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Evidence for stability of cardiac troponin T concentrations measured with a high sensitivity TnT test in serum and lithium heparin plasma after six-year storage at -80°C and multiple freeze-thaw cycles

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Abstract

Objectives: As high-sensitivity cardiac troponin T (hs-cTnT) is making the transition from diagnostic to prognostic use, a long-term stability study of 5th generation hs-cTnT according to EFLM CRESS recommendations was set up for investigation of frozen clinical specimens (two matrices).

Methods: Study samples collected in serum tubes and lithium heparin tubes with gel from patients admitted for suspected minor myocardial damage were measured directly after completion of the study (0 years), and after 3-year and 6-year storage at -80°C , and recovery of hs-cTnT concentrations after long-term storage (%hs-cTnT concentration compared to 0-year) was calculated. Hs-cTnT changes were also compared to decisive delta changes, such as the ones proposed in the ESC NSTEMI 0 h/1 h algorithm (<3 or >5 ng/L for ruling out and ruling in suspected NSTEMI patients).

Results: Eighty-six patients were included in the study, whereof 28 both lithium heparin plasma and serum samples were collected simultaneously, in others only serum ($n=30$) or plasma ($n=28$). Multiple aliquots per patient were made, so that 479 serum and 473 plasma samples were available for analysis. Across the overall hs-cTnT measuring range, median recovery after 6 years was 105.4 % and 106.2 % for serum and plasma, respectively. Based on these decisive delta changes, serum showed consistent results upon long

term storage (max 0.8 % of samples above delta threshold of >5 ng/L) as compared to heparin plasma (up to 19.2 % of samples above threshold).

Conclusions: Over 6 years of storage at -80°C , recovery of hs-cTnT in serum and heparin plasma was similar and within common lot-to-lot variation. Yet, when evaluating absolute delta increments around hs-cTnT clinical decision points, long-term stored sera displayed better clinical performance compared to heparin plasma samples.

Keywords: high sensitive cardiac troponin T; preanalytical stability study; CRESS checklist; serum; lithium heparin plasma

Introduction

High-sensitive cardiac troponin T (hs-cTnT) is a specific and sensitive biomarker for myocardial injury and is a cornerstone laboratory test for diagnosis of non-ST-segment elevation myocardial infarction (NSTEMI) [1, 2]. A fast 0/1 h algorithm with specific rule-out and rule-in criteria and assay-specific delta values for high-sensitivity troponins (hs-cTnT and hs-cTnI) was introduced in the 2015 guideline of the European Society of Cardiology (ESC) [2], and is also described in the 2023 update of this guideline [3]. Currently, hs-cTnT test indications further expand from diagnostic to prognostic use, which often implies biobanking over several years, and thus the need to grant both reagent and sample stability over such time. Whereas the analysis of reagent stability has been traditionally very well standardized (e.g. CLSI EP25), this is much less the case for sample stability, where some efforts have been recently undertaken by the various clinical chemistry societies [4]. Determination of long-term stability of hs-cTnT in frozen clinical specimens is crucial for evaluating the potential of hs-cTnT as prognostic biomarker, making it possible to use long term stored bio-bank samples for research on prognostic value of hs-cTnT. Therefore, in this study, the impact of long term storage on

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hs-cTnT concentrations at relevant clinical decision points was investigated.

Earlier studies focusing on long term storage or freeze-thaw cycles of TnT samples, include a study that showed that TnT levels are stable up to 12 months freezing at -70°C , using a third generation TnT test [5]. Another small study by *Mansour et al.* focused on freeze-thaw cycles and showed that a pooled sample (EDTA plasma) had stable hs-cTnT concentrations after three freeze-thaw cycles (performed within one week after freezing, so short-term storage) [6]. A large study published in 2011 had availability of 7.600 samples that were collected between 1996 and 1998 (time period of 3 years) and then stored at -70°C for 11–14 years before measured in 2009 and 2010 using the current hs-cTnT assay (5th generation). As total storage time differed between samples, it was assessed whether storage resulted in recovery loss, which showed only minor change (0.36 ng/L per year) [7]. However, their study design was an indirect approach for assessing long-term stability, as there was no direct comparison of hs-cTnT concentrations within the same patient samples [7]. Lastly, a study using the 5th generation hs-cTnT assay, measured hs-cTnT levels in heparin-plasma, EDTA-plasma and serum from 30 patients, directly after blood draw and after 6 months (one freeze-thaw cycle) and one year (both one freeze-thaw cycle in another aliquot and two freeze-thaw-cycles in the aliquot that was also measured at 6 months). The authors concluded that samples were stable for at least one year and after two freeze-thaw cycles [8]. The studies described above used either an older generation hs-cTnT assay [5], investigated freeze-thaw cycles in short-term storage [6], investigated long-term stability up to one year [5, 8] or did not investigate within-sample stability [7]. Our current study addresses all these issues, investigating up to six-year storage within-sample stability with the 5th generation hs-cTnT assay.

A large dataset of around 900 aliquots, from almost 90 patients, was available for this analysis. Previous results of this present biobank, focusing on different pre-analytical conditions, have been published by *Gillis et al.* [9]. The present analysis includes additional analyses on long term storage (up to six years storage at -80°C), investigating long term stability of hs-cTnT samples in both serum and lithium heparin plasma.

Materials and methods

Blood samples were collected from patients admitted to the catheterization laboratory for suspected minor myocardial damage (patients undergoing a cardiac catheterization due to suspicion of a narrowing or possibly even an occlusion of

one of the coronary arteries). Samples were collected specifically for this study. Medical ethical approval was obtained from Amphia Hospital, Breda, the Netherlands and informed consent was obtained from all individuals in this study.

Ninety patients were included in the study, of which 30 patients participated in part A and 60 patients in part B. Patients were included in the period from June to October 2009. In Figure 1 an overview of the study setup is depicted.

Part A of the study evaluated the effect of storage of whole blood on hs-cTnT concentrations. Of 30 patients venous blood was collected by venipuncture into 6 blood tubes each, of which 3 serum tubes with gel (10 mL Venosafe™ plastic tubes; serum gel; Terumo) and 3 lithium-heparin tubes with gel (10 mL Venosafe™ plastic tubes; plasma gel with lithium heparin; Terumo). Mixing of the tubes was done by inversion directly after drawing the blood. These tubes were either centrifuged (2500 g, 5 min, 4°C) and processed immediately (fresh frozen, reference condition) or the tube containing whole blood was stored at room temperature for 3 h or 6 h, after which they were centrifuged. Serum and plasma supernatants were then aliquoted into 2 mL cryovials (screw cap; BIOplastics) and immediately frozen at -80°C . The temperature of the -80°C freezers was monitored continuously using a centralized temperature registration system. Samples were thawed for analysis directly after completion of the study (0-year condition), after 3 years at -80°C (3-year condition) and 6 years at -80°C (6-year condition). After each analysis the plasma or serum was refrozen, resulting in three freeze-thaw cycles of the samples.

Part B of the study evaluated the effect of storage of serum or plasma on hs-cTnT concentrations. In 30 additional patients, venous blood by venipuncture was collected in 5 serum tubes and from 30 different patients in lithium heparin tubes. Tubes were processed within 45 min of blood collection, by centrifuging, pooling the serum or plasma for each individual patient and then aliquoting in vials. The vials were then stored according to different conditions (14 in total): directly frozen at -80°C (reference condition), 1 h, 2 h, 5 h or 24 h at room temperature; 1 h, 2 h, 5 h or 24 h at $2-8^{\circ}\text{C}$; 24 h, 1 week, 4 weeks and 3 months at -20°C and 3 months at -80°C . At the end of each time and condition of storage, the aliquot was transferred to the -80°C . After each analysis the plasma or serum was refrozen, resulting in three freeze-thaw cycles of the samples.

First analysis of the samples (0-year condition) was performed at the Amphia Hospital, Breda. After that, samples were transferred to the Leiden University Medical Center (LUMC) in frozen conditions on dry ice and analyzed after intermittent temperature-controlled storage at the

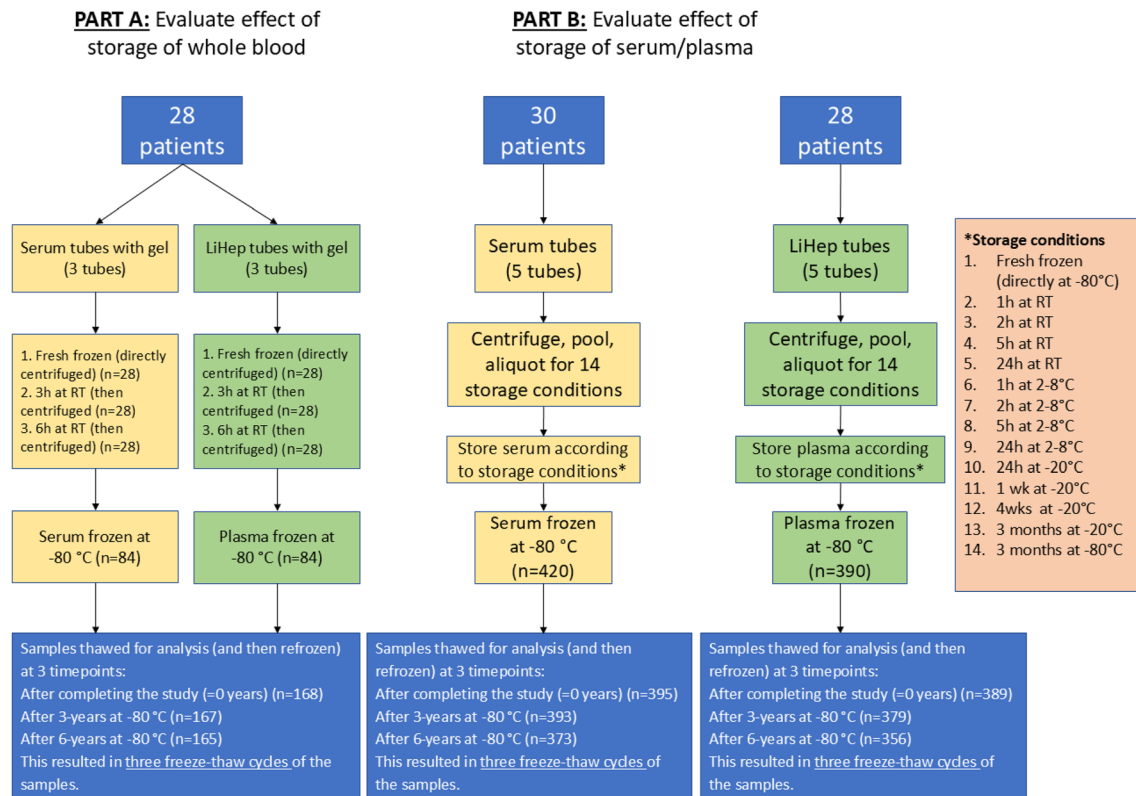


Figure 1: Setup of the preanalytical study. Results on storage conditions have previously been published by Gillis et al. [9]. In this current study only the effect of 6 year-storage and three freeze-thaw cycles is investigated (in samples from both part (A) and (B) of the study). Two patients had hs-cTnT levels below limit of blank (3 ng/L) leading to 28 included patients instead of 30. LiHep, lithium heparin; RT, room temperature; hr, hour(s); wk, week; wks, weeks.

LUMC after 3 and 6 years. Samples for both part A and part B were measured in duplicate in a single batch per analysis, using the 5th generation Roche Diagnostics assay (Amphia hospital, Cobas e601 and LUMC, Modular E170), with a limit of blank of 3 ng/L, limit of detection of 5 ng/L, limit of quantification of 13 ng/L, and 99th percentile (upper reference limit) of 14 ng/L. Samples below 3 ng/L were not included in the analysis [10]. For catalog and lot numbers of reagents and calibrators, see Supplementary Table S1. In terms of traceability, the high sensitive troponin T assay has been standardized against the high sensitive troponin T STAT assay, which was in turn originally standardized against the Enzymun-Test Troponin T (CARDIAC T) method [10].

Statistical analysis was performed using Wilcoxon signed-rank tests (test for paired data, nonparametric, for long term -80 °C storage time) and Spearman's r (nonparametric test, for correlation between matrices). Of duplicate measurements, means were calculated. $p < 0.05$ was considered statistically significant. Median recovery was calculated as the median result of percentual recoveries (concentration after long term storage/original concentration*100 %). The Checklist

for Reporting Stability Studies (CRESS) of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) working group for the preanalytical phase, developed as a guide for standardized reporting of stability studies, was used as guidance for the writing of this manuscript [11]. See Supplementary Table S2 for the application of this checklist in our study.

For calculation of long term recovery, the absolute difference and between measurements at 0 years and 6 years within the same sample was calculated, and recovery after 6 years (depicted as %) was calculated.

To place the results in an additional clinical context, the data of this long-term stability study were compared with the delta thresholds that are used in the ESC guideline for NSTEMI as rule-in and rule-out criteria for myocardial infarction in the 0–1 h algorithm (5 ng/L and 3 ng/L respectively) [1, 2]. Only samples in the relevant concentration range (5–52 ng/L for rule-in and 5–12 ng/L for rule-out) were included in this exploratory analysis. It was calculated how many samples had an increase that exceeded the delta threshold.

Results

Part A

Matrix comparison. Of 30 included patients in this part of the study, two patients had hs-cTnT levels <3 ng/L (below limit of blank) and were excluded from further analysis. The 28 patients (22 male, 6 female) had a median age of 67 years (range 46–80 years). As in this part of the study, serum and plasma were collected within the same patient group simultaneously, a direct comparison between serum and plasma was possible and this showed that results of hs-cTnT concentrations correlated well (Spearman $r=0.9958$), see Figure 2. Median hs-cTnT in serum was 12.7 ng/L and median hs-cTnT in plasma 12.2 ng/L. Difference plots showed that especially at lower concentrations differences between plasma and serum were very small (Figure 2C and D). In Figure 2C a group of data points that seem to be outlier points with a percentual difference of $\sim 20\%$ can be seen, these data points represent a single patient under different conditions.

Part B

Storage comparison. Part B was originally designed to investigate 14 different storage conditions. Results of this analysis have been published previously [9]. As in this part serum and plasma samples were drawn from a separate study population, these data were not included in the above matrix comparison. For serum, samples from 30 patients ($n=395$ samples) were available at 0-year condition, for plasma two out of 30 patients were excluded from further analysis as hs-cTnT levels were <3 ng/L and 389 samples were available at 0-year condition. The 30 patients for the serum analysis (23 male, 7 female) had a median age of 62.5 years (range 33–81 years). The 28 patients of the plasma analysis consisted of 24 males and 4 females (median age 66 years, range 51–81 years).

Part A+B

Samples for both part A and part B were stored and refrozen at 0 years, 3 years and 6 years under identical conditions.

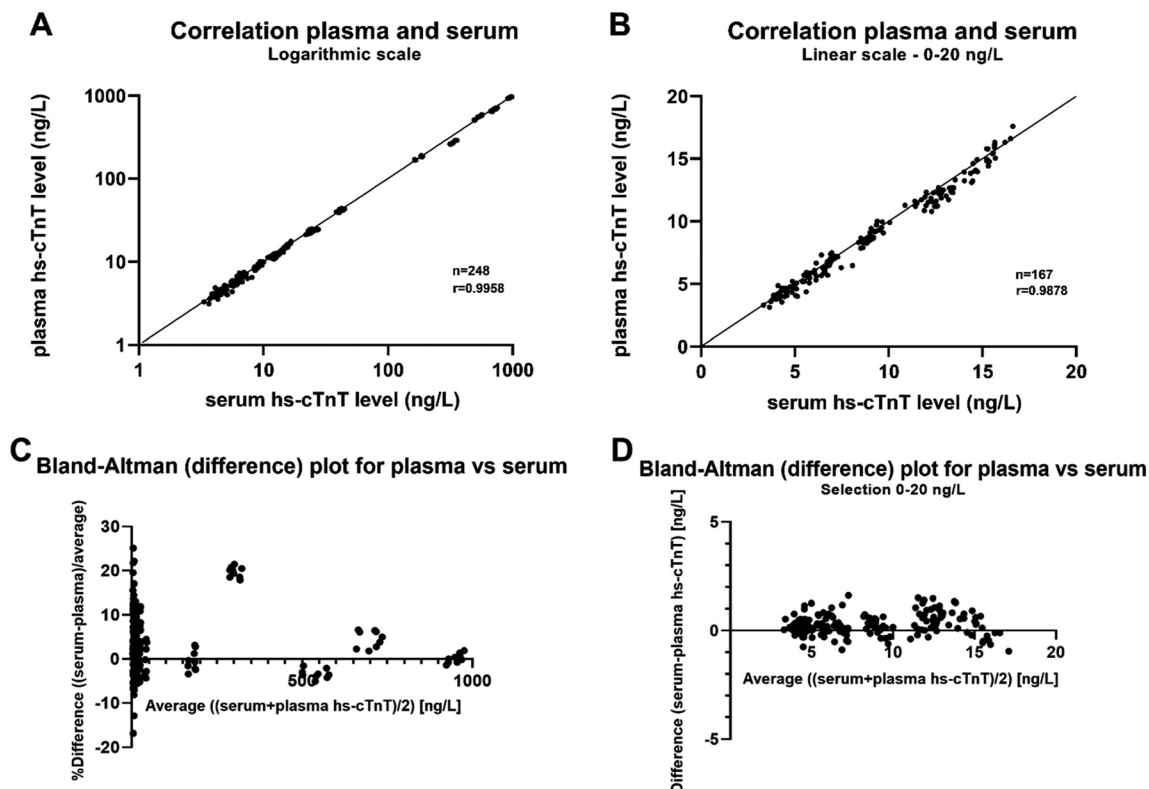


Figure 2: Comparison of 5th generation hs-cTnT levels in serum and plasma. (A) all measurements are included (logarithmic scale); (B) only results between 0 and 20 ng/L are plotted (linear scale). This figure covers all storage periods (0, 3 and 6 years in -80°C) and three storage conditions (fresh frozen, 3 h RT and 6 h RT) before processing the blood taken together. This comparison contains only results of part A of the study, as the plasma and serum tubes were drawn within the same patients. (C) and (D) Bland-Altman plots (difference plots) for all measurements (C) and results between 0 and 20 ng/L (D). hs-cTnT, high sensitive cardiac troponin T.

Therefore, the comparison of long term storage conditions (0 years, 3 years and 6 years at -80°C), was performed in all samples taken together. Data are shown in Figure 3 and Table 1. In Table 1 data are also shown for part A and part B separately. Overall analysis showed that results at 3-years or 6-years differed significantly from 0-years ($p < 0.001$ for both storage times). The same accounted for Part B of the study. However, when only taking results of part A into account, there were no statistically significant differences after 3 or 6 years storage, for both plasma and serum (p -values between 0.21 and 0.54). Median recovery results for serum and plasma

are depicted in Table 2, showing that median recovery is below 110 % for all concentration ranges.

Results in relation to 0–1 h algorithm

Results were compared with the delta thresholds for the ESC guideline for NSTEMI (rule-in and rule-out-criteria for myocardial infarction, 5 ng/L and 3 ng/L). Results are depicted in Table 3. It shows that for serum samples in the relevant concentration range a maximum of 0.8 % of samples exceeded

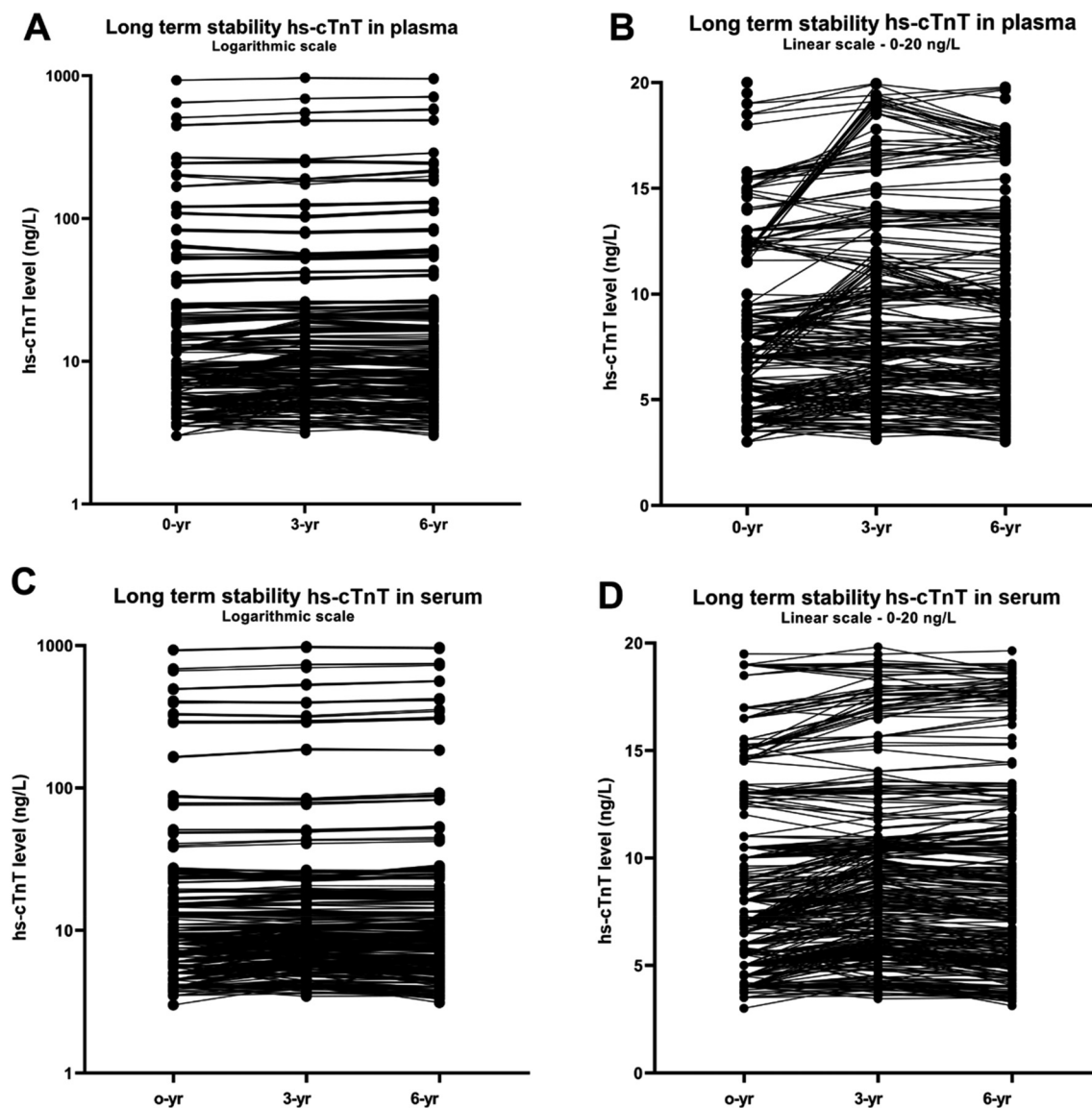


Figure 3: Fifth generation hs-cTnT levels at 0-year, 3-year and 6-year storage. (A) and (B) depict measurements in plasma samples, (C) and (D) measurements in serum samples. (A) and (C) contain all measurements in the specific matrix (logarithmic scale); in (B) and (D) only results between 0 and 20 ng/L are plotted (linear scale). In this figure all preanalytical conditions (times before processing, different short term conditions of both part (A) and part (B) of the study) are taken together. hs-cTnT, high sensitive cardiac troponin T; yr, year.

Table 1: Stability of 5th generation hs-cTnT-levels at 0-year, 3-year and 6-year storage time at -80°C .

Condition	Median	Interquartile range	Comparison to 0-years (reference) ^a
Overall – serum			
0 years (n=479)	9.2 ng/L	6.0–23.5 ng/L	–
3 years (n=477)	10.3 ng/L	6.6–22.8 ng/L	p<0.001
6 years (n=456)	10.5 ng/L	6.4–23.7 ng/L	p<0.001
Overall – plasma			
0 years (n=473)	15.0 ng/L	7.0–53.5 ng/L	–
3 years (n=462)	17.2 ng/L	7.5–54.7 ng/L	p<0.001
6 years (n=438)	16.9 ng/L	7.6–59.0 ng/L	p<0.001
Part A – serum			
0 years (n=84)	12.8 ng/L	7.0–25.7 ng/L	–
3 years (n=84)	12.2 ng/L	6.6–25.0 ng/L	p=0.41
6 years (n=83)	12.7 ng/L	6.3–25.7 ng/L	p=0.21
Part A – plasma			
0 years (n=84)	12.4 ng/L	7.0–24.5 ng/L	–
3 years (n=83)	11.6 ng/L	6.5–23.7 ng/L	p=0.22
6 years (n=82)	11.8 ng/L	5.9–24.5 ng/L	p=0.54
Part B – serum			
0 years (n=395)	8.5 ng/L	5.5–23.0 ng/L	–
3 years (n=393)	10.2 ng/L	6.5–22.5 ng/L	p<0.001
6 years (n=373)	10.3 ng/L	6.4–23.6 ng/L	p<0.001
Part B – plasma			
0 years (n=389)	18.0 ng/L	7.0–62.5 ng/L	–
3 years (n=379)	19.4 ng/L	7.9–55.4 ng/L	p<0.001
6 years (n=356)	17.5 ng/L	7.7–59.4 ng/L	p<0.001

^a0 years at -80°C (directly after completion of the study) is taken as a reference value, and 3 years and 6 years at -80°C are compared to this reference condition using Wilcoxon signed ranks test. p-values in bold are statistically significant. Data are shown for part A and part B taken together, but also separately for each study part.

this delta threshold, while this was a maximum of 19.2 % for plasma samples.

Discussion

This study investigated the effect of long term storage and up to three freeze-thaw-cycles of both serum and lithium heparin plasma samples on hs-cTnT concentrations, according to EFLM CRESS recommendations. Over 6 years of storage at -80°C , median recovery of hs-cTnT (5th gen) was 105.4 % and 106.2 % for serum and plasma, respectively. When looking at smaller absolute changes at clinical decision points, we observed that serum showed better consistency

Table 2: Recovery of 5th generation hs-cTnT-levels after 6-years storage with 0-years as reference value. Results are depicted both overall, and for the samples in the ranges 0–20 ng/L, 20–100 ng/L and >100 ng/L separately.

Matrix	Median absolute change 6-years vs. 0-years (IQR)	Median recovery 6-years vs. 0-years (IQR) ^a
Serum		
Overall – serum (n=456)	0.8 ng/L (–0.1–2.0 ng/L)	105.4 % (98.1–112.2 %)
Serum – range 0–20 ng/L (n=331)	0.4 ng/L (–0.2–1.3 ng/L)	105.3 % (97.5–116.2 %)
Serum – range 20–100 ng/L (n=83)	2.0 ng/L (0.6–3.5 ng/L)	105.0 % (101.2–109.0 %)
Serum – range >100 ng/L (n=42)	20.2 ng/L (17.7–26.0 ng/L)	106.1 % (104.3–108.2 %)
Plasma		
Overall – plasma (n=437)	1.3 ng/L (–0.1–3.6 ng/L)	106.2 % (99.1–111.3 %)
Plasma – range 0–20 ng/L (n=258)	0.6 ng/L (–0.3–1.6 ng/L)	105.8 % (96.2–122.3 %)
Plasma – range 20–100 ng/L (n=101)	2.2 ng/L (0.5–3.3 ng/L)	105.9 % (100.6–110.7 %)
Plasma – range >100 ng/L (n=78)	11.5 ng/L (4.9–30.8 ng/L)	107.1 % (103.7–108.5 %)

^aMedian recoveries vary between 105 and 107 %. The analytical performance goals are a desirable analytical variation of $\leq 7\%$ and a minimal analytical variation of $\leq 10\%$ [12]. IQR, interquartile range.

and apparent stability after 3-year and 6-year storage than plasma. Shown previously is that time before centrifugation or short term storage in different conditions did not affect hs-cTnT concentrations significantly in this study [9].

As, in a subpart of this study, paired plasma and serum samples were available from the same patient population, the effect of plasma vs. serum on hs-cTnT concentrations could be investigated. An excellent correlation ($r=0.9958$) was identified. Multiple studies have been published focusing on potential serum vs. plasma differences in hs-cTnT measurements. In the study by Donato et al. that had the availability of over 2000 paired serum and plasma samples, good correlations for both the fourth generation ($r^2=0.998$) and fifth generation assay ($r^2=0.999$) were found [13]. However another study by Saenger et al., which was an international multicenter trial evaluating the high-sensitivity troponin T assay (fifth generation) compared to the fourth generation assay showed that hs-cTnT levels in plasma were on average 4 % lower than in serum (n=140 samples) [14, 15]. A factor that could contribute to differences in TnT concentrations between matrices are differences in protein cleaving. A proteomic analysis by Katrukha et al. showed that heparin

Table 3: Effect of sample matrix on clinically relevant hs-cTnT (5th gen) changes after 3-year and 6-year of storage at -80°C .

ESC criterion	Delta threshold 0–1 h (ESC)	Relevant measuring range	n samples within measuring range at 0 years	n samples with increase between 0 and 3 years exceeding delta threshold ^a	n samples with increase between 0 and 6 years exceeding delta threshold ^a
Rule-in	5 ng/L	5–52 ng/L	Serum: 337 Plasma: 271	Serum: 0/337 (0 %) Plasma: 18/271 (6.6 %)	Serum: 3/337 (0.8 %) Plasma: 18/271 (6.6 %)
Rule-out	3 ng/L	5–12 ng/L	Serum: 199 Plasma: 151	Serum: 0/199 (0 %) Plasma: 29/151 (19.2 %)	Serum: 1/199 (0.5 %) Plasma: 24/151 (15.9 %)

^aClinically relevant changes were defined as exceeding the delta threshold at relevant clinical decision limits, based on the European Society of Cardiology (ESC) guideline criteria for rule-in and rule-out of non-ST-segment elevation myocardial infarction (NSTEMI) [1–3]. ESC, European Society of Cardiology.

plasma samples contained mainly full-sized TnT, while in serum samples a smaller form (29 kDa instead of full-sized 35 kDa) was present, probably due to *in vitro* thrombin-mediated cleavage during the clotting process in the serum tube [15, 16]. Another older study that measured TnT with the third generation assay, found 15 % lower mean TnT concentrations in heparin plasma compared to serum. The authors suggested that binding of heparin to troponin decreases immunoreactivity by causing conformational changes of the troponin molecule or covering analytical epitopes, making it more difficult for the analytical antibodies to bind [17]. Over the years, the TnT assay keeps being evolved and became more matrix independent with newer generations. The current fifth generation hs-cTnT assay with high demands in terms of analytical and clinical sensitivity, is very robust as shown here.

In our study and Table 3, heparin plasma samples measured with the fifth generation assay, displayed less consistent results around the clinical decision limits and generated more delta increases during long term storage. The mechanism behind this is not known. It could be that fragmentation is different in the specific matrices. As hs-cTnT is measured in mass units, not in molar units, it is not exactly known what specific analyte is measured.

We compared the effects of hs-cTnT storage changes during three and six year storage with delta thresholds from the ESC guideline. Although these delta thresholds are used in a different clinical context, i.e. in the acute setting for ruling-in or ruling-out NSTEMI, we found this comparison relevant, as it gives insight in the usability of long term stored biobanked samples. As an additional caveat, the serial samples of a patient would be affected by a similar storage effect, i.e. if one sample would be measured immediately, and the second sample only after time. In this comparison, serum turned out to be a more stable matrix than heparin plasma. For diagnosing NSTEMI, the guidelines demand for troponin assays a maximum of 10 % analytical variation (coefficient of variation, CVA) at the 99th percentile of the assay [10, 12]. This requirement led to an improved analytical

performance with the introduction of hs-cTnT assays. In combination with the increased sensitivity at the low end, this enabled the possibility to predict long-term cardiovascular risk in the general population [12]. To define the necessary analytical performance for tests, three different models have been defined in the Milan Hierarchy EFLM paper [18]: based on clinical outcome, biological variation or state-of-the-art. For hs-cTnT, the model selected depends on the intended use and the clinical context in which the test is used. If based on biological variation, analytical variation should only add minor changes, and a CVA with a maximum of half of the within-subject biological variation is regarded desirable and three quarters of the biological variation is regarded minimal [12]. As most studies suggest a within-subject biological variation of 15 %, the desirable analytical variation is approximately 7 % and minimal analytical variation 10 % [12].

Long term stability of test performance, i.e. minimizing lot-to-lot variations in reagents and calibrators, is an important factor that can influence results of a stability study. Previously, we showed that our local stringent policy using human pool-based internal quality control (IQC) samples around the 99th percentile for hs-cTnT, aimed at monitoring longitudinal accuracy, resulted in no differences in IQC results after lot changes of reagents of calibrators during that study period [19]. One of the limitations of current study is that troponin T has been analyzed with a specific troponin T reagent (5th generation assay from Roche). Because the various immunoassays target various protein epitopes, our results cannot be generalized to other troponin subunits (cTnI) or different immunoassays. Because the prognostic performance is often analyzed in biobanks of much longer storage times, a future aim of our lab is to document the effect of >10-year storage on the same samples in the future. Samples that have not been thawed previously are available for this as well, which makes it possible to investigate the effect of multiple freeze-thaw cycles.

In conclusion, hs-cTnT measurements in stored serum and lithium heparin plasma samples had median recoveries

of 105–107 %. Consequently, Roche hs-cTnT tests meet minimal analytical performance goals and can still be used after 6 years of storage at -80°C and multiple freeze-thaw cycles. When both serum and plasma samples are available in biobanks for prognostic clinical studies, we recommend the use of serum over that of heparin plasma, as serum produces more consistent results upon long-term storage leading to adequate rule in/rule out of NSTEMI patients when applying the 0/1 h ESC algorithm with a change in classification of $\leq 0.8\%$ for serum as compared to up to 15.9 % for heparin plasma.

Research ethics: Research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with the Helsinki Declaration (as revised in 2013), and has been approved by the Medical Ethical Committee of Amphia Hospital, Breda, the Netherlands.

Informed consent: Informed consent was obtained from all individuals included in this study, or their legal guardians or wards.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Use of Large Language Models, AI and Machine Learning Tools: None declared.

Conflict of interests: The authors state no conflict of interest.

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Data availability: The raw data can be obtained on request from the corresponding author.

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