Is it possible to make a common reference standard for D-dimer measurements? Communication from the ISTH SSC Subcommittee on Fibrinolysis

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Abstract

Background: D-dimer antigen is a heterogeneous mixture of fibrin degradation products that when present at high levels in plasma indicate ongoing coagulation and fibrinolysis. The heterogeneous nature of the target D-dimer antigen and the variety of assay systems means that it is difficult to compare results from different methods.

Objectives: To identify a universally agreed D-dimer standard that could help harmonize results from different methods.

Methods: A pool of patient plasma with high D-dimer levels was freeze-dried and investigated as a long-term World Health Organization international standard for D-dimer. Fibrin degradation products from clot lysis reactions were also freeze-dried in various formulations and investigated in commutability studies with patient plasma.

Results: Problems of instability of D-dimer plasma emerged suggesting loss of reactivity after freeze-drying and storage at −20°C of 10%–18% per year. Freeze-dried fibrin degradation products added to plasma were also unstable, but the sugar trehalose was found to improve stability. However, this preparation was not suitable as a standard in widely used assay platforms. Previous studies suggest fibrin degradation products are prone to structural rearrangements and amyloid formation, which may explain the instability of candidate D-dimer standards.

Conclusions: The known difficulties of D-dimer standardization are compounded by instability of D-dimer antigen after freeze-drying, described in this report. Fibrin degradation products added to plasma and stabilized by trehalose are not suitable as a standard for D-dimer measurement harmonization. Trehalose stabilization of pooled patient plasma containing high D-dimer levels may produce a useful standard, but this requires confirmation.

KEYWORDS
amyloid, D-dimer, fibrin degradation, protein aggregation, standardization
1 | INTRODUCTION

Elevated plasma D-dimer is a marker for ongoing coagulation and fibrinolysis. The antigen target in D-dimer tests is actually a heterogeneous mixture of fibrin degradation products (FD), of varying size, that are generated by plasmin following activation of the fibrinolytic system. These fibrin fragments contain cross-linked D domains, formed by activated factor XIII (FXIIIa), a transglutaminase produced by the action of thrombin on the zymogen precursor FXIII. Early work on the identification and use of fibrin breakdown products as a diagnostic marker were boosted by the introduction of monoclonal antibody technology and subsequently led to the development of a large diagnostic testing industry. However, common D-dimer tests are characterized by high sensitivity but low specificity, which means the results have good negative predictive value (NPV), but poor positive predictive value (PPV, a high rate of false positives). Therefore, D-dimer testing in clinical practice is widely used to exclude thrombosis, but confirmatory testing is required if patient values exceed a predefined cut-off. NPV can be improved by various algorithms such as the Wells’ score, which aim to reduce the likelihood of positive results in the population tested. Elevated D-dimer levels are seen in a number of other pathological states, such as disseminated intravascular coagulation, cardiovascular disease, cancer, inflammatory disease, trauma, and infections, including SARS-Cov- and are used to monitor therapy in patients with previous thrombosis. Measurement and interpretation of D-dimer assay results are complicated by the poor PPV and the establishment of reliable cut-off values to make clinical decisions.

Further problems in routine D-dimer testing, reporting, and interpretation are the use of different units: D-dimer units or fibrinogen equivalent units (FEU), both expressed as mass/volume, but which can be ng/ml or µg/ml or µg/L etc. Different methods have different cut-off values or recommend users define their own cut-off values, sometimes with adjustment for age. The diverse antibodies used in available methods react in different ways to the heterogeneous mixture of D-dimer antigens in samples found in the various conditions in which elevated D-dimer is of interest. All these factors mean it is impossible to directly compare raw results from different methods, but it may be possible to harmonize results between methods by using a common standard. A pooled plasma with elevated D-dimers from a large number of patients might be suitable as a common reference material. Alternatively, a mixture of FDP prepared from digested fibrin added to plasma may work, be easier to produce reproducibly at scale, and be more comparable with calibrators prepared by manufacturers. The work described below reports attempts to generate a potential international standard for D-dimer from pooled plasma or in vitro-generated cross-linked FDP, which might improve harmonization and reliability of results. However, a problem of instability of D-dimer antigen in plasma after freeze-drying was found, which further complicates development of reliable reference materials and harmonization of D-dimer measurements. These observations also suggest the stability of D-dimer antigen in clinical samples should be investigated.

2 | MATERIALS AND METHODS

2.1 | D-dimer measurement

Several different methods were used and compared when studying D-dimer in patient plasma or FDP added to plasma, including the Dade Behring Innovance D-dimer assay (Siemens Healthcare); HemosIL D-dimer HS on an ACL Top 500 (Instrumentation Laboratory SpA); Hyphen Zymutest DDimer (HYPHEN BioMed); Stago Asserachrom D-DI (Diagnostica Stago SAS), Technoclone Technozyme D-dimer ELISA (Technoclone). Hyphen and Stago methods were ELISA-based and were performed using a Softmax M5 plate reader (Molecular Devices) and results were analyzed using GraphPad Prism Software (GraphPad Software). Additional details for methods and platforms used for commutability studies are described below.

2.2 | Preparation of SS258

Patient plasma left over from routine testing and with high levels of D-dimer was collected at the Royal Hallamshire Hospital UK and its use in standard development was approved by the National Institute for Biological Standard and Control (NIBSC) ethics committee. Thirty plasma pools were provided of 10–12 ml and D-dimer in each pool was determined using the Dade Behring Innovance D-dimer assay. D-dimer values for these samples ranged from approximately 3 to >20 µg/ml (FEU units) and gave a volume of 360 ml with an expected estimated overall value of approximately 6 µg/ml, using the Dade Behring Innovance method. The pooled plasma was chilled on ice and 1 M Hepes buffer, pH 7.4 was added to the plasma to a final concentration of 40 mM before the pool was dispensed in 0.5 ml aliquots into 3 ml ampoules for freeze-drying over 4 days using eight steps from −50 to +25°C and 150 to 25 mTorr. Finally, ampoules were backfilled with nitrogen gas and temporarily stoppered before sealing using a Bausch Strobel sealer. This batch of ampoules is referred to as SS258.
2.3 Preparation of SS444 and SS523

FDP were generated in a such way to produce a heterogenous mixture of fibrin fragments, from a purified system. Clots were formed using purified fibrinogen and thrombin, which included FXIIIa, plasminogen activator, and plasminogen. Plasmin was generated from plasminogen by tissue plasminogen activator (tPA, code, 98/714; NIBSC) or urokinase plasminogen activator (code 11/184; NIBSC). All solutions were made up in 10 mM Hepes buffer pH 7.4, containing 0.15 M NaCl and 0.01% Tween 20. Clot lysis mixtures were prepared by mixing 0.2 ml of activator solution (containing thrombin and FXIIIa [code 02/170; NIBSC] plus tPA or urokinase [uPA]) with 1.0 ml of fibrinogen solution (also containing plasminogen). The final mixtures contained 4 nM thrombin (code 01/578, NIBSC) plus 1 IU/ml activated FXIIIa and 2.5 mg/ml fibrinogen (Calbiochem) and 0.22 µM plasminogen, either glu-plasminogen (HYPHEN BioMed) or lys-plasminogen (Immunogen) with 2 nM tPA or 1 nM uPA. Clot lysis at 37°C was followed in four mixtures of tPA or uPA with glu- or lys-plasminogen (abbreviated to TG, TL, UG, UL) for varying amounts of time and 1 ml samples were taken at four time points: the moment the clot collapsed, t₀ (6–12 minutes), 2x t₀, 60 min and 300 min. Reactions were stopped by cooling immediately on ice and adding concentrated aprotinin (Baxter) to a final concentration of 7 µM (see Longstaff and Locke[25]) before adding to plasma and snap freezing for storage at −80°C. Assays on samples from these reaction mixtures were performed using the Technoclone Technozyme D-dimer ELISA, the Hyphen Zymutest DDimer ELISA method, and the Stago Asserachrom D-DI ELISA method and the HemosiIL D-dimer HS latex automated method (ACL TOP). A larger pool of FDP in plasma was prepared by combining equal volumes of these different reaction mixtures into 100 volumes of buffer or plasma and additional excipients to prepare samples A, B, C, and D, as shown in Results. This set of freeze-dried ampoules was labeled SS444 A to D. Sucrose and trehalose (the same as SS444 D) was prepared for commutability studies and is labeled batch SS523.

2.4 Thioflavin T assays

A selection of 10 World Health Organization (WHO) standards were used when available as part of accelerated degradation studies taking place at NIBSC. Standards were reconstituted as directed in their instructions for use and four-point, 2-fold dilution ranges prepared in phosphate-buffered saline (PBS). A working solution of 50 µM Thioflavin T (ThT) was prepared in PBS and 175 µl of this solution mixed with 25 µl of the protein solution from the WHO standards in Corning Costar black microtiter plates (Corning Life Sciences). After 30 min incubation at room temperature, the fluorescence was read in an M5 fluorescent plate reader (Molecular Devices) using an excitation wavelength of 435 nm and emission wavelength of 485 nm. A linear dose response was obtained, and results are presented for the highest reading (most concentrated protein) in Results, after subtraction of background in no-protein blanks.

2.5 Commutability studies

All assays were conducted by Roche Diagnostics. Three different D-dimer assay systems were used: Roche Tinaquant on a Cobas c501 platform; Stago STA LIA D-dimer plus on a STA platform; Innovance D-Dimer assay on Sysmex CS-5100 platform. All methods included the same internal reference calibrators plus candidate standards SS258 and SS523. A selection of 54 patient plasma samples, labeled 1–54, containing varying levels of D-dimer were included. Results are presented below as from anonymized methods A, B, and C, not necessarily in the order of methods listed above.

2.6 Degradation analysis

Degradation studies were performed by measuring the change in relative potency (D-dimer antigen concentration) over time of freeze-dried candidate standards stored at a range of temperatures, −70, −20, 4, 20, 37, 45°C. Degradation rates at each temperature were modeled using the linear form of the Arrhenius equation, Equation (1), by a specially designed computer program previously established at NIBSC. This modeling assumes a first order decay of activity, which is reasonable from previous studies, shown in Equation (2), or in the linearized form as Equation (3). Rate constants can also be calculated directly by fitting degradation data over time at each temperature using Equation (2) or (3) by non-linear or linear regression, respectively. These calculations were performed using R.

The linear transformation of the Arrhenius equation:

\[
\ln k = \ln A - \Delta E/RT
\]

First-order reaction A→P

\[
[A]_t/[A]_o = \exp(-kt)
\]

linear transformation:

\[
\ln[A]_t = -kt + \ln[A]
\]

3 RESULTS

A pool of plasma with a high level of a heterogeneous mixture of D-dimer antigen was collected with the aim of preparing a batch of freeze-dried plasma in ampoules that could be investigated as a candidate standard to be used to calibrate manufacturer or other working standards. The characteristics of this pool and the freeze-dried material are shown in Table 1.
The pool was made up of many small pools of 5–12 ml of patient plasma with D-dimer levels ranging between approximately 3 to >20 µg/ml. Hepes buffer, pH 7.4 was added to the plasma, as commonly done with WHO plasma standards before freeze-drying. There was no prior indication that the D-dimer antigen present in this freeze-dried plasma was likely to be unstable, but stability studies, using accelerated degradation methods, must be performed on potential WHO International Standards before they can be established. Therefore, in line with usual practice, ampoules were stored at temperatures of −20, 4, 20, 37, and 45°C for extended periods so that the amount of activity loss could be determined, and predictions made of likely stability on long-term storage at −20°C. The D-dimer content of these ampoules was measured after 0.55, 1.24, 1.91, 2.47 years of storage. Unexpectedly, a significant loss of potency (measurable D-dimer antigen) was observed, even at the earliest time points. Several different D-dimer assay methods were included in these studies (Innovance D-Dimer Assay, HemosIL D-dimer HS, Zymutest DDimer, Stago Assechrom D-DI), which gave similar results on the same samples. A bar chart summarizing the loss of activity for D-dimer in SS258 over time at each storage temperature is shown in Figure 1.

This degree of instability is much greater than seen in other freeze-dried plasma standards prepared by NIBSC as WHO International Standards. Accelerated degradation results are analyzed by established methods\(^{25,26}\) to calculate rate constants for loss of activity at each temperature investigated and to predict the loss of activity per year with confidence limits. However, the theoretical basis for these calculations assumes first order kinetics for the loss of activity, and that the reaction follows the Arrhenius equation (see Materials and Methods).

Results from the Degtest program\(^{26}\) produced values for the rate constant for loss of activity at the temperatures shown, but the fitting deviated from Degtest predictions based on assumptions of first order reaction kinetics and validity of the Arrhenius equation for this system (compare Figure 1A and B). Other possible sources of error were the inclusion of different methods and the long time period of study, which meant that the lot number and calibrators of kits changed, or kits exceeded their stated shelf-life. A summary of results from Degtest analysis is presented in Table 2. It is also possible to use time and activity data at each temperature and fit the results to linear equations for first order reaction kinetics (see Equation (3)). It is striking that whichever analysis method was used there was a significant loss of activity of 10–18% after 1 year on storage at −20°C. In practice these results mean that this freeze-dried plasma containing D-dimer is not suitable as a WHO Standard due to this marked instability.

### 3.1 Generation of FDP from fibrin

FXIIIa crosslinked FDP were made from clotted fibrinogen in the presence of tPA or uPA and glu- or lys-plasminogen as described

### Table 1 Characteristics of the freeze-dried candidate standard SS258

| Plasma Volume        | 290 ml with 40 mM Hepes buffer |
|----------------------|--------------------------------|
| D-dimer content      | Estimated >6 µg/ml              |
| Ampoules filled @ weight (CV) | 556 @ 0.51 g (0.72%) |
| Dry weight (CV)      | 0.038 g (1.61%)                 |
| Residual moisture (CV)| 0.19 mg (13.5%)                 |

### Table 2 Fitted rate constant for loss of D-dimer activity on storage at various temperatures

| Temperature °C | Degtest k year\(^{-1}\) | % loss | First order k year\(^{-1}\) | % loss |
|----------------|------------------------|--------|-----------------------------|--------|
| −20            | 0.11                   | 10.4   | 0.2                         | 18.3   |
| 4              | 0.27                   | 23.8   | 0.24                        | 21.5   |
| 20             | 0.46                   | 36.8   | 0.19                        | 17.6   |
| 37             | 0.75                   | 52.9   | 0.25                        | 22     |
| 45             | 0.92                   | 60.2   | 0.48                        | 37.9   |

Note: Rate constants were derived from fitting using the Degtest program or to a linearized first order reaction equation at each temperature.
in Materials and Methods to give four reaction time courses designated as TG, TL, UG, UL where activator was tPA (T) or uPA (U) with glu-plasminogen (G) or lys-plasminogen (L). Samples were taken from each clot lysis mixture at the point of clot collapse, at \( t_c = 6-12 \) min, \( 2 \times t_c \), 60 min and 300 min, the reactions were stopped and FDP added to plasma. This approach was developed to generate a diverse mixture of FDP that might reflect the mixture of D-dimer structures present in clinical samples from different disease conditions. D-dimer values were measured in all these mixtures using the Technoclone Technozyme D-dimer ELISA, the Hyphen Zymutest DDimer ELISA, the Stago Asserachrom D-DI ELISA, and the HemosIL D-dimer HS latex automated method (ACL TOP) and results are shown in Figure 2.

Results shown in Figure 2 indicate there was no clear difference between reaction mixtures with plasminogen activators tPA or uPA; or with glu- or lys-plasminogen. However, this group of methods provides a clear indication of the different sensitivities observed for FDP generated over time corresponding to large sizes at early times and small FDP fragments at longer times. The Stago ELISA method gave a reasonably consistent response from 6 min to 5 hours of fibrin digestion, whereas other methods showed changing responses over the period of clot lysis. The absolute concentration of D-dimer is not consistent across methods, peaking around 0.7 mg/ml for HemosIL, at the lower end and reaching around 2.0 mg/ml for the Hyphen Zymutest ELISA. These discrepancies highlight the lack of intermethod standardization. These results are corrected for dilutions into the different assay methods and so report D-dimer formation in the clot lysis mixture which contained 2.5 mg/ml fibrinogen. The diverse results shown in Figure 2 emphasize the need for a heterogeneous mixture of D-dimer fragments in any standard aimed at harmonizing different assay methods.

3.2 | Stabilization of fibrin degradation products

In order to explore the stabilization of freeze-dried FDP, experiments were performed on a mixture of 10 \( \mu \)g/ml FDP from the reactions used in Figure 2, which were added to four different formulations for freeze-drying and stability testing, as shown in the legend to Figure 3. Subsequently, a second FDP formulation in plasma with added trehalose, the same as sample D of SS444, was prepared and this was labeled SS523.

Immediately after freeze-drying, samples were placed at elevated temperatures for storage and periodic measurement of D-dimer antigen using the HemosIL kit method on the ACL TOP platform. Results are shown in Figure 3 for assays after 0.3, 0.65, 1.33, and 1.65 years of storage (or only up to 1.33 years for formulations A and B because there was little change between 0.3 and 1.33 years). It is apparent from the results shown in Figure 3 that there was an initial loss of activity in samples A-C at all temperatures from −70 to +45°C. Maximum activity was close to 2.0–2.5 \( \mu \)g/ml or only 20%–25% of the expected value. Therefore, formulations A-C with sugars, trehalose or sucrose, or with plasma and HEPES buffer do not give good recovery after freeze-drying. Recovery was much better, at around 75%, in formulation D containing plasma, HEPES buffer, and 10 mg/ml trehalose. On extended storage there was further loss of activity in formulations C and D but only at higher temperatures, 20, 37, and 45°C.

This loss of activity in formulation D was explored further by analyzing the decay over time using the Degtest program. The analysis produced a set of decay constants and loss per year shown in Table 3, although the results are based only on changes in values seen at 20, 37, and 45°C. Good agreement between observed and predicted data, as percent of starting value, is also shown in Figure 4. There is obviously clustering of values around 100% (very little loss of activity), which come from the samples stored at −70, −20, and 4°C, but overall, the slope of 0.86 and intercept of 11.8%, \( R^2 \) of 0.92 are satisfactory. Without the clustered values around 100% the linear fit is better, intercept = 1.3%, slope = 1.08, and \( R^2 = 0.93 \), suggesting a good fit of model and data.

The conclusions from Table 2 are that this preparation is stable at temperatures below zero and could serve as a standard for long-term use on this basis. However, it is not known whether the FDP in plasma in the presence of trehalose could act as a standard for plasma D-dimer from patient plasma, which is explored below.

3.3 | Commutability

A reference material (standard) is said to be commutable if the same results are obtained for clinical samples between methods that will use the reference material. Commutability studies are performed with clinical samples when new reference materials are being introduced, or when a new method is introduced. Therefore, in the case of D-dimer standards it is important to investigate potential reference materials with patient samples in established methods. A series of such studies are shown in Figure 5.

There was good agreement between the common standard (green circle) and the candidate standard SS258 (which had been stored for >5 years at −40°C at this point). Conversely, preparation SS523, FDP in plasma with trehalose, showed very different behavior between assays indicating that method A detected lower levels of D-dimer in this sample than methods B and C. Optimal agreement between pairs of methods would lead to a slope of 1 and intercept of zero on linear plots, and in this series of experiments the slopes ranged from 0.52 to 1.65 for the internal standard, SS258, and plasmas. However, the slopes for candidate SS523 in the three-method pairings shown in Figure 5 were 3.19, 4.20, and 1.38, for methods A versus B, A versus C, and B versus C, respectively. The distribution of plasma samples was generally consistent; however, there are several extreme samples with variable behavior between methods as shown circled in purple (plasma numbers 48, 49, 52, 53) or in the gray box (31, 35, 45, 46) and indicated by the arrow (47). These results suggest that overall the internal standard used in this series of testing performed in a broadly similar way to the freeze-dried plasma SS258, but the mixture of FDP added to plasma and
freeze-dried in the presence of trehalose was not appropriate as a standard. Candidate standard SS523 is certainly not commutable between methods; and the individual plasma samples highlighted are also problematic between methods and all standards. These results suggest that no combination of methods or standards will be completely commutable for all D-dimer plasma samples.

### 3.4 | Mechanism of loss of D-dimer antigen

It is possible that aggregation of D-dimer fibrin fragments could explain the loss of D-dimer signal over time as aggregation during fibrinolysis is known. It is known that fibrin aggregation during fibrinolysis is accompanied by cross beta sheet formation and amyloid development as demonstrated by the binding of ThT assessed by increased fluorescence. However, it is not known whether this process can take place in freeze-dried proteins. To explore this question, a selection of 10 freeze-dried preparations that were undergoing accelerated degradation studies were investigated, providing 99 data points from various times of storage at temperatures between −70 and 45 °C. The results shown in Figure 6 clearly show a trend of increasing ThT fluorescent signal over baseline indicating some structural rearrangement and amyloid structure formation with increasing storage temperature. In most cases the major protein present in the samples was albumin either purified or in plasma, but there were also preparations of purified immunoglobulin or alpha-1-proteinase inhibitor. Therefore, it is entirely possible that freeze-dried D-dimer could undergo the same structural rearrangement and aggregation on storage after freeze-drying, which would explain the loss of D-dimer antigen response noted above. Conservative statistical analysis using Tukey’s method for comparing this family of estimates gave statistically significant differences for all pairwise comparisons above and including 20°C. Interestingly, if only samples that included trehalose in the freeze-dried formulation were analyzed, the only statistically significant difference was for the −20 and 45°C pair.

### 4 | DISCUSSION

Freeze-dried WHO international standards are generally very stable and have shelf lives of years to decades, and stability at −20°C must be confirmed by degradation studies (for example, the study to establish the 4th International Standard for Streptokinase found no loss of activity of standards in collaborative studies over a 30-year period). The unusual instability of D-dimer or FDP was manifested in two ways as shown in Figures 1 and 3. In the case of preparation SS258, the patient plasma with elevated D-dimer, there was a
rapid decline in measured D-dimer at all temperatures during storage. The rate of loss was estimated to be 10%–18% per year, though these estimates rely on an assumption of simple first order kinetics, which may not be justified. In the case of SS444, the freeze-dried FDP, there was a poor recovery of added D-dimer antigen in formulations A–C compared to formulation D, which included plasma, Heps buffer, and trehalose. Longer term, D-dimer measurements were stable in sample D during storage at 4°C and below. It is not clear what the mechanism might be for the loss of D-dimer antigen in these freeze-dried samples, but based on previous studies it is known that FDPs have a tendency to aggregate, which could mask available D-dimer antigen sites.

**TABLE 3** Derived rate constants and calculated % loss of activity per year for storage of SS444 formulation D at various temperatures

| Temperature | k (SE) year\(^{-1}\) | % Loss per year |
|-------------|---------------------|----------------|
| -70         | 0                   | 0              |
| -20         | 0.00016 (0.00012)   | 0.016          |
| 4           | 0.008 (0.0033)      | 0.801          |
| 20          | 0.076 (0.018)       | 7.32           |
| 37          | 0.64 (0.043)        | 47.4           |
| 45          | 1.62 (0.067)        | 80.2           |

**FIGURE 3** D-dimer measurements over time in samples of SS444, freeze-dried fibrin degradation product (FDP) in four formulations (A–D), stored at various temperatures at the times shown. The panels correspond to results for 10 µg/ml FDP added to 10 mM HEPES buffer, pH 7.4, containing 0.15 M NaCl, with additions: (A) 10 mg/ml trehalose, (B) 10 mg/ml sucrose, (C) plasma, and (D) plasma + 10 mg/ml trehalose. Note: no measurements were made after 1.33 years for samples A and B or the 45°C sample at 1.65 years because of insolubility.

**FIGURE 4** Observed versus predicted percent activity remaining in ampoules of SS444 stored at elevated temperatures over time.

The propensity of fibrinogen to aggregate, sometimes with the formation of amyloid structures, is well known. For example, there are a group of hereditary fibrinogen amyloidosis disorders associated with mutations that cause increases in β-sheet regions and self-assembly of fibrinogen with amyloid fibril deposition. Other heterogeneous amyloid-promoting molecules such as β-amyloid or serum amyloid A can bind to fibrinogen and cause oligomerization.
with associated amyloid structure development. Aggregation or gelation of fibrinogen or FDP by histones has also been observed.\textsuperscript{40,41} Fibrin polymerization catalyzed by thrombin does not normally result in development of $\beta$-sheet from fibrinogen $\alpha$-helix, although stressed fibrin will show such secondary structure rearrangements.\textsuperscript{42,43} However, aggregates formed by FDPs during fibrinolysis have been observed in many studies,\textsuperscript{29-32,44} and in some cases these have been associated with development of $\beta$-sheet amyloid structures that bind ThT.\textsuperscript{33,34} Using a column clot perfusion system to generate and analyze FDPs from lysing clots, Walker and Nesheim\textsuperscript{32} identified the structures and molecular weights of large fibrin fragments. Significantly, they also concluded that FDPs bound to fibrin with an affinity proportional to their mass. Therefore, the conclusion from these diverse studies using a variety of methods is that plasmin digestion of fibrin leads to rearrangement of the fibrin secondary structure and generation of large fragments with some amyloid structure. These fragments tend to aggregate, and the larger fragments have higher binding affinity. In the context of D-dimer antigen measurement, it is plausible that this aggregation would lead to a loss of binding sites and reduced D-dimer measurement. This type of association would explain the loss of D-dimer signal over time in the case of SS258 and SS444 preparations. How association of D-dimer fragments in patient samples might affect measurement in clinical samples is worthy of further investigation.

Results from Figure 3 suggest trehalose stabilizes D-dimer measurements in plasma, at least on low temperature storage. If high molecular weight D-dimer fragments are more prone to aggregation,\textsuperscript{32} stabilization with trehalose may lead to an enrichment of these fragments in the freeze-dried preparations SS523 and SS444 (D).
could explain the results in Figure 4 in which the FDP + plasma + trehalose preparation, SS523, behaved very differently to the other standards: the internal standard (a commercial preparation of unknown composition) and SS258 plasma, which were not formulated with trehalose. Alternatively, the FDP preparation that was formulated to contain a heterogeneous mixture of D-dimer fragments may be unrepresentative of D-dimer in clinical samples and hence unsuitable for this reason. In either case, it appears that a synthetic mixture of degraded fibrin products is not a straightforward option as a standard for D-dimer assays. The results shown in Figure 5 comparing the internal standard and SS258 in these three assay pairings suggest that SS258, D-dimer plasma may be a useful standard for harmonization purposes. However, the freeze-dried preparation of SS258 was more than 5 years old (stored at −40°C) by the time this set of experiments was performed, and based on the results of Figure 1, would be a different preparation from that immediately after freeze-drying. Thus, although the results from Figure 5 suggest SS258 would be potentially a useful standard, the instability is not acceptable. Figure 6 includes data from 10 diverse freeze-dried standards that indicate the development of amyloid-like structures that bind ThT, particularly in samples stored at higher temperatures. As mentioned above, these structures are seen in fibrin in solution, but these results suggest that structural rearrangements and aggregation can take place in freeze-dried preparations. Previous studies on pharmaceutical protein solutions have suggested that protein misfolding with amyloid structure formation and aggregation is a general mechanism to explain immunogenicity in aged or stressed therapeutic drug preparations. Our data may be the first time freeze-dried proteins have been shown to generate these structures. Trehalose is widely used as a stabilizer for freeze-dried proteins, but the mechanism of action is not fully understood. Nevertheless, it is interesting that trehalose has been shown to inhibit amyloid plaque formation in the case of β-amyloid peptide that is associated with Alzheimer’s disease.

In summary, it has not so far been possible to make a standard for D-dimer that would be useful in harmonizing results from different assay methods. The current work has highlighted a previously unforeseen problem of stability in freeze-dried D-dimer preparations, but also presents a potential solution to the problem using trehalose as a stabilizer. Further work is required to develop such a standard and prove its utility.

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**CONFLICTS OF INTEREST**

The authors have no conflicts of interest.

**AUTHOR CONTRIBUTIONS**

CL conceived the work, performed experiments, and wrote the manuscript. SB performed experiments and helped with the manuscript.

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