

## Toward standardization of carbohydrate-deficient transferrin (CDT) measurements: I. Analyte definition and proposal of a candidate reference method

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### ABSTRACT

An alcohol-associated change in the serum transferrin glycoform pattern, carbohydrate-deficient transferrin (CDT), is used as a biomarker for chronic moderate to heavy alcohol consumption. A current limitation in CDT analysis is the lack of standardization, which hampers clinical and analytical comparison between studies. This situation prompted initiation of a Working Group (WG) on CDT standardization under the auspices of the IFCC. The standardization work aims to define and validate the analyte, select a reference method, work out procedures for the production of reference materials, and make suggestions for the clinical usage of CDT. The first recommendation of the WG is that disialotransferrin should be the primary target molecule for CDT measurement and the single analyte on which the CDT standardization is based. It is further recommended that HPLC should be the analytical principle considered as the basis of an interim reference method until a suitable mass spectrometric reference method is established. In clinical use, CDT should be expressed in a relative amount (% CDT) to compensate for variations in the total transferrin concentration.

### HISTORICAL BACKGROUND: CDT AND ANALYTICAL METHODS

Transferrin, the main iron transport glycoprotein in humans, shows a distinct microheterogeneity owing to variations in the amino acid sequence, the oligosaccharide (N-glycan) structure, and the iron load (1, 2). In the 1970s, Stibler et al. compared transferrin patterns in serum and cerebrospinal fluid using the newly developed isoelectric focusing (IEF) technique, and identified an abnormal transferrin isoform (glycoform) pattern that was related to alcohol abuse (3). The alcohol-associated glycoforms showed isoelectric points at or above pH 5.7 in the iron-saturated state and were demonstrated to possess lower sialic acid content compared with the major, tetrasialotransferrin, glycoform; hence the name "carbohydrate-deficient" transferrin, or CDT for short (4). CDT was originally defined as the sum of the asialo-, monosialo- and disialotransferrin glycoforms. Later studies revealed that disialo- and asialotransferrin are missing not only the terminal sialic-acid residues, but one complete N-glycan in the case of disialotransferrin, or

both N-glycans in the case of asialotransferrin (5, 6).

Hundreds of studies have since been published on various clinical applications of CDT as a biomarker for detection and follow-up of chronic heavy alcohol consumption, while there has been less focus on the analytical issues (2, 4, 7-9). Determination of CDT traditionally has relied on the differences in charge (i.e., isoelectric point) between iron-saturated transferrin glycoforms, with IEF of serum proteins followed by immunofixation using anti-transferrin antibodies long considered the standard method. It was not until 1992 that a commercial test kit for CDT (CDTect™, Pharmacia Diagnostics) was launched (10). In the CDTect method, which is no longer in use, CDT was separated from the non-CDT glycoforms using small anion-exchange columns, followed by measurement of absolute amounts of CDT (in mg/L or U/L) using a transferrin immunoassay. Subsequent CDT test kits (e.g., %CDT TIA, %CDT) have retained the original principle of column separation followed by a transferrin immunoassay, but involved modifications in the separation procedure, leading to the inclusion of variable proportions of trisialotransferrin in the CDT fraction, or in the

immunochemical, quantitative part of the test (11-13). Inclusion of trisialotransferrin was later demonstrated to be disadvantageous for CDT testing, as serum samples containing a high level of this glycoform, which is unrelated to alcohol abuse, can produce falsely high CDT immunoassay results (14-17).

Another important change compared to the original CDTEct method was expressing CDT in a relative (% CDT) instead of absolute concentration. Normalization of CDT values to the total transferrin concentration contributed to a significant improvement in CDT analysis (18-21), by compensating for falsely high and falsely low values in cases of increased (anemia, pregnancy, oral contraceptives) or decreased (chronic disease, carcinoma) transferrin concentrations. A drawback of the column separation-transferrin immunoassay combinations is that they separate CDT from non-CDT glycoforms based on differences in isoelectric point and charge, without any control of the individual subfractions eluted. Accordingly, these methods may give inaccurate results for any shift in isoelectric point, such as that occurring in genetic transferrin polymorphisms and rare congenital disorders of glycosylation (CDG) (2, 4, 15, 22). In a limited but meaningful number of cases, this might lead to falsely high or falsely low CDT results that could cause false-positive or false-negative identifications of alcohol abuse with unintended clinical and forensic consequences.

Other analytical techniques have also been introduced for routine determination of CDT, including both commercial and non-commercial HPLC (23-26) and capillary electrophoresis (CE) (27-29) methods. Although generally more laborious and time-consuming than immunoassays, specific advantages of the HPLC and CE methods are the visible documentation of transferrin glycoform patterns, and therefore the possibility of detecting potential analytical interferences such as genetic transferrin variants, CDG, and elevated relative amounts of mono- and trisialotransferrin (15, 17, 22, 30). In differentiating between moderate and heavy drinking, ROC curve analysis also demonstrated that the area under the curve for HPLC was significantly greater than for a minicolumn method (CDTEct) (24).

In addition to these methods, the first direct CDT immunoassay (N Latex CDT) for use on the Dade Behring BN<sup>TM</sup> systems was recently launched (31, 32). N Latex CDT is a direct immunonephelometric assay based on a monoclonal antibody that specifically recognizes the structure of transferrin glycoforms that lack one or both of the complete N-glycans, corresponding to disialo-, monosialo-, and asialotransferrin (i.e., the CDT glycoforms), and on a simultaneous total transferrin immunoassay. The relative values (% CDT) are calculated automatically.

## NEED FOR STANDARDIZATION OF CDT MEASUREMENTS

Over the last 15 years or so, the introduction of a wide range of analytical techniques and methods for

CDT quantification in various absolute (mg/L or U/L) or relative (e.g., % CDT to total transferrin or tetrasialotransferrin) amounts, each showing different analytical sensitivity and specificity (2, 4, 9), as well as the inclusion of various transferrin glycoforms in "CDT", has complicated the comparability of analytical and clinical results between studies (2, 15, 33). The associated wide range of cutoff values for CDT (ranging from 0.8% to 6.0% for relative values) used to detect harmful alcohol intake has also caused much confusion among medical staff when this test is used clinically for screening and follow-up of heavy alcohol intake. Furthermore, the structural complexity of the CDT glycoforms has hindered the development of reference materials on which to base the inter-assay standardization necessary for regulatory oversight of clinical laboratory proficiency and validity of results.

These problems prompted the initiation of a Working Group on Standardization of CDT (WG-CDT) under the auspices of the IFCC. The initial goals for this work are to define the most appropriate CDT analyte, select a reference method for CDT measurement, and work out procedures for the production and distribution of reference materials for the selected analyte. Subsequent goals are to validate the analyte, the reference method and reference materials in multicenter studies. In addition, the WG will provide recommendations on clinical applications for CDT.

The WG had its first meeting in 2005 at the joint IFCC/AACC Congress in Orlando, Florida, and an analytical subgroup meeting was held in March 2006 in Ingelheim, Germany. The initial work toward standardization of CDT measurements has focused on definition of the analyte and proposal of a reference method. Future work will deal with the preparation of CDT reference materials based on purified human serum disialotransferrin, and using such materials for evaluation and comparison of different CDT methods.

## DEFINITION OF THE ANALYTE

Largely for analytical reasons related to the separation of transferrin glycoforms by the early methods, CDT was originally defined as the sum of asialo-, monosialo- and disialotransferrin (4). Among these, monosialotransferrin is not considered suitable as a target analyte for CDT measurement, because the amount of this glycoform in serum is mainly associated with a high trisialotransferrin level of the sample and not with alcohol intake (15).

Asialo- and disialotransferrin, on the other hand, are both clearly related to chronic heavy alcohol consumption, although showing different sensitivity and specificity (25, 28, 34, 35). Disialotransferrin increases with alcohol intake and elevated concentrations are observed after sustained moderate to heavy alcohol consumption (36). With the current analytical methods for CDT, asialotransferrin is not measurable in abstinent and socially drinking subjects, but becomes elevated by chronic heavy alcohol use (37), and then accompanies an already elevated level of disialotransferrin (25, 26). Accordingly, the

diagnostic sensitivity of asialotransferrin is lower when compared with disialotransferrin, and too low for detection of chronic moderate alcohol use. Because any slight improvement in diagnostic specificity obtained by measuring asialotransferrin alone does not justify the significant reduction in diagnostic sensitivity in comparison with disialotransferrin (25, 26), and because the standardization work should focus on a single substance, it was agreed that disialotransferrin should be the primary target analyte for the CDT standardization.

The iron saturation level of transferrin also needs to be considered in the definition of the analyte, as this influences the isoelectric point and the structure, and hence the chemical and analytical (and also possibly the antigenic) properties. Accordingly, for CDT analytical methods that rely upon differences in charge (isoelectric point) between the target molecule disialotransferrin and the other glycoforms, measurement should be carried out after complete iron saturation of transferrin in the sample. Furthermore, because disialotransferrin may theoretically be glycosylated at two distinct positions (at Asn-413 or Asn-611), it is considered important to also elucidate the structure of the analyte.

#### PROPOSAL OF A REFERENCE METHOD FOR CDT

It is recommended that any reference method for CDT should be "public" or independent of proprietary technologies; it should also be applicable to samples from patients with genetic transferrin variants and other transferrin glycoforms, or at least be able to identify such samples. Mass spectrometry is probably the best technique for a reference method, but at present HPLC is considered the best candidate as an interim reference method for CDT, because:

- measurement of the iron-transferrin absorbance at 460-470 nm makes this technique specific and sensitive with a low risk of analytical interference (38);
- it allows for adequate separation of the transferrin glycoforms;
- quantification of either the absolute or relative disialotransferrin concentration can be achieved by measuring peak area or peak height; and
- the HPLC trace gives an easily understood record of peak identification.

A well-defined HPLC method for quantification of transferrin glycoforms using commercially available materials has been published (25) and could easily be set up in an international network of reference laboratories.

CE was considered less suitable as a reference method because it relies on the rather unspecific measurement of the absorbance of the peptide bond at ~200 nm, at which many other biomolecules with similar isoelectric points (e.g., C-reactive protein, light chains, and complement factors) could interfere when present at increased concentrations (28, 39, 40). The analytical sensitivity of CE has also been reported to be lower than for HPLC (29), although improved CE methods are being developed.

#### CONCLUSIONS

The definition of disialotransferrin as the primary target molecule for CDT measurement and the single analyte for CDT standardization, and the recommendation of HPLC as the analytical principle for an interim reference method until a mass spectrometric method becomes available, represent the first steps toward standardization of CDT measurements. In clinical use, CDT values should be expressed as a relative amount (% CDT) to compensate for falsely high and falsely low values in cases of increased or decreased total transferrin concentrations. The WG-CDT hopes that the recommendations presented here will improve the diagnostic performance of CDT as a biomarker of chronic moderate to heavy alcohol consumption and advance the comparability of clinical and analytical trials on CDT. The outcome of this will be better laboratory capability to assist in forensic work, more validly assist medical staff in diagnosis, and last, but not least, improve patient care.

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