

Status Report

Toward standardization of carbohydrate-deficient transferrin (CDT) measurements: II. Performance of a laboratory network running the HPLC candidate reference measurement procedure and evaluation of a candidate reference material

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Abstract

Carbohydrate-deficient transferrin (CDT) is a descriptive term used for a temporary change in the transferrin glycosylation profile caused by alcohol, and used as a biomarker of chronic high alcohol consumption. The use of an array of methods for measurement of CDT in various absolute or relative amounts, and sometimes covering different transferrin glycoforms, has complicated the comparability of results and caused confusion among medical staff. This situation prompted initiation of an IFCC Working Group on CDT standardization. This second publication of the WG-CDT covers the establishment of a network of reference laboratories running a high-performance liquid chromatography (HPLC) candidate reference measurement procedure, and evaluation of candidate secondary reference materials. The network laboratories demonstrated good and reproducible performance and thus can be used to assign target values for calibrators and controls. A candidate secondary reference material based on native human serum lyophilized with a cryo-lyoprotectant to prevent protein denaturation was found to be commutable and stable during storage. A proposed strategy for calibration of different CDT methods is also pre-

sented. In an external quality assurance study involving 66 laboratories and covering the current routine CDT assays (HPLC, capillary electrophoresis and immunoassay), recalculation of observed results based on the nominal values for the candidate calibrator reduced the overall coefficient of variation from 18.9% to 5.5%. The logistics for distribution of reference materials and review of results were found to be functional, indicating that a full reference system for CDT may soon be available.

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Introduction

Carbohydrate-deficient transferrin (CDT) is a descriptive term used for a temporary change in the glycosylation pattern of transferrin caused by, and used as a biomarker of sustained moderate to high alcohol consumption. Several glycoforms of transferrin are normally present in blood of which tetrasialotransferrin, containing two biantennary disialylated N-glycans (1–3), is the most abundant and accounts for approximately 80% of serum transferrin (4). Two normally minor glycoforms, disialotransferrin which is missing one N-glycan, and asialotransferrin missing both glycans (1–3), occur in increased amounts in response to heavy drinking and are considered the main CDT glycoforms (5). When excessive alcohol consumption is discontinued or much reduced, the transferrin profile normalizes with a half-life of approximately 1.5 weeks (6), and takes approximately 2–5 weeks to return to baseline levels (7).

Many different analytical techniques and methods have been used for measurement of “CDT”, often measuring various transferrin glycoforms and sometimes showing different sensitivity and specificity for alcohol related changes (8, 9). The CDT assays in current routine use are based on high-performance liquid chromatography (HPLC) (10–12), capillary electrophoresis (CE) (13–15), or immunochemistry (16). CDT analysis by HPLC and CE relies on charge-based separation of the glycoforms followed by photometric detection. The relative concentrations to total transferrin are calculated based on peak areas. For immunochemical measurement, at present there is only one CDT immunoassay on

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the market (N Latex CDT, Siemens) since all the indirect methods based on anion-exchange minicolumn separation followed by immunoassay have been withdrawn. N Latex CDT relies on a monoclonal antibody that recognizes a structural change in transferrin missing one or both N-glycans, corresponding to asialo-, monosialo-, and disialotransferrin, followed by nephelometric quantification of these glycoforms (16). The relative CDT values are calculated using a simultaneous total transferrin immunoassay.

CDT methods based on HPLC and CE offer the advantage of graphic visualization of the transferrin profile, and allow qualitative and quantitative determination of individual forms (17). The glycoform pattern also enables detection of potential analytical interferences with CDT as an alcohol-specific biomarker. These potential interferences include genetic transferrin variants (11, 18), congenital disorders of glycosylation (CDG) (19–21), and other interferences that are apparently unrelated to heavy drinking (22–24). Although generating a similar graphic image, HPLC and CE utilize quite different principles for detection of CDT. HPLC methods rely on the selective absorbance of the iron-transferrin complex at approximately 460–470 nm (25), whereas CE detection is based on UV absorption by the peptide bond at approximately 200 nm. The rather non-specific detection principle employed in CE occasionally hampers the correct determination of CDT due to co-migrating interferences (14, 26, 27).

The use of a wide range of methods for CDT measurement and expressing the result in various absolute or relative amounts, covering either a distinct species (typically disialotransferrin) or a mixture of glycoforms, has complicated the comparability of analytical and clinical results (5, 11, 28). The associated array of method-related cut-off thresholds also has caused much confusion among medical staff when CDT is used for routine screening and the follow-up of heavy drinking. The structural complexity of transferrin glycoforms has delayed development of reference materials on which to base interassay standardization.

These problems prompted the initiation of a Working Group on Standardization of CDT (WG-CDT) under the auspices of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). The goal of standardization is that different measurement procedures should produce comparable results, permitting the use of a common cut-off threshold. However, a problem with CDT testing is that some methods differ in the measurand used. In the first publication of the WG-CDT (5), disialotransferrin (i.e., disialylated mono-glycan transferrin) (3) was defined as the single measurand for CDT standardization and the primary, but not sole (29), target analyte for CDT measurements. The basis for choosing disialotransferrin over asialotransferrin or the sum of disialotransferrin and asialotransferrin, is that disialotransferrin represents the major glycoform measured with all current CDT methods. Also, standardization is difficult to perform for a mixture of two analytes. Moreover, because asialotransferrin is only detected following heavy drinking and thus always accompanies an already increased disialotransferrin concentration (4, 11), measurement of asialotrans-

ferrin alone will yield low diagnostic sensitivity (30). HPLC was considered the best currently available candidate for a reference measurement procedure, due to high analytical sensitivity and an inherent low risk for test interference (5). Furthermore, reporting disialotransferrin or CDT values as a percentage of the total transferrin concentration was strongly recommended because this strategy compensated for falsely high and falsely low absolute values observed in cases of increased or decreased total transferrin concentrations (23).

This publication covers the continuing CDT standardization activities of the WG-CDT, including the establishment of a network of reference laboratories and evaluation of candidate reference materials. The results of a ring trial and a proposed strategy for calibration of CDT methods are also presented.

Establishment of a network of CDT reference laboratories

Based on the WG-CDT recommendation for HPLC as the analytical principle for a candidate reference measurement procedure (5), the organization of a network of reference laboratories using the proposed HPLC method (11) was initiated. The network laboratories are intended to establish target values for calibrator materials that can be used for preparation of CDT controls and external quality assurance (EQA) materials that can be applied with the different routine methods. At present, there are five CDT network laboratories; four located in European countries (in France, in The Netherlands, and two in Sweden) and one in the USA. For reason of independence, the network does not involve diagnostic companies.

CDT analysis by the HPLC candidate reference measurement method is done according to a published procedure (11). In this method, pre-treatment of the serum sample includes iron-saturation with FeNTA and precipitation of lipoproteins, followed by chromatographic separation of the transferrin glycoforms with an anion-exchange column (SOURCE 15 Q, GE Healthcare) using salt gradient elution. Quantification of individual glycoforms is performed by monitoring the absorbance of the transferrin-iron complex at 470 nm. The method uses baseline integration for all peaks from asialo- until hexasialotransferrin, because the results obtained in this manner are less sensitive to variations in peak resolution. In accordance with the WG-CDT recommendation (5), disialotransferrin is the single measurand and the amount is calculated as the relative amount (%) to total transferrin based on peak areas. In the event that asialotransferrin is detected, it is also included in the total transferrin peak area. To ensure robust performance, in addition to published information, an extended standard operating procedure and a technical maintenance appendix have been delivered to the network laboratories.

To verify that the disialotransferrin results originating from the network laboratories show acceptable and reproducible agreement, an intercomparison study was initiated. Starting in 2007 and proceeding until the beginning of 2010, five

Table 1 Results of a CDT network laboratory intercomparison study for the HPLC candidate reference measurement procedure.

Year-Nr	Network laboratories (n)	Sample type	Sample origin	Disialotransferrin		
				Range, %	Mean (95% CI); median, %	CV ^a , %
2007-1 ^b	3	Lyophilized	Serum (Bio-Rad low calibrator)	1.61-1.89	1.76 (1.70-1.83); 1.81	6.0
	3	Lyophilized	Serum (Bio-Rad high calibrator)	5.28-5.39	5.34 (5.32-5.36); 5.34	0.7
2008-1	5	Serum	Serum	0.84-1.12	1.00 (0.95-1.05); 1.01	8.9
	5	Serum	Serum	2.40-2.89	2.60 (2.52-2.68); 2.63	5.5
	5	Serum	Serum	4.04-4.55	4.23 (4.15-4.31); 4.19	3.3
	5	Lyophilized	Serum (Bio-Rad low control)	1.11-1.58	1.33 (1.26-1.41); 1.31	10.0
	5	Lyophilized	Serum (Bio-Rad high control)	3.11-3.50	3.32 (3.24-3.40); 3.31	4.1
2009-1	5	Serum	Serum	1.11-1.52	1.34 (1.28-1.40); 1.37	8.1
	5	Serum	Serum	3.04-3.38	3.18 (3.12-3.23); 3.20	3.2
	5	Lyophilized	Serum (Bio-Rad low control)	0.95-1.18	1.05 (1.01-1.09); 1.04	6.9
	5	Lyophilized	Serum (Bio-Rad high control)	3.04-3.44	3.22 (3.15-3.29); 3.20	3.7
	5	Serum	Serum	2.26-2.50	2.42 (2.37-2.48); 2.44	3.2
2009-2	5	Lyophilized 1 ^c	Same serum conventionally lyophilized	2.26-2.54	2.39 (2.33-2.46); 2.42	3.8
	5	Lyophilized 2 ^c	Same serum lyophilized with a cryo-lyoprotectant	2.30-2.65	2.43 (2.36-2.51); 2.40	4.2
	5	Lyophilized 3 ^c	Same serum lyophilized after delipidation	2.25-2.54	2.39 (2.32-2.46); 2.40	4.1
	5	Serum	Serum	1.03-1.22	1.11 (1.07-1.16); 1.10	5.4
	5	Lyophilized-2	Same serum lyophilized after delipidation	1.02-1.29	1.13 (1.06-1.19); 1.14	8.0
2010-1	5	Serum	Serum	2.77-3.01	2.90 (2.84-2.96); 2.91	3.0
	5	Lyophilized-2	Same serum lyophilized after delipidation	2.78-3.04	2.91 (2.85-2.96); 2.92	2.6

^aCalculations are based on all results of triplicate or duplicate measurements. ^bResults for ring trials 2007-1 until 2009-1 are based on triplicate measurements, 2009-1 until 2010-1 on duplicate measurements. ^cThree candidate reference materials were evaluated; Material 1, serum with conventional lyophilization; Material 2, serum lyophilized with a cryo-lyoprotectant; Material 3, serum lyophilized after delipidation. All lyophilized materials were reconstituted in water.

trials have been conducted, involving different sets of ready-for-use and lyophilized serum materials. Some high-CDT specimens have been native serum collected from heavy drinkers and some were control serum spiked with isolated disialotransferrin (3). All analyses were performed in blinded fashion and the results returned blinded for evaluation. Starting with the second ring trial in 2009, the distribution of samples and review of results has been performed by the WG-CDT network coordinator. The serum samples used within this part of the CDT standardization work are pools prepared from deidentified leftover serum from routine samples, as approved by the Local Ethics Committee.

To further improve harmonization of disialotransferrin measurements between the network laboratories, copies of HPLC chromatograms have been returned for fine-tuning of peak integration and, in cases where it was indicated not to be optimal, individual feedback. Variability in defining the start and end of small peaks can make a significant difference for minor glycoforms, such as disialotransferrin. The CDT network laboratories use automated integration settings followed by visual checks and, if required, manual re-integration according to the standard procedures for clinical samples.

The results of the intercomparison study with the network laboratories are shown in Table 1. As expected, imprecision depended on the CDT concentration in the sample with the highest coefficient of variation (CV) obtained at low CDT concentrations, and vice versa. Nevertheless, the interlaboratory CV has been gradually improving during the course of the quality control scheme (Figure 1). At concentrations near and above the upper limit of the reference interval for the HPLC candidate reference method (the mean +2 SD is

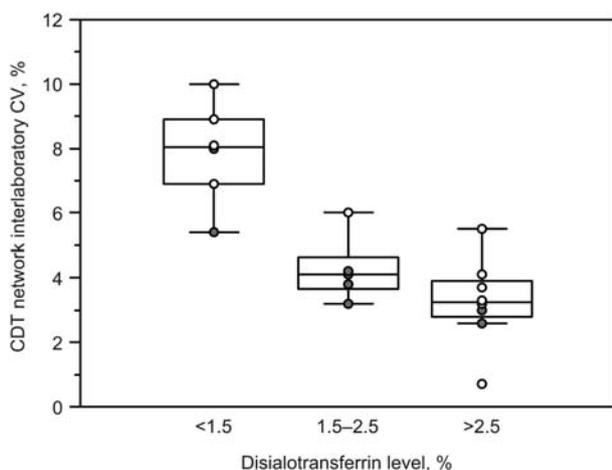


Figure 1 Box-and-Whisker plots of mean values from a CDT network laboratory intercomparison study for ready-for-use and lyophilized serum materials with low, intermediate and high disialotransferrin concentrations according to the HPLC candidate reference measurement procedure.

The Box-and-Whisker plot shows the 25th–75th percentile (box), the median (horizontal line in box), range, and outside values. Filled symbols represent the results obtained in the last two intercomparison trials.

approx. 1.7% disialotransferrin) (11, 31), the CV has typically been <5%. This implies that the network laboratories are able to assign values for CDT reference materials with an analytical uncertainty <0.1% disialotransferrin near the upper level of the reference interval. However, based on the results of the 2009–2 network intercomparison (Table 1), which comprised one native serum pool and three lyophilized preparations (i.e., essentially four identical samples), the intra-laboratory CV (range 1.0%–2.4%) was lower than the interlaboratory CV (3.2%–4.2%), demonstrating that there is still some room for further improvement.

Commutability and stability of CDT candidate reference materials

One important issue within the CDT standardization activities is to establish procedures for the production and distribution of reference materials for the selected analyte. A reference material should be suitable for use with any measurement procedure and stable when stored. The commutability of three lyophilized CDT candidate reference materials prepared from the same native human serum pool with a disialotransferrin concentration of approximately 2.4% (Material 1: the serum was frozen at -80°C followed by conventional lyophilization for 2 days at 5°C ; Material 2: the serum was added with a patented cryo-/lyoprotectant CLP No. 5 to prevent denaturation of protein, frozen at -80°C and lyophilized for 3 days at 20°C ; Material 3: the serum was delipidized with dextran to prevent turbidity, frozen at -80°C and lyophilized for 2 days at 5°C ; the materials were prepared by the network coordinator according to in-house procedures) were evaluated in a ring trial (2009–2), including the network laboratories. In addition, 66 laboratories participating in the Swedish EQUALIS ($n=52$) and the Dutch SKML ($n=14$) EQA schemes for CDT and six in-house laboratories of the CDT manufacturer's taking part in the standardization work were also included. In this way, all current routine methods for CDT measurement were covered.

There was no evidence for marked differences in CDT results between the ready-for-use serum and three lyophilized materials with any of the methods. The interlaboratory CV (based on single measurements) for the EQA participants ranged from 3.6% to 4.4% ($n=18$) for the non-commercial HPLC methods [i.e., methods based on (6, 11)], 5.0%–7.7% ($n=11$) for the Bio-Rad %CDT by HPLC assays, 2.4%–5.7% ($n=5$) for Capillarys CE (Sebia), 3.7%–5.8% ($n=3$) for CEofix CE (Analis), and from 5.6% to 6.4% ($n=26$) for the N Latex CDT immunoassay (Siemens). Statistically significant differences in results between the native and lyophilized materials were only noted for N Latex CDT. Compared with the ready-for-use serum, the conventionally lyophilized serum (Material 1; Figure 2A) yielded slightly higher N Latex CDT values ($p<0.05$), while serum lyophilized following delipidation (Material 3; Figure 2C) gave slightly lower values ($p<0.05$). These effects were more noticeable when the results for these two materials were plot-

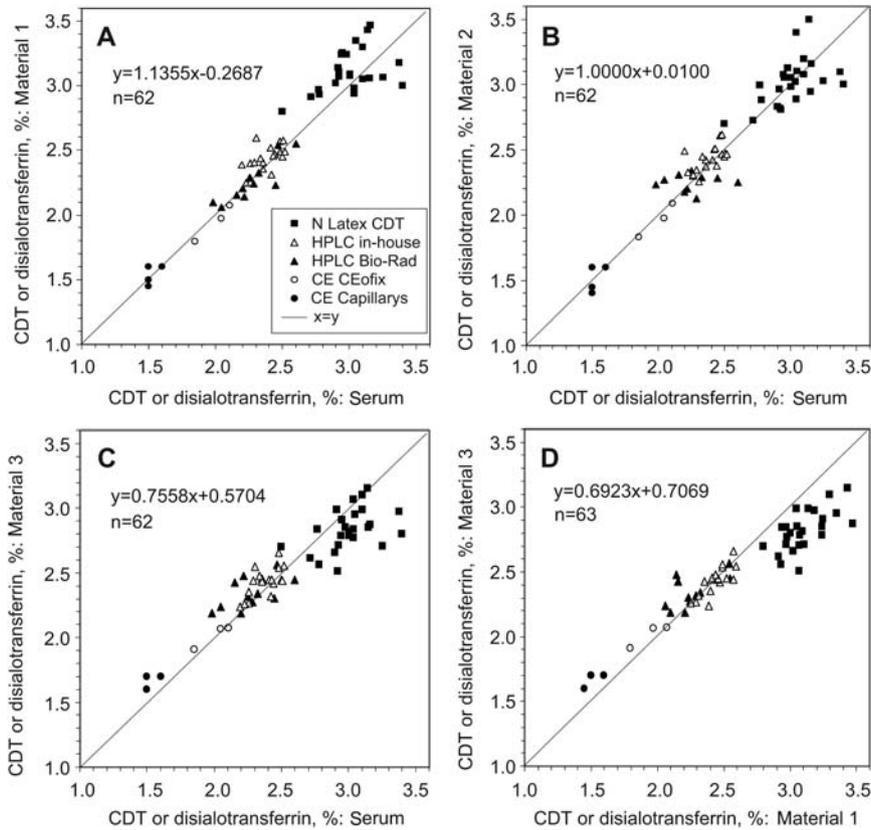


Figure 2 CDT results (% CDT or %disialotransferrin) for three lyophilized CDT candidate reference materials (Material 1, serum with conventional lyophilization; Material 2, serum lyophilized with a cryo-/lyoprotectant to prevent denaturation of protein; Material 3, serum lyophilized after delipidation to prevent turbidity) plotted against the results with the original native human serum sample. For the N Latex CDT immunoassay, there was a statistically significant ($p < 0.05$) positive bias for Material 1 (A), and a significant ($p < 0.05$) negative bias for Material 3 (C). This effect is further highlighted in graph D. Only Material 2 was found to be commutable (B). It should be noted that the various assay procedures produce different values for %CDT or %disialotransferrin, with the N Latex CDT method producing higher values and the Capillarys CE method producing lower values than the HPLC candidate reference and other methods. The results for one in-house CE method and one using the Recipe HPLC kit were not included. Each symbol represents the result for one laboratory. The Passing-Bablok regression equations are given.

ted separately (Figure 2D). Based on these results, only the serum lyophilized with a cryo-/lyoprotectant to prevent protein denaturation (Material 2) was indicated to be commutable, the slope of the regression line was one with a minimal intercept (Figure 2B).

An interesting observation from the EQA study was that the results measured in-house by the CDT manufacturers sometimes differed considerably from the corresponding results reported by their customers. The results from both CE methods showed good overall agreement, but the Bio-Rad manufacturer’s laboratory obtained a 6% higher HPLC value compared with the average for their customers. For the N Latex CDT immunoassay, the in-house value from Siemens was 17% lower. These observations highlight the value of diagnostic assay manufacturers taking part in EQA programs, and the need to confirm that customers perform their methods according to the instructions.

To examine the long-term stability of the three lyophilized CDT candidate reference materials, the material was stored at various temperatures and analysed periodically with the HPLC candidate reference measurement procedure, the

Capillarys CE assay, and the N Latex CDT immunoassay. Measurements were performed blinded and comparisons were made with the results obtained with the original serum sample stored frozen, where CDT is known to be stable (11, 32). After storage for 3 months, all materials were shown to be stable at -84°C with all three methods (Table 2). Using the N Latex CDT, there was no indication of decreased results under any storage condition. However, the HPLC method gave slightly lower results (89% and 93%, respectively, of values for frozen serum) compared with the conventionally lyophilized Material 1, and the material lyophilized after delipidation (Material 3) when stored at room temperature (20°C). With the Capillarys CE assay, all lyophilized materials produced lower values when stored at 20°C , and this was also seen at 4°C for Material 1 which was lyophilized conventionally. These results indicate that lyophilization of serum with a cryo-/lyoprotectant to prevent protein denaturation (Material 2) is the best method for production of a commutable and stable CDT secondary reference material. The stability assays will be repeated following storage for 6, 12, 24 and 36 months.

Table 2 Stability of three lyophilized CDT candidate reference materials after storage for 3 months at different temperatures^a.

Specimen	Storage temperature, °C	CDT (% of values for serum stored at -84°C)		
		N Latex CDT (Siemens)	Capillarys CE (Sebia)	HPLC candidate reference measurement procedure
Original serum	-84	100 ^b	100	100
	-20	105	100	100
Lyophilized Material 1: Serum with conventional lyophilization	-84	100	100	100
	-20	97	103	99
	4	102	85	99
	20	101	79	89
Lyophilized Material 2: Serum lyophilized with a cryo-/lyoprotectant against denaturation	-84	100	100	100
	-20	98	100	102
	4	99	100	98
	20	100	88	102
Lyophilized Material 3: Serum lyophilized after delipidation	-84	100	100	100
	-20	98	94	98
	4	98	94	97
	20	98	79	93

^aMeasurements were performed at Meander Medical Center, Amersfoort, The Netherlands. ^bResults are mean values of duplicate determinations.

A proposed strategy for calibration of different CDT assays

In accordance with many previous observations, CDT values generated by different analytical procedures, and sometimes also for assays using the same technology, are not interchangeable. However, the aim of the standardization work is that different methods should produce comparable results, emphasizing the importance of having access to reference materials. Standardization would be achieved if all measurement procedures were linked to a commutable calibrator with

an assigned, traceable CDT or, rather, disialotransferrin value.

To simulate such an approach, a preliminary one-point calibration was done for the results of the EQA ring trial. The calculations were based on nominal disialotransferrin values assigned by the network laboratories for the native serum and the candidate reference Material 2 which was lyophilized with a cryo-/lyoprotectant to prevent denaturation of protein. The observed results and those obtained following virtual calibration (Figure 3) demonstrated that the interassay and interlaboratory CV was reduced considerably, producing

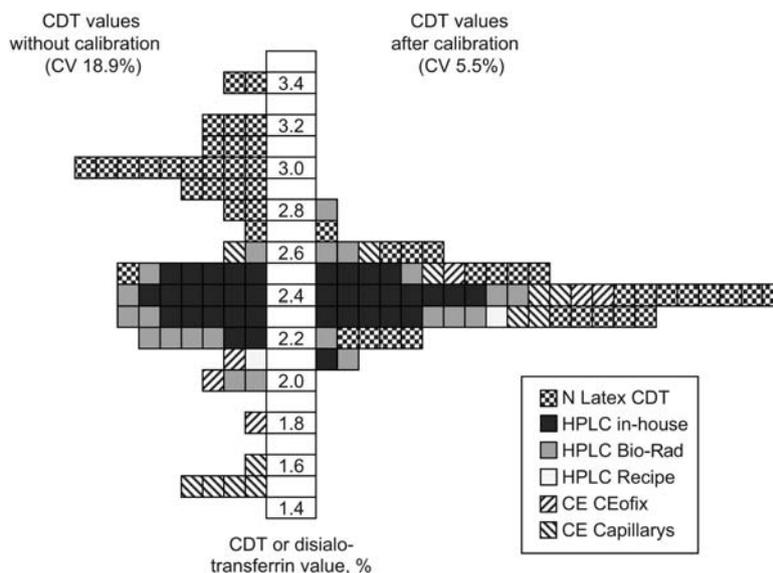


Figure 3 Observed results for CDT or disialotransferrin by different methods originating from 64 laboratories participating in the EQA programs (left), and the corresponding values after a one-point virtual calibration based on the relationship between observed and nominal values for the commutable Material 2 [i.e., $\text{sample}_{\text{observed value}} \times (\text{calibrator}_{\text{assigned value}} / \text{calibrator}_{\text{observed value}})$]. Each square represents the result from one laboratory.

more comparable results due to the elimination of systematic differences between methods. During routine use, the calibration curve needs to include several levels of a commutable and traceable calibrator, not only to comply with official guidelines, but also due to non-linear correlation and deviating CDT concentrations between some methods (15–17).

Conclusions

The first publication of the IFCC Working Group on CDT standardization was devoted to defining the measurand and proposing a candidate reference method. In this second publication, further standardization activities are presented, including the establishment of an international network of reference laboratories using the HPLC candidate reference measurement procedure and evaluation of candidate secondary reference materials. The network laboratories demonstrated good and reproducible performance with no outliers. Thus, their results can be used to assign target values for calibrators and controls. A candidate secondary reference material based on native human serum lyophilized with a cryo-/lyoprotectant to prevent denaturation of protein was demonstrated to be commutable and stable on storage. A preliminary strategy for calibration of different CDT methods was also presented, based on the results from laboratories participating in EQA programs. The logistics for distribution of reference materials and collection and review of the results by the network coordinator were found to be functional, indicating that the tools for a full CDT reference system may soon be available.

Remaining issues of the CDT standardization work include focus on the development of a traceable primary reference material (3), and for an approved CDT reference measurement procedure. It might also be necessary to adjust the unit from percentage to an SI unit. Finally, the WG-CDT will make recommendations for the clinical and forensic use of the test.

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Conflict of interest statement

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