cTnT and cTnI have been reported but the mechanism of such discrepancy has not been investigated. This study investigates the clearance of cTn with the aid of an in vitro model of haemodialysis (HD).

Methods: Serum was obtained before and after a single session of dialysis from 53 patients receiving HD and assayed for cTnT and cTnI. An in vitro model of the dialysis process was used to investigate the mechanism of clearance of cTn during HD.

Results: Serum cTnI was significantly lower ($p=0.043$) following a session of HD whereas cTnT concentrations were similar to those obtained before HD.

Using an in vitro model of dialysis, it was demonstrated that cTnI is not dialysed from the vascular compartment but adheres to the dialyser membrane.

Conclusions: The adherence of cTnI to the dialyser membrane is responsible for the observed decrease in serum cTnI following a session of dialysis. The adherence of cTnT or T-I-C complex to the dialyser membrane could not be demonstrated and supports the observation that pre-HD and post-HD serum concentrations of cTnT are similar.

INTRODUCTION

The cardiac troponins (cTn) T (cTnT) and I (cTnI) are the gold standard tests for the diagnosis¹ and risk stratification² of acute myocardial infarction (AMI). Elevated cTn can occur in other conditions including patients with chronic kidney disease (CKD). The diagnosis of AMI in patients with CKD,

KEY MESSAGES

- ▶ Serum cTnI decreases following a session of haemodialysis.
- ▶ Serum cTnT does not decrease following a session of dialysis.
- ▶ Cardiac troponin I adheres to polysulphone dialyser membrane.

especially those receiving dialysis, is problematic. The atypical presentations and severity of the CKD itself often masks the classical signs and symptoms of AMI. As the cardiac biomarkers are cleared via the kidney, the interpretation of such biomarkers including cTn in those with renal impairment is a great clinical challenge. Patients with CKD have a reduced lifespan compared with patients without renal disease and cardiovascular mortality accounts for the majority of renal deaths.³ Cardiovascular morbidity is also increased with 55% of patients receiving haemodialysis (HD) renal replacement therapy demonstrating concomitant congestive heart failure.⁴

In 1995, it was reported that serum cTnT was elevated in patients with renal failure in the absence of elevated cTnI⁵ and this was considered to be a false positive. The reason for this is twofold. First, the assay suffered interference due to adsorption of skeletal TnT (sTnT) to the assay tube wall. This adsorbed sTnT was subsequently detected by the non-specific signal antibody.⁶ This phenomenon was abolished by redesigning the assay with two cardiospecific antibodies for capture and detection.⁷ This assay demonstrated fewer cTnT elevations in patients with CKD (5/40, 13%); however, persistent



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Department of Chemical Pathology, Clinical Blood Sciences, St George's Hospital & Medical School, London, UK

Correspondence to

Dr David C Gaze;
david.gaze@stgeorges.nhs.uk

elevations were observed in some patients with CKD suggesting it is not simply a false-positive result. Second, negative cTnI were assigned based on inappropriately high upper reference limits for the diagnosis of AMI. This artificially raised the diagnostic efficiency of cTnI.⁸ The use of more appropriate cut-offs demonstrated that cTnT and cTnI were detectable in patients with renal disease.⁹ The use of recently introduced high sensitivity assays in CKD gives rise to comparable rates of detection of cTnT and cTnI in patients with renal dysfunction.¹⁰ Prognostic studies and meta-analysis data confirm that elevated cTn in patients with CKD is of prognostic value.^{11–22}

There have been a number of published papers investigating changes in cTnT^{16, 23–27} and cTnI^{16, 25–28} in samples taken before and after a dialysis session; however, there is a lack of consensus in the results.

In the present study, pre-HD and post-HD serum samples from patients undergoing HD were assayed for cTnT and cTnI and the mechanism of clearance of cTn was investigated using an *in vitro* model of HD.

METHODS

Pre-HD and post-HD serum samples

Renal patients attending the HD were invited to participate and informed consent was obtained. Whole blood was obtained before (pre-HD) and immediately post-HD therapy in Vacutainer serum separator gel (SST) tubes (Becton-Dickinson, Oxford, UK). Following clotting, centrifugation at 3000 rpm for 10 min and routine biochemical analysis for clinical patient management, residual serum samples were aliquoted and stored at -70°C until further analysis of cTn.

Immunohistochemical staining of cellulose acetate membranes

The experimental design occurred in two phases. In phase 1, passive diffusion of cTn from a commercial source in cut dialyser membranes was examined. This was followed by phase 2, in which the counter-current flow of dialyser fluid and fluid in the blood compartment was used to replicate the process of dialysis to demonstrate the location of cTnI binding to the cuprophane membrane. Capillary middle-flux polysulfone Helixone, FX80 (Fresenius Medical Care, Nottinghamshire, UK) HD membranes were used throughout.

Phase 1: passive diffusion in dialyser membrane

A sterile cuprophane dialyser was flushed with reagent grade distilled water. The plastic casing was cut with a hacksaw to remove the inlet and outlet caps. Twenty-millimetre long sections of membrane tubing were cut using a scalpel and the bundle was loosely placed in a 0.5 mL Eppendorf tube. Each bundle was washed twice with 0.1 M phosphate buffered saline (PBS), pH 7.2. The membrane sections were then incubated overnight in 1000 μL of serum spiked with free

cTnT, free cTnI and cTn I-T-C complex (HyTest Ltd, Finland). The concentrations were 78 000 and 29 150 $\mu\text{g}/\text{L}$ for free cTnI and cTnT, respectively. For the human cTn complex, the cTnT and cTnI concentrations were 27 420 and 30 370 $\mu\text{g}/\text{L}$, respectively.

A 1:100 concentration of M7 anti-cTnT MAb (Roche Diagnostics, Tutzing, Germany) was incubated in one Eppendorf tube, the other was incubated with a 1:100 dilution of 19C7 anti-cTnI MAb (HyTest Ltd, Finland) for 1 h. The membranes were rinsed three times with 0.1 M PBS, pH 7.2, to remove any excess unbound primary antibody. The membranes were then incubated with biotin-labelled mouse anti-IgG (1:1000) for a further hour, washing unbound secondary antibody again with PBS. The membranes were then incubated with the fluorescent marker streptavidin-labelled fluorescein isothiocyanate (FITC). Membranes were transferred to cork tissue boards and orientated either longitudinally (LS) or transversely (TS). The entire block was covered in cryo-embedding media (OCT, Tissue-Tek, Lamb Ltd, East Sussex, UK) and snap frozen by submerging in liquid nitrogen. The frozen tissue board was transferred to a cryotome cryostat (-70°C) and allowed to equilibrate to environmental temperature for 3 min prior to sectioning. LS and TS sections, 5 μm thick, were cut and mounted on aminoalkylsilane-coated (Silane-Prep, Sigma Diagnostics) slides. The slides were allowed to air dry before being visualised using fluorescent microscopy (Olympus BX-40-FLA, Olympus Optical Ltd, London, UK) and video images were captured using Image Grabber PCI V.2.05, (Neotech Ltd, London, UK) for Microsoft Windows.

Phase 2: replication of counter-current flow of dialysis

In order to ascertain if cTnI crosses the dialyser membrane during the dialysis process or if it remains within the vascular compartment of the dialyser, dialysis membranes were examined after simulating the process of dialysis. Dialysate fluid and a volume of serum spiked with cTn (representing the blood compartment) were passed through the cuprophane membrane in counter-current flow, similar to that of dialysis. A water-driven vacuum pump was attached via the dialysate outlet and the tubing cannulated for effluent dialysate sample collection. The inlet was connected to a 5 L reservoir containing dialysate fluid (figure 1). Renalyte acid concentrate bicarbonate dialysate fluid (Fresenius Medical Care, Nottinghamshire, UK) was reconstituted 1 to 1.26 to 32.78 in 8.4% sodium hydrogen carbonate and reagent grade water, respectively, as per the recommended protocol of the manufacturers. The final solution had the following composition: Na^+ 103 mmol/L, K^+ 2 mmol/L, Ca^{++} 1.25 mmol/L, Mg^{++} 0.5 mmol/L, Cl^- 108.5 mmol/L, CH_3COO^- 3 mmol/L and glucose 5.6 mmol/L. An aliquot of the renalyte dialysate fluid working solution was tested for possible interference in the cTnT and cTnI assays. The counter-current flow rate of dialysis fluid (Qd) was controlled at 200 mL/min.

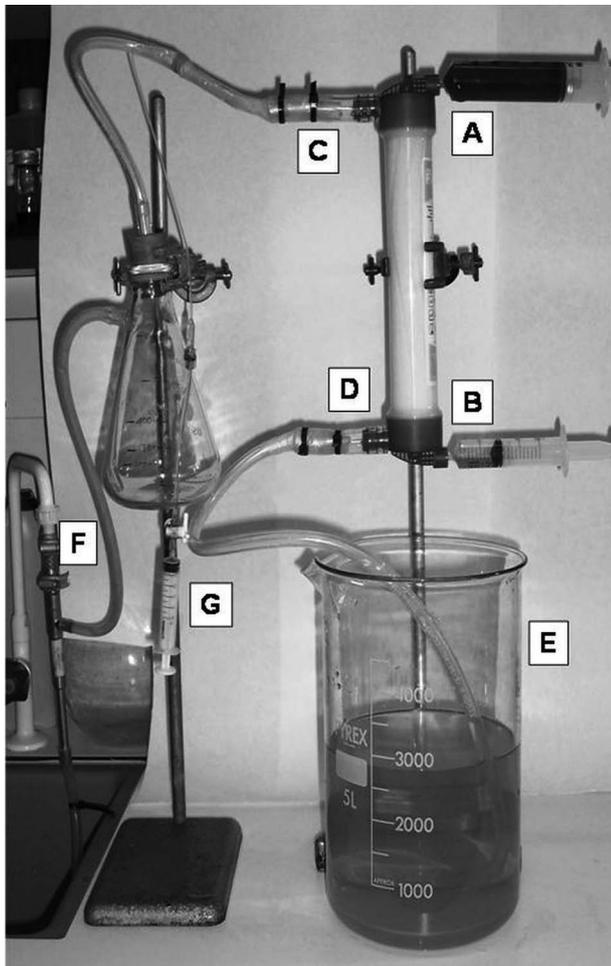


Figure 1 In vitro setup of simulated haemodialysis. (A) Blood compartment inlet; (B) blood compartment outlet; (C) dialysate outlet; (D) dialysate inlet; (E) dialysate reservoir; (F) water-driven vacuum pump; (G) efferent dialysate collection syringe.

The membranes were primed with 200 mL dialysate and an effluent sample was collected for cTnT and cTnI assay testing before introduction of the blood component.

Five serum pools of 50 mL volumes were constructed from healthy volunteers free from a history of AMI and who did not demonstrate serum cTn positivity. The serum pools were filtered and further centrifuged at 3000 rpm for 10 min to remove any particulate matter. The pools were prepared as follows: pool A: troponin free serum pool (no spiking); pool B: unbound free cTnI; pool C: unbound free cTnT; pool D: troponin I-C; pool E: troponin T-I-C complex.

For each pool constructed, a fresh sterile membrane filter was used in the simulation. The serum pools were injected into the blood compartment of the HD membrane using a 20 mL syringe. Two-millilitre aliquots were drawn from the blood compartment outlet repeated at 2 min intervals. In addition, 2 mL aliquot fractions of efferent dialysate were also collected at 2 min intervals

for a period of 15 min. Samples from the blood compartment outlet and the efferent dialysate were assayed for cTnT and cTnI.

Following simulated HD procedures, all membranes were disconnected and the outer plastic shell was cut using a hacksaw at the collar of the housing to remove the inlet and outlet caps. Two to 4 cm long bundles of fibres were cut from the mid-section of the dialyser with a sterile scalpel and placed into 0.5 mL Eppendorf tubes and incubated with anti-cTn antibodies and visualised with fluorescent microscopy as described above.

cTnT assay

cTnT was determined using the fourth generation Troponin T STAT assay on an Elecsys 2010 (Roche Diagnostics, Haywards Heath, UK). The assay total imprecision was 5.4–9.3% in the range 0.47–11.5 µg/L. The measuring range was 0.01–25 µg/L. The 10% CV was at 0.03 µg/L with a 99th centile of <0.01 µg/L.

cTnI assay

cTnI was determined using the TnI-Ultra assay for the ADVIA Centaur (Siemens Healthcare Diagnostics, Frimley, UK). The detection limit of the instrument was 0.006 µg/L, upper limit 50 µg/L. The manufacturers claim was 10% CV at 0.03 µg/L with a 99th centile of 0.04 µg/L.

Data handling and statistics

All data were exported to Microsoft Excel (Microsoft Corporation). All statistical analyses will be performed using the Analyse-it, add-in software for Excel. Data were examined for normal distribution. Box and whisker plots were constructed to demonstrate the distribution of pre-HD and post-HD cTnT and cTnI concentrations and formally tested for statistical significance with non-parametric Wilcoxon signed-ranks testing. A $p \leq 0.05$ was deemed significant. All biomarker concentrations were measured in triplicate and are reported as mean \pm SD.

RESULTS

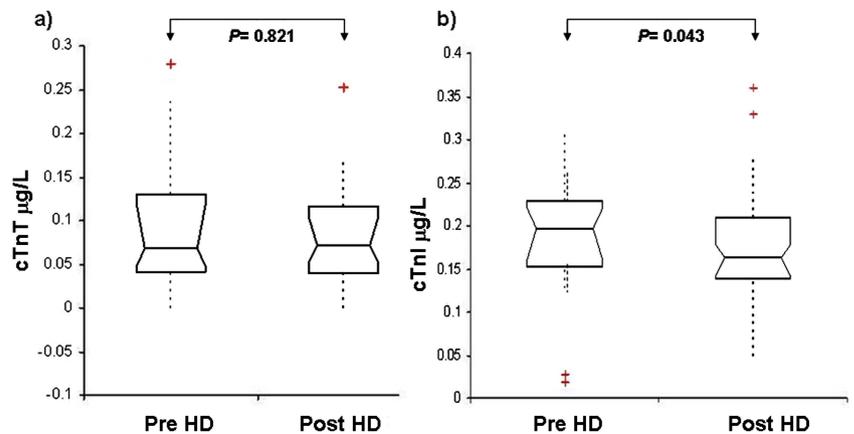
Serum concentrations of cTn before and after dialysis

The mean pre-HD cTnT serum concentration was 0.06 µg/L which increased to 0.08 µg/L post-HD, however this was not statistically significant ($p=0.821$, figure 2A). The mean pre-HD cTnI concentration was 0.197 µg/L which was significantly higher than the post-HD concentration of 0.163 µg/L ($p=0.043$, figure 2B).

Phase 1: passive diffusion in dialyser membrane

Using cut cellulose membranes and simple passive diffusion following incubation of the polysulfone membrane in an Eppendorf tube with very high concentrations of serum containing cTnT, cTnI and T-I-C tertiary complex, immunofluorescent signal can be seen within the lumen in the TS (figure 3A) and LS sections of the membrane (figure 3B) incubated with free cTnI. There

Figure 2 Box and whisker plot demonstrating the concentrations of (A) cardiac troponin T (cTnT) and (B) cTnI in serum obtained immediately prehaemodialysis (pre-HD) and post-HD.



was a lack of immunofluorescent signal when membrane was incubated with either free cTnT (figure 3C) or T-I-C binary complex (figure 3D). Autofluorescence of the fleece fibre packing material occurs (figure 3C marked with*) which adsorbs the streptavidin-labelled FITC and is an artefact.

Phase 2: replication of counter-current flow of dialysis

Samples of renalyte dialysate fluid were below the detection limit for cTnT (<0.01 µg/L) and cTnI (<0.02 µg/L) indicating no interference with the antibodies in the immunoassay. Fifty millilitres of spiked serum

representing the blood (vascular compartment) which does not come in contact with the dialysate fluid was introduced into the dialyser. The dialysate fluid was pumped counter-currently at a flow rate of 200 mL/min, typical of the dialysis procedure.

After dismantling the dialyser membrane and exposure of the cellulose acetate core, it can be demonstrated that cTn-free serum does not produce any fluorescent signal (figure 4A1). To demonstrate that the TS sections of membrane had adequately adhered to the Silane-Prep slide, the corresponding light micrograph is shown in figure 4A2.

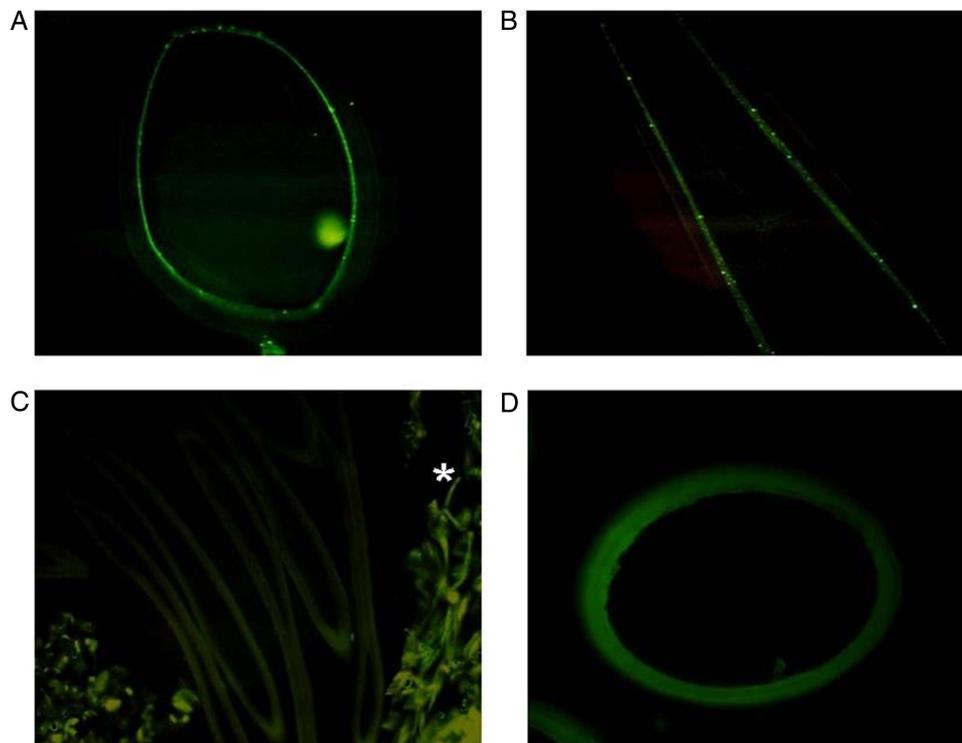
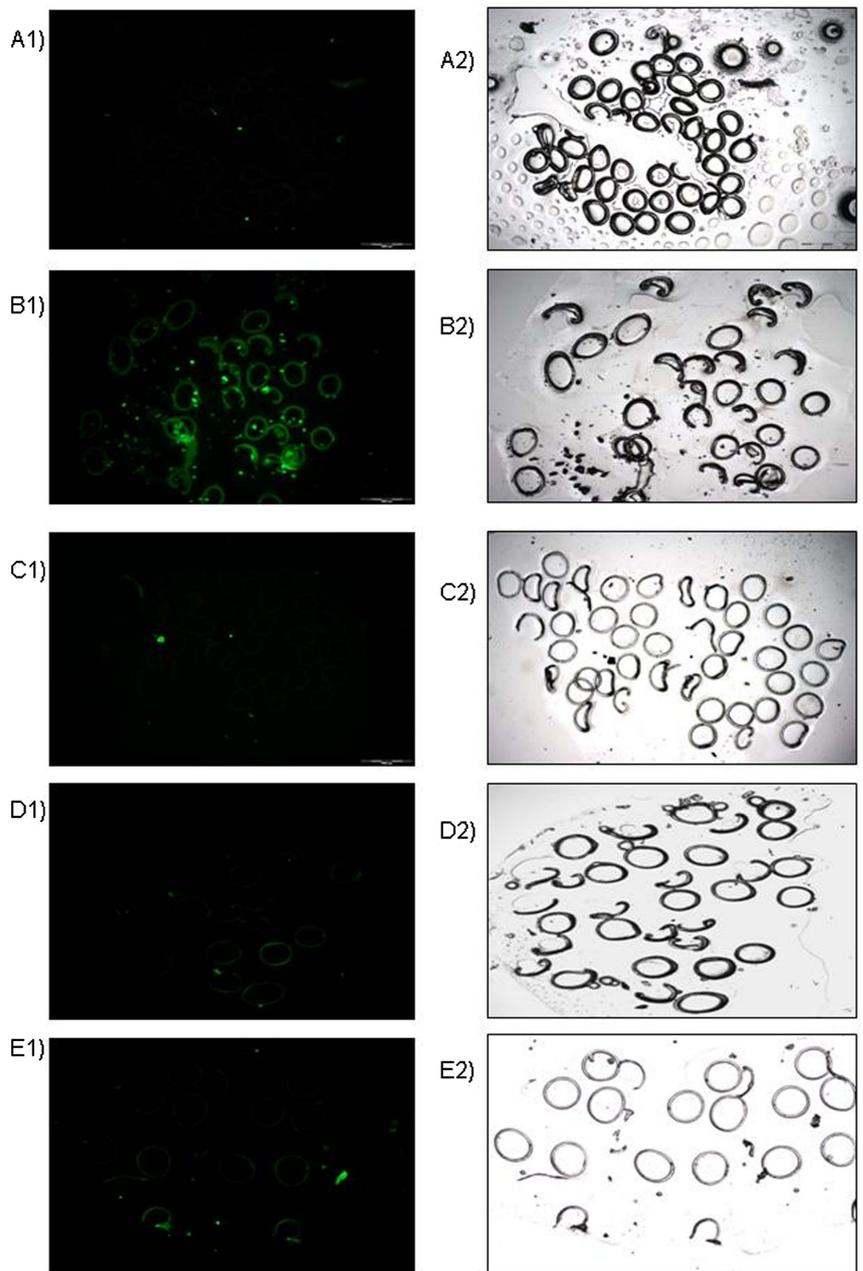


Figure 3 Immunofluorescence of polysulfone dialysis membranes. (A) Transverse section of membrane tube incubated with free cardiac troponin I (cTnI) and stained with anti-cTnI MAb. (B) Longitudinal section of membrane tube incubated with free cTnI and stained with anti-cTnI MAb. (C) Transverse section of multiple tubes incubated with free cTnT and stained with anti-cTnI M7 MAb. (D) Transverse section of a single polysulfone tube incubated with Tn T-I-C binary complex incubated with anti-cTnI and anti-cTnT MAb ($\times 100$ magnification).

Figure 4 Immunofluorescence of polysulfone haemodialysis membranes. Series A1–E1: immunofluorescent images; series A2–E2, corresponding standard bright field light microscopy images. (A) Troponin (Tn) free serum pool; (B) free cardiac Tn I (cTnI) spiked pool; (C) free cTnT spiked pool; (D) I-C binary complex spiked pool; (E) T-I-C complex spiked pool (all images are transverse sections at $\times 40$ magnification).



However, when serum spiked with cTnI was circulated through the dialysis simulation, it can be observed that a strong fluorescent signal was obtained. This signal represents cTnI from the vascular compartment which adheres to the exterior of the cellulose acetate tubes (figure 4B1). These data suggest that the cTnI from the patient's circulation has remained within the vascular compartment of the dialysis system rather than passing across the membrane to the dialysate.

Further evidence to support this hypothesis is that an aliquot of the efferent dialysate fluid was obtained at the end of the experiment and assayed again for cTnI. The dialysate fluid had an undetectable cTnI concentration. Similar analysis revealed an undetectable concentration of cTnT in the efferent dialysate.

When samples of cTnT spiked serum (figure 4C1), I-C binary complex spiked serum (figure 4D1) or T-I-C

tertiary complex spiked serum (figure 4E1) were circulated through the dialysis simulation, there was no evidence of fluorescent signal, thus suggesting that all of these cTn entities remain within the vascular compartment and in the clinical scenario are reintroduced into the patient's circulation during the dialysis session.

DISCUSSION

cTnT and cTnI are the gold standard diagnostic tests for the detection and management of acute cardiac disease²⁹ and are central components of the third universal definition of myocardial infarction.¹ Contemporary and high-sensitivity assays demonstrate comparative clinical sensitivity and specificity for diagnosis of AMI.^{30–31} Elevation of cTnT and cTnI occurs outside of AMI including patients with CKD.

There have been a number of published papers investigating changes in cTn concentration following an episode of dialysis but there is a lack of consensus. Some authors claim there are no significant differences in pre-dialysis and postdialysis cTn concentrations while others refute this. Non-significant increase in cTnT has been demonstrated in post-HD samples^{23 24} whereas others have found no change in concentrations^{16 25} or decreases in cTnT.^{26 27} For cTnI either no change in concentrations^{16 25} or decreases in concentration is observed.^{26–28} Many of the early studies (pre-2005) utilised high cut-off concentrations to define cTn positivity, equivalent to the WHO derived cut point for AMI. This results in a reduction in the number of positive samples in CKD and the observation of non-significant changes or no change between pre-HD and post-HD cTn concentrations. In the present study, a significant reduction in serum cTnI post-HD was observed; however, cTnT concentrations were similar to those before the initiation of dialysis.

When adopting lower clinical cut-off values equivalent to either 10% total assay coefficient of variation or the upper 99th centile of a healthy reference population, significant changes are often seen with cTnT increasing and cTnI decreasing following HD treatment. It should also be noted that Lippi *et al*²⁷ further classified patients according to type of dialyser used. Pre-HD and post-HD cTnT and cTnI were determined from a single dialysis session from 18 patients using low-flux and 16 patients using high-flux haemodialysers. High-flux membranes cleared cTnT and cTnI more efficiently than low-flux membranes.

Solute removal in dialysis occurs through a combination of three processes: diffusion, convection and adsorption. Adsorption is the adhesion of proteins and macromolecules to the surface of the membrane without penetration. Adsorption depends on the internal pore structure and membrane hydrophobicity.³² Presently, it is not known how cTnI is removed during dialysis. In this proof of concept study, dialyser membranes were isolated and incubated with serum containing cTnT and cTnI. Following incubation and immunofluorescent antibody detection it was demonstrated that cTnI is detectable on the membrane whereas cTnT is not and suggests a reason for the disparity between pre-cTnI and post-cTnI serum concentrations observed in this study and by others.^{26–28 33} The proof of concept study could not demonstrate if cTnI is dialysed out of the serum during HD as the dialyser membrane tubes were submerged in the serum during the incubation period.

By replicating the counter-current model of HD, the results of the proof of concept study were confirmed. There was no evidence of cTnT adhering to the polysulfone cellulose acetate dialyser membrane. It was; however, possible to demonstrate that cTnI is not cleared by the dialysis process from the circulation to the dialysate, but is adsorbed to the membrane in the

vascular compartment. This probably occurs due to the high theoretical isoelectric point (pI) of cTnI (9.87). The pI is the pH at which a molecule carries no net electrical charge. A high pI gives the cTnI molecule a high positive charge³⁴ making the molecule 'sticky' and able to interact freely by adsorbing to the polysulfone membrane. One limitation to the present study is the lack of positive control as demonstrated by other proteins with similar pI values. It has been demonstrated that protein adsorption occurs at much lower isoelectric points. Tomisawa and Yamashita³⁵ demonstrated adsorption of albumin (pI=4.4) to polymethylmethacrylate membranes. Mares and colleagues eluted adsorbed proteins from polysulfone dialysis membranes, observing 84 proteins eluted from five dialysed patients. Of these, 57 were identified by mass spectrometry and included ficolin-2 (pI 6.1), clusterin (pI 3.7), complement fragment C3c (pI=6.29) and apolipoprotein A1 (pI=5.56). A further limitation is the ubiquitous use of a single dialyser membrane surface from a single manufacturer. Tomisawa and Yamashita³⁵ demonstrated approximately 20% higher fractional adsorption of albumin using polymethylmethacrylate membranes compared with polyester polymer alloy membranes. Given that many different membranes are used clinically, the adsorption of cTnI to these surfaces may differ and affect post-cTnI serum concentrations.

CONCLUSION

Using immunofluorescent microscopy, it has been possible to demonstrate that cTnI is not dialysed from the vascular compartment like other waste products but remains within the vascular compartment and adheres to the polysulfone cellulose acetate membrane. These novel data provide for the first time, a mechanism by which cTnI decreases following dialysis while cTnT remains similar to predialysis concentrations.

Contributors DCG devised the study design, carried out the experimental work and wrote the manuscript. POC coauthored and approved the manuscript.

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Competing interests None.

Ethics approval The Wandsworth Research Ethics Committee approved the study protocol, in agreement with the declaration of Helsinki.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement No additional data are available.

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