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Standardisation and use of the alcohol biomarker carbohydrate-deficient transferrin (CDT)☆

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ABSTRACT

Carbohydrate-deficient transferrin (CDT) is a glycoform profile of serum transferrin that increases in response to sustained high alcohol intake and over the last decades has become an important alcohol biomarker with clinical and forensic applications. However, the wide range of CDT measurement procedures has resulted in lack of uniform results and reference limits, and hampered comparison of results. In 2005, the IFCC therefore founded a special working group (WG) aiming for standardisation of CDT measurement. This review summarises the history of CDT and the actions taken by the WG-CDT. Initial steps included the definition of the measurand (serum disialotransferrin to total transferrin fraction expressed in %), and the determination of a well-defined anion-exchange HPLC procedure as the candidate reference measurement procedure (cRMP). Subsequent achievements were the establishment of a network of reference laboratories to perform the cRMP, setting a reference interval, and development of a reference material based on human serum for which the laboratory network assign values. Using a set of reference materials for calibration allowed for achieving equivalence of results of all present CDT measurement procedures. The final steps of the WG-CDT have been a full validation of the cRMP to make it an IFCC approved RMP, and providing guidance for international standardisation of all CDT measurement procedures.

1. Transferrin glycoforms as alcohol biomarker — background on CDT

A substantial part of the global burden of disease is attributable to excessive alcohol consumption, being ranked among the top five risk factors for disease, disability and death throughout the world [1]. In attempts to reduce harmful alcohol use and its negative health, social and economic consequences, early detection of those engaged in heavy drinking is essential. Questionnaires on the frequency and quantity of drinking and other self-report measures are employed for this purpose, due to their ease of use and low cost [2,3]. Although convenient in routine clinical use, the data obtained in this way often suffer from poor reliability [4,5] and are not suitable for follow-up of heavy drinkers or for medico-legal purposes.

A large number of laboratory tests have therefore been evaluated as objective measures of acute and chronic alcohol consumption or alcohol-related toxicity. In addition to standard blood or breath alcohol testing that can only detect alcohol intake in the past hours [6], blood tests traditionally employed as biomarkers for long-term heavy drinking are "liver function tests" (the enzymes gamma-glutamyltransferase (GGT), and alanine and aspartate aminotransferase (ALT and AST)), and the mean corpuscular volume of erythrocytes (MCV) [7,8]. A limitation with these tests is that they suffer from poor specificity for alcohol-related effects. Today, however, a panel of sensitive and specific direct and indirect long-term

☆ Previous publications from the IFCC WG have alternatively referred to standardisation and harmonisation of CDT. We now use the term standardisation, as discussed in the text.

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[1] Current WG-CDT members are AH, JWi (chair), RA, VB, JD, JWh, CW and FS.

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(chronic) and short-term (acute) alcohol consumption related biomarkers are available, including the transferrin glycoform profile known as carbohydrate-deficient transferrin (CDT) [9], phosphatidylethanols (PEth) that are formed from phosphatidylcholines and ethanol by enzymatic action [10,11], and conjugated ethanol metabolites (ethyl glucuronide and ethyl sulphate) [12–14].

The iron transport glycoprotein transferrin shows natural microheterogeneity, owing to variations in iron load, amino acid sequence (i.e. genetic variants), and the structure of the two N-linked oligosaccharides (N-glycans) that can be bi-, tri-, or tetra-antennary [15,16] (Fig. 1). In human serum, tetrasialotransferrin is usually the most abundant glycoform (~80%), followed by pentasialo- (~14%), trisialo- (~4%), disialo- (~1%), and hexasialotransferrin (~1%) [17]. In the mid-1970s, a temporary change in the transferrin glycoform profile in serum and cerebrospinal fluid was demonstrated to be associated with sustained heavy alcohol consumption [18], corresponding to daily intake averaging at least 40–60 g ethanol over at least 1–2 weeks [19]. The abnormal serum transferrin pattern, initially presenting as an increased amount of transferrin bands with pI ≥ 5.7 (corresponding to disialo-, monosialo- and asialotransferrin) in isoelectric focusing (IEF) (Fig. 2), normalised on abstinence with a half-life of ~10 days [20]. Measurement of this protein fraction, later named CDT, was suggested as a specific biomarker to identify sustained heavy alcohol consumption and monitor abstinence during treatment [9].

2. Need for standardisation of CDT

Development of a CDT method suitable for routine clinical use turned out to be challenging and the first commercial test kit (CDTect) [21] for use on serum samples was not introduced until 1992. In the CDTect and similar assays, a CDT fraction was separated from the other transferrin glycoforms by ion-exchange chromatography on disposable mini-columns, followed by immunochemical measurement of the transferrin amount in the CDT fraction [22,23]. Subsequently, a direct CDT immunoassay [22,24], and methods based on high-performance liquid chromatography (HPLC) [20,25] and capillary electrophoresis (CE) [26–29], intended for routine quantification of serum CDT have replaced the indirect immunoassays.

A problem related to CDT measurement is that different methods cover variable fractions of the transferrin glycoforms (i.e. different analytes) normally present in blood (Fig. 3). In addition, because CDT measurement was not standardised, a wide array of method-dependent cut-off values have been used. Another inconsistency between CDT methods is that the test result has been expressed as either absolute (e.g. mg/L or U/L) or relative (e.g. % of total transferrin or % of disialotransferrin) amounts [30]. Because these differences complicated interpretation of CDT results across methods and studies, and limited its clinical and forensic use as alcohol biomarker, the need for standardisation of CDT measurement became obvious. The goal is that different methods should produce identical results, permitting use of common cut-offs. An example of the value of such work was obtained from a successful national harmonisation of CDT measurement by

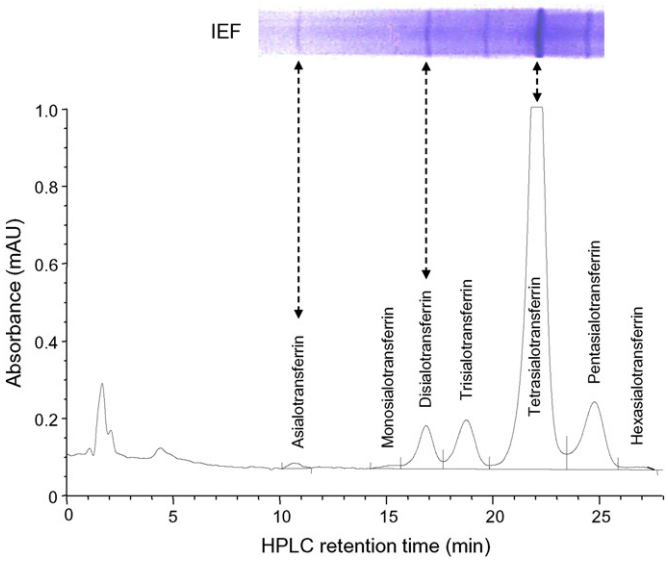

Fig. 2. Separation of transferrin glycoforms in a serum sample from a heavy drinker by the high-performance liquid chromatography (HPLC) method employed as candidate reference measurement procedure (cRMP) for carbohydrate-deficient transferrin (CDT) [50]. In the HPLC cRMP, the disialotransferrin to total transferrin fraction (%) is determined based on peak areas. Separation of serum transferrin glycoforms by thin-layer isoelectric focusing (IEF) is also shown.

HPLC methods (i.e. agreement on a standard operation procedure and use of baseline instead of valley-to-valley peak integration) performed in Sweden in 2003 within the Equalis external quality assurance (EQA) scheme, that reduced the between-laboratory coefficient of variation from ~16% to ~8% (unpublished data).

Taken together, this prompted the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) to initiate a Working Group on Standardisation of CDT (WG-CDT) in 2005. Initial terms of reference for the IFCC WG-CDT were to define and validate the measurand and standardise the nomenclature, select a reference method and set up a network of reference laboratories, work out procedures for the production of commutable reference materials, and make suggestions for the clinical usage of CDT including establishment of reference intervals.

3. Definition of the analyte and measurand

The deglycosylated (i.e. "carbohydrate-deficient") transferrin forms were initially believed to miss only the terminal sialic acid residues on one or both N-glycans. However, subsequent studies revealed that, compared with tetrasialotransferrin, disialo- and asialotransferrin are missing one and both complete N-glycans, respectively [31–33].

The variable combinations of asialo-, monosialo-, disialo- and trisialotransferrin measured by different CDT methods (i.e. different analytes) posed a significant problem for standardisation of CDT

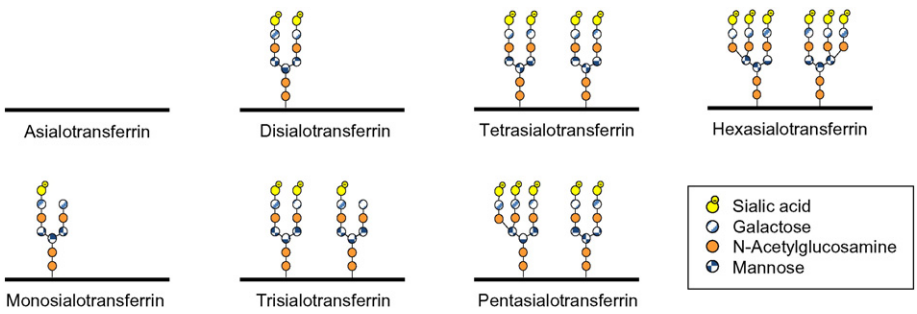

Fig. 1. Structural illustration of different human transferrin glycoforms. Following sustained heavy alcohol consumption, the amounts of disialo- and asialotransferrin to total transferrin fraction (%) in serum become increased while the amount of tetrasialotransferrin is correspondingly reduced [17].

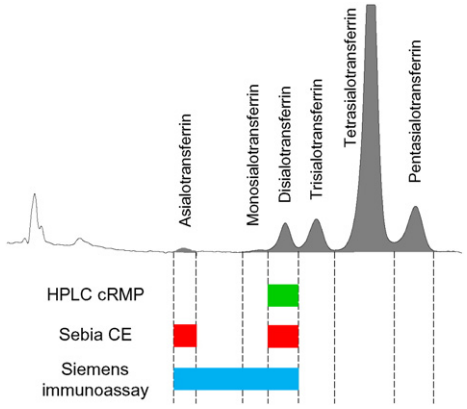

Fig. 3. Visualisation of the transferrin glycoforms measured by a selection of the currently used CDT measurement procedures, to highlight the differences in the "CDT" analyte. It should be noted that most but not all HPLC and CE methods can selectively measure disialotransferrin, or the sum of disialo- and asialotransferrin. HPLC cRMP, high-performance liquid chromatography candidate reference measurement procedure for CDT [50]; Sebia CE, Sebia CAPILLARYS CDT capillary zone electrophoresis assay (Sebia, Lisses, France); Siemens immunoassay, Siemens N Latex CDT immunonephelometric assay (Siemens, Marburg, Germany).

measurement. Trisialotransferrin was later demonstrated to be unrelated to alcohol consumption [17,34,35]. Inclusion of this glycoform was therefore problematic for the accuracy of CDT testing, since serum samples containing a high trisialotransferrin level, not related to heavy alcohol use, could generate false positives (high CDT test results) [36,37]. Furthermore, the level of monosialotransferrin, albeit usually making up only a very small proportion of total serum transferrin, is unrelated to alcohol use but correlates well with the trisialotransferrin level [17]. Hence, in cases with a markedly elevated serum trisialotransferrin concentration [38], inclusion of monosialotransferrin in the definition and measurement of CDT could also generate a false positive result.

Asialo- and disialotransferrin, on the other hand, are both causally and directly related to heavy alcohol consumption in a dose-related manner [17,19]. Still, asialotransferrin is not measurable in serum samples from abstinent and socially drinking subjects by the currently used routine CDT methods, but becomes elevated by chronic heavy drinking and is only detectable when the disialotransferrin concentration is already increased [17,34]. The diagnostic sensitivity of asialotransferrin is therefore lower than for disialotransferrin. Furthermore, because the standardisation work should focus on the clinically most relevant and for simplicity reasons a single analyte, serum disialotransferrin (i.e. disialylated mono-glycan transferrin) (Fig. 1) [33] was chosen as the single target for CDT standardisation [39]; it is also the primary but not sole transferrin fraction for CDT determination [40].

The use of CDT to total transferrin ratio values, instead of disialotransferrin concentration values only, was demonstrated to significantly reduce variability of CDT analysis results, as this compensated for variations in sample properties due to differences in the serum total transferrin concentration that occur in certain conditions (e.g. anemia, pregnancy, use of oral contraceptives, and carcinoma) [41]. Accordingly, the measurand for CDT standardisation by the WG-CDT was defined as disialotransferrin to total transferrin fraction (%) in serum, measured by HPLC with spectrophotometric measurement of iron-saturated transferrin at 470 nm [39].

4. Selection of a candidate reference method for CDT

Testing for drugs of abuse often involves a two-step approach with initial preliminary (immunoassay) screening followed by confirmatory analysis of "preliminary positive" results by methods based on mass spectrometry (MS). Due to its high selectivity, MS is usually the preferable technique for a reference method. However, although qualitative and semi-quantitative MS measurement procedures for serum transferrin glycoforms were published [31,33,42], there is no method allowing for quantitative measurement of all different glycoforms.

Among the chemical principles and laboratory techniques employed for measurement of serum CDT, specific advantages of measurement procedures based on HPLC and CE include the separation and quantification of the full spectrum of transferrin glycoforms. Furthermore, the visible documentation of the transferrin profile (in chromatograms or electropherograms) makes it possible to detect potential analytical interferences, including a variety of genetic transferrin variants [34,43], acquired changes in transferrin glycosylation particularly caused by severe liver pathology [44,45], and subtypes of rare congenital disorders of glycosylation (CDG) [46,47].

Of these two techniques, HPLC with spectrophotometric detection was considered the best candidate as a reference method, because the identification and measurement relies on the relatively specific absorbance of iron-saturated transferrin at ~470 nm [48]. In contrast, CE relies on the rather unspecific measurement of the UV absorption by the peptide bond at ~200 nm, making such methods susceptible for analytical interference by other biomolecules with similar electrophoretic characteristics (e.g. CRP, free light chains, and intact immunoglobulins). In addition, the analytical sensitivity (detection limit) of CE was reported to be lower than for HPLC [49].

A well-defined HPLC method for identification and quantification of serum transferrin glycoforms based on peak areas, and using a commercially available column and chemicals [50], was selected as a candidate reference measurement procedure (cRMP) for serum CDT (disialotransferrin to total transferrin fraction (%)), until a potential MS method with better performance may become available [39]. In the HPLC cRMP method, pre-treatment of the serum sample includes iron-saturation with ferric nitrilotriacetic acid (FeNTA) and precipitation of lipoproteins, followed by chromatographic separation of the transferrin glycoforms on an anion-exchange column using salt gradient elution. The quantification of individual glycoforms is performed by monitoring the specific absorbance of the transferrin–iron complex at 470 nm. In serum samples pre-treated with an anti-transferrin antibody to remove all transferrin, no co-migrating interfering peaks were seen [50], proving high specificity for transferrin glycoforms. The method uses baseline integration for all peaks from asialo- until hexasialotransferrin (Fig. 2), and the amount of disialotransferrin is calculated as the relative amount (%) to total transferrin, based on peak areas, whereby total transferrin represents the sum of the peak areas for asialo- until hexasialotransferrin.

Further evaluation of the HPLC cRMP within the WG-CDT revealed a very good agreement of the measured results for a transferrin-free serum spiked with the different isolated transferrin glycoforms and the theoretical (i.e. gravimetric) values. A close agreement (r^2 close to 1) between measured and calculated disialotransferrin to total transferrin fraction (%) values was also obtained, for a serum pool spiked with different amounts of isolated disialotransferrin [33]. These results indicated that different transferrin glycoforms have similar molar absorption coefficients, and that the relative (%) peak area of iron-saturated serum disialotransferrin to total transferrin is expected to be numerically close to the amount of substance fraction of disialotransferrin to total transferrin expressed in % as it would be determined by an MS-based method. Accordingly, because the cRMP does not require pure substance calibrators, target values for secondary calibrators can be assigned by the network of CDT reference laboratories (Schellenberg et al., manuscript in preparation) or other experienced users of the cRMP.

5. Establishment and performance of a network of CDT reference laboratories

Based on the goals for the WG-CDT, an international network of experienced CDT laboratories running the HPLC cRMP was organised, to ensure long-term stability of reference measurement services. The network laboratories establish target values for calibrator materials that

can be used for preparation of other CDT reference materials and in EQA schemes. In order to further improve agreement of CDT measurement between the network laboratories, extended standard operation and technical maintenance procedures were introduced.

To verify that disialotransferrin to total transferrin fraction (%) results generated by the HPLC cRMP show reproducible agreement between network laboratories, a between-laboratory comparison scheme using blinded samples (i.e. external quality assurance) was initiated and carried out throughout the WG-CDT efforts. Since the initial ring trial in 2007, several rounds involving different sets of ready-for-use and lyophilized serum materials (native serum from controls and heavy drinkers, and control serum spiked with isolated disialotransferrin) [33] have been conducted [51–53]. A good agreement of results was obtained already in the initial ring trials (Fig. 4), and, during the course of the WG-CDT standardisation work, the between-laboratory imprecision has been further improved [51]. For CDT values by the HPLC cRMP in the range 1.5–2.5% disialotransferrin to total transferrin fraction (i.e. near the upper limit of the reference interval) [50] or higher, the CV has typically been <5% of the target value [51,52].

6. Calibration of CDT measurement procedures

A reference material should be suitable for use with as many as possible measurement procedures, homogenous, and stable on storage. In the CDT standardisation work, various frozen and lyophilized candidate reference materials based on either native human serum samples containing different disialotransferrin to total transferrin fraction (%) levels, or on a low CDT serum pool spiked with isolated disialotransferrin, were tested. Among these candidates, frozen native serum was eventually found to be the most suitable as reference material for use with all currently utilized routine CDT assays [52].

While all CDT routine methods demonstrated good linear results in the calibration range, the calibration curves for methods based on CE and immunonephelometry were not parallel with the HPLC cRMP but showed different slopes and intercepts with the origin [52,53]. These discrepancies are explained by the different analytical principles, the calibration methods employed, and/or the differences in analyte. Hence, multi-level calibration over the entire measuring range was found necessary, to eliminate systematic differences between the diverse CDT methods.

A multi-level CDT calibration study using two series of six-level disialotransferrin to total transferrin fraction (%) candidate serum-based calibrators, one being constituted from a pool of native human serum samples and the other formed by spiking a low CDT sample pool with isolated disialotransferrin, was shown to significantly reduce the between-method imprecision [53]. These promising results were later confirmed in additional experiments based on serum samples from individual patients (Fig. 5), demonstrating that standardisation of the different CDT measurement procedures was achievable.

The term "harmonisation", which was used in a previous publication from the WG-CDT [52], is appropriate when results are equivalent either by being traceable to a reference material or based on a consensus approach, such as agreement to an all methods mean, but neither a higher-order reference material nor a RMP exists [54]. However, after further validation of the CDT cRMP (Schellenberg et al., manuscript in preparation), the WG-CDT is currently in the process of having this HPLC method become an accepted RMP, and therefore use the term "standardisation". Following approval as an IFCC RMP, a common and unique identifier for the standardised CDT measurements (CDT_{IFCC}) will be launched, whether they are obtained by the HPLC RMP, or by other HPLC, CE, or immunochemical measurement procedures.

7. Clinical use of CDT testing

Since its introduction as a biomarker of heavy alcohol consumption, CDT testing has become increasingly employed in many parts of the world for objective indication of sustained moderate to high alcohol consumption and to monitor reduction in drinking and/or abstinence. CDT tests are used both for routine clinical and forensic applications (e.g. in outpatient treatment, traffic medicine, and company healthcare testing), as well as in hundreds of clinical trials and experimental studies [9,30,55].

The major benefit of CDT over other laboratory tests traditionally used as alcohol consumption biomarkers, such as GGT, ALT and AST [7,56,57], is its higher specificity. However, it should be noted that serum disialotransferrin to total transferrin fraction (%) also increases slightly during pregnancy and may reach levels near the upper limit of the reference interval during the third trimester, returning to baseline after delivery [58,59]. Other factors that can also influence the transferrin glycoform profile, and might hence interfere with the interpretation of CDT testing for heavy alcohol use, are a variety of genetic transferrin

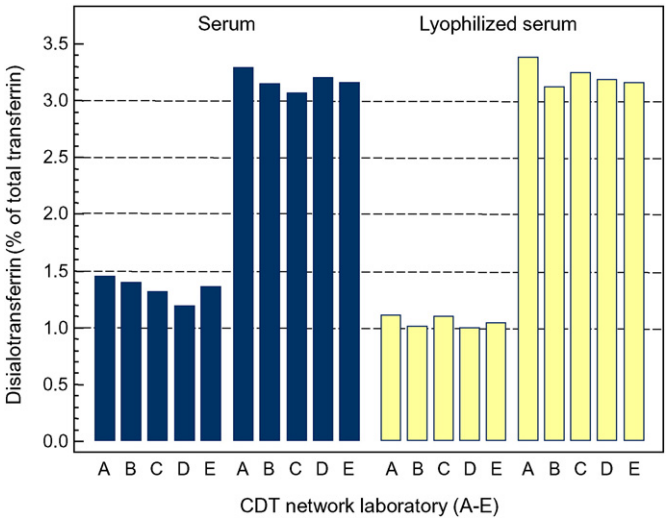

Fig. 4. Agreement of disialotransferrin to total transferrin fraction (%) values by the HPLC candidate reference measurement procedure from one initial ring trial involving five WG-CDT network laboratories (*A–E*) and two native and two lyophilized serum samples. All measurements were performed blinded. In the WG-CDT ring trial scheme, the between-laboratory coefficient of variation has typically been <5% of the measured value, at disialotransferrin to total transferrin fraction (%) values around the upper limit of the reference interval (i.e. 1.5–2.5% disialotransferrin) or higher.

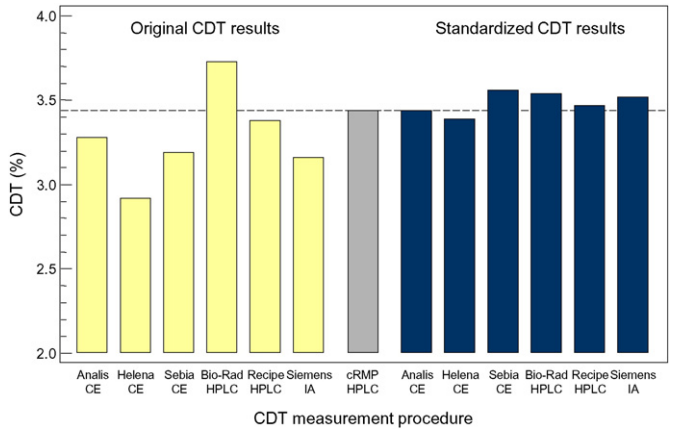

Fig. 5. Comparison of uncalibrated CDT and calibrated disialotransferrin to total transferrin fraction (%) values by six CDT routine measurement procedures based on capillary zone electrophoresis (CE) of Analis (Suarlée, Belgium), Helena (Beaumont, TX, USA), and Sebia (Lisses, France), HPLC of Bio-Rad and Recipe (both Munich, Germany), and immunonephelometry (IA) of Siemens (Marburg, Germany), compared with the HPLC candidate reference measurement procedure (cRMP) [50]. Data are mean values for 20 different patient serum samples.

variants [34], serious liver disease (e.g. cirrhosis) that may result in poor chromatographic separation between disialo- and trisialotransferrin ("di-tri bridging") [44,45], and rare types of CDG [46,60].

Based on results from studies of different populations using the HPLC cRMP, the average serum disialotransferrin to total transferrin fraction (%) level was ~1.20% with a standard deviation (SD) of ~0.20% [17,50]. These results further indicated that the differences in serum transferrin glycoform levels noticed in relation to factors such as ethnicity, age, sex, body mass index, and smoking were small and considered clinically insignificant, meaning that adjustment of CDT reference intervals for these reasons was not required [17]. Based on the mean value plus 2 SD for healthy controls [17,50], and the 97.5th percentile for non-drinkers and light/heavy drinkers combined [56], the upper limit of the reference interval for the disialotransferrin to total transferrin fraction (%) was set at 1.70%.

For forensic use of CDT (e.g. in traffic and occupational medicine) [61–63], different approaches are used in several countries, based on a cut-off higher than the upper limit of the reference interval aiming at decreasing the risk for false positives.

8. Consideration of biological variability of CDT levels

Based on the results of the WG-CDT between-laboratory comparison work, the network laboratories were able to assign values to CDT reference materials with a measurement uncertainty < 0.1% disialotransferrin to total transferrin fraction near the upper limit of the reference interval. The total intraindividual (i.e. analytical plus biological) variability in CDT levels can be estimated from a study where weekly blood samples were collected from teetotallers over ~5 months [64]. The results of this study demonstrated a considerable variation in CDT baseline levels between individuals without drinking any alcohol, but also that the CDT values were fairly constant within each individual. When these samples were reanalysed using the HPLC cRMP (Fig. 6), the values were demonstrated to vary randomly over time but always within ±0.2% disialotransferrin to total transferrin fraction (%). These results indicate that during follow-up of alcohol patients with serial CDT testing [65], obtaining values that exceed the individual baseline disialotransferrin to total transferrin fraction (%) level by more than 0.2% of total transferrin can be taken as an indication of increase in drinking (e.g. detection of relapse). However, this change in individual disialotransferrin to total transferrin fraction (%) level needs to consider the interassay measurement uncertainty in the laboratory performing the testing.

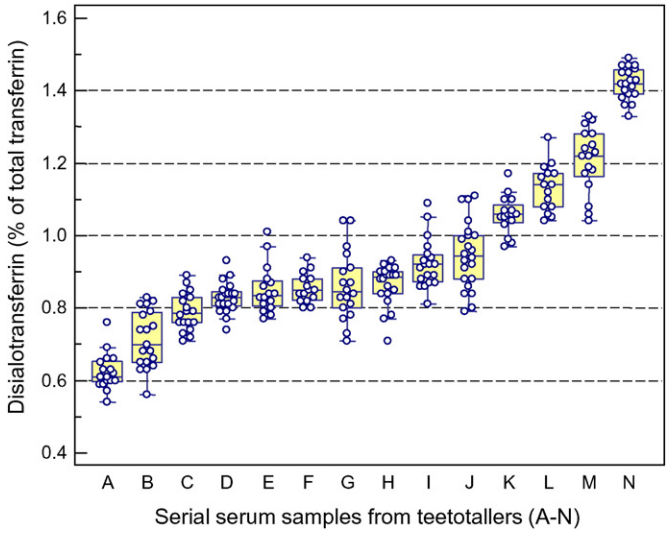

Fig. 6. Box-and-Whisker plots and individual values for disialotransferrin to total transferrin fraction (%) in serial weekly serum samples (N = 15–22/person) collected from 14 teetotallers (*A–N*) over ~5 months [64]. Measurement (Helander, unpublished) was done using the HPLC candidate reference measurement procedure [50].

9. Conclusions

The overall main goal of the IFCC WG-CDT to establish international standardisation of CDT analysis was not fully achieved to the extent that traceability of measurement results to the SI could not be established due to the assumption of identical molar extinction coefficients for all transferrin glycoforms; however, as the presented data show, this assumption is likely close to the truth. Nevertheless, the WG-CDT demonstrated that the different steps taken in this process, including defining the measurand (disialotransferrin to total transferrin fraction (%) in serum), selection of a well-defined HPLC candidate reference method allowing for relative quantification of the transferrin glycoforms, and organisation of an international laboratory network using the cRMP that assigns values to CDT calibrators based on frozen native human serum, contributes to the standardisation of CDT measurements.

The achieved standardisation and introduction of a common unique identifier for standardised CDT measurements (CDT_{IFCC}) allows for direct comparison of values obtained by different routine measurement procedures, and use of a common reference interval. This will improve the diagnostic performance of CDT measurement as a biomarker of chronic heavy/excessive alcohol consumption, advance the ability to compare clinical and analytical trials on CDT, and last, but not least, improve patient care.

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