Development and Multicenter Evaluation of the N Latex CDT Direct Immunonephelometric Assay for Serum Carbohydrate-Deficient Transferrin

Joris R. Delanghe,1* Anders Helander,2 Jos P.M. Wielders,3 J. Maurits Pekelharing,4 Heinz J. Roth,5 François Schellenberg,6 Catherine Born,7 Eray Yagmur,8 Wolfgang Gentzer,9 and Harald Althaus9

Background: Carbohydrate-deficient transferrin (CDT) is a promising biomarker of alcohol abuse. We describe the development and multicenter evaluation of N Latex CDT (Dade Behring), an automated, particle-enhanced, homogeneous immunonephelometric assay for directly determining CDT.

Methods: N Latex CDT uses a monoclonal antibody that recognizes the structure of transferrin glycoforms lacking 1 or 2 complete N-glycans [i.e., disialo-, monosialo-, and asialotransferrins (CDT glycoforms)] in combination with a simultaneous assay for total transferrin. The Dade Behring BN II™ and BN ProSpec® systems automatically calculate the CDT value as a percentage of total transferrin (%CDT). No preanalytical sample treatment is used.

Results: Total imprecision values for serum pools containing 1.8%–8.7% CDT were 3.4%–10.4% (mean, 6.8%). The mean (SD) %CDT for 561 serum samples from healthy control individuals was 1.76% (0.27%; range, 1.01%–2.85%). No marked sex or age differences were noted. The 97.5th percentile was at 2.35%. Transferrin genetic variants did not interfere with measurements. High transferrin concentrations did not falsely increase %CDT values, but increased %CDT values were noted for some samples with transferrin concentrations <1.1 g/L. N Latex CDT results correlated with those of a commercial CDT immunoassay involving column separation ($r^2 = 0.862$) and an HPLC candidate reference method ($r^2 = 0.978$).

Conclusion: N Latex CDT is the first direct immunoassay for quantifying %CDT in serum. The specificity of N Latex CDT for identifying alcohol abuse may be higher than for immunoassays that use column separation, because transferrin genetic variants do not interfere with measurements.

Alcohol abuse causes physical injuries, neuropsychiatric defects, social problems, and substantial costs to society (1). Identifying individuals with alcohol-related problems is important but is not easy. Conventional laboratory tests such as the γ-glutamyltransferase assay focus mainly on identifying individuals engaged in long-term, chronic alcohol abuse. These tests indicate organ damage and show low diagnostic sensitivity and specificity before the clinical manifestations of alcohol abuse develop (2, 3). Carbohydrate-deficient transferrin (CDT)10 is considered the most accurate biomarker for identifying sustained heavy alcohol consumption and for monitoring abstinence (3, 4). Transferrin, which occurs at concentrations of 2.0–3.5 g/L in serum, exhibits a degree of

1 Department of Clinical Chemistry, Ghent University Hospital, Ghent, Belgium.
2 Alcohol Laboratory, Karolinska Institute and Karolinska University Hospital, Stockholm, Sweden.
3 Department of Clinical Chemistry, Meander Medical Center, Amersfoort, The Netherlands.
4 Reinier de Graaf Groep, Diagnostic Center SSDZ, Delft, The Netherlands.
5 Limbach Laboratories, Heidelberg, Germany.
6 Laboratory of Clinical Chemistry, Hospital Trousseau, Centre Hospitalier Régional Universitaire, Tours, Tours, France.
7 Institut Regional pour la Sante, La Riche, France.
8 Central Laboratory, University Hospital, Rheinisch Westfälische Technische Hochschule, Aachen, Germany.
9 Research Laboratories, Dade Behring Marburg GmbH, Marburg, Germany.

* Address correspondence to this author at: Department of Clinical Chemistry, De Pintelaan 185, B-9000 Ghent, Belgium. Fax 32-9-240-4985; e-mail joris.delanghe@ugent.be.

Received December 11, 2006; accepted March 8, 2007.
Previously published online at DOI: 10.1373/clinchem.2006.084459

10 Nonstandard abbreviations: CDT, carbohydrate-deficient transferrin; CDG, congenital disorders of glycosylation; mAb, monoclonal antibody; %CDT, CDT as a percentage of total transferrin.
microheterogeneity that depends on iron saturation (~30%), amino acid sequence, and/or carbohydrate content (5–7). Amino acid sequence variation is observed in individuals with genetic variants B, C, and D (8), whereas transferrin glycoforms with variable carbohydrate content and/or branching of the maximum 2 N-linked oligosaccharide chains (N-glycans) are always present (4, 7, 9). Typically, the major serum transferrin glycoform, tetrasialotransferrin, contains 2 disialylated biantennary glycans. Other, less abundant glycoforms are pentasialo-, trisialo-, and disialotransferrins (10). Disialo- and asialotransferrin fractions increase after sustained heavy drinking (10–12). These glycoforms, together with monosialotransferrin (10), have collectively been referred to as CDT (4). A regular intake of ~50–80 g ethanol/day for a minimum of ~1–2 weeks is required to increase the serum CDT concentration in ~80% of individuals (4, 13). The half-life of the CDT marker is ~1–1.5 weeks, and a return to the usual glycoform pattern requires ~2 weeks of abstinence (4, 14, 15).

Early methods for assaying CDT used isoelectric focusing followed by immunofixation (16–18). Alternative procedures used chromatofocusing (19), HPLC (10, 14, 20), fast protein liquid chromatography (21), and capillary electrophoresis (22–24). Immunoassays include an initial chromatographic separation of CDT glycoforms from non-CDT glycoforms on disposable minicolumns (25–27). Drawbacks with this last approach are the labor involved and the fact that transferrin genetic variants may cause falsely high or falsely low results (28). We present data on the development and multicenter evaluation of the 1st direct immunoassay for CDT (N Latex CDT; Dade Behring) and on possible interference by transferrin genetic variants and congenital disorders of glycosylation (CDG) (29).

Materials and Methods

DEVELOPMENT OF A MONOCLONAL ANTIBODY

We raised monoclonal antibodies (mAbs) against CDT by means of a recombinant nonglycosylated transferrin, which was a gift from Anne B. Mason, Ph.D. (Department of Biochemistry, College of Medicine, University of Vermont, Burlington, VT) (30), in which the 2 asparagine residues at the carbohydrate-linkage sites were mutated to aspartic acid (Asn413Asp and Asn611Asp). We immunized BALB/c mice with recombinant transferrin in complete Freund adjuvant. A booster with an emulsion prepared in incomplete adjuvant was given after 4 weeks, and another booster without adjuvant was administered after 8 weeks. During the final 3 days before the fusion, we gave the mice daily intravenous boosters.

After the mice were killed, we removed the spleens and cloned the B-cells with myeloma cells. Single hybrid cells that produced antibodies specific for CDT (i.e., binding to nonglycosylated transferrin but not to typical transferrin) were cloned, and appropriate clones were expanded. After removing the cells, we concentrated the solution and purified the antibodies with Protein A Sepharose Fast Flow (GE Healthcare/Amersham Biosciences). The CDT mAb (98/84-011) with the highest specificity for nonglycosylated transferrin but no affinity for typical human transferrin was selected for assay development (see the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue6).

We diluted the selected CDT mAb to a concentration of 80 mg/L in blocking buffer (10 g/L bovine serum albumin and 0.5 mL/L Tween 20 in Tris-buffered saline (0.02 mol/L Tris, 0.15 mol/L NaCl, pH 7.5) and then added alkaline phosphatase-linked secondary antibodies (Bio-Rad Laboratories) in blocking buffer. p-Nitro tetrazolium blue and 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich) were added as substrates (31). We used sera from 1 control individual and 1 alcoholic proband to compare the specificity of the CDT mAb to that of polyclonal antibodies directed against several transferrin epitopes (Dade Behring).

We further evaluated the specificity of the CDT mAb by investigating its reaction with CDT, other transferrin glycoforms, and enzymatically modified transferrin. Transferrin lacking the terminal sialic acid residues was obtained by treating serum with neuraminidase (2.5 mU/mg transferrin; Dade Behring) in phosphate-buffered saline (0.048 mol/L Na2HPO4, 0.02 mol/L KH2PO4, 0.145 mol/L NaCl, 0.015 mol/L NaNO3, pH 7.2) for 18 h at 37 °C. We obtained transferrin lacking entire N-glycan moieties by treating transferrin with peptide-N-glycosidase F (500 mU/mg transferrin; Roche Diagnostics) for 4 h at 37 °C in phosphate-buffered saline (0.048 mol/L Na2HPO4, 0.02 mol/L KH2PO4, 0.145 mol/L NaCl, 0.015 mol/L NaNO3, pH 7.2) containing 10 mmol/L EDTA and 1 g/L sodium dodecyl sulfate. After this incubation, proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Immunodetection was performed with the CDT mAb and a polyclonal antibody against human transferrin.

CDT mAb–based immunoassay

N Latex CDT is based on an mAb that specifically recognizes transferrin glycoforms that lack one or both of the complete N-glycans [i.e., disialo-, monosialo-, and asialotransferrins (the CDT glycoforms)] in combination with a simultaneous assay for total transferrin (N Antibody to Human Transferrin; Dade Behring). Polystyrene particles coated with the CDT mAb are agglutinated by CDT-coated polystyrene particles. CDT inhibits this reaction in a dose-dependent manner, allowing nephelometric CDT quantification over 18 min on the Dade Behring BN II™ and BN ProSpec™ systems. No sample pretreatment is required. Because the degree of iron saturation of transferrin influences the binding affinity of the antibody, the transferrin-bound iron is stripped with a chelating agent in the first incubation step. The simultaneous determination of total transferrin allows an automatic calculation of
the amount of CDT as a percentage of total transferrin (%CDT). The measurement range is ~20–640 mg/L or 0.77%–25% CDT.

MULTICENTER EVALUATION
We evaluated the N Latex CDT assay at 8 sites (A–H) with 2 independent reagent lots (01 and 03). The reagent lots were distributed so that each lot was used at least once on each analyzer. Imprecision was determined according to the Clinical and Laboratory Standards Institute EP5-2A guideline. The 2 reagent set controls (N CDT Control SL/1 and SL/2; Dade Behring) and 4 different human serum pools were run in duplicate in 2 runs/day, over 20 days. Dade Behring provided 2 serum pools (R1 and R2). R1 contained samples with an increased %CDT, and R2 contained samples with low and increased %CDT values. Two other serum pools ("low" and "high" %CDT pools) were produced individually by each laboratory. We used the N Latex CDT assay to analyze a proficiency panel of 29 serum samples that were provided in frozen aliquots at 6 of the sites.

We obtained informed consent and approval of the local ethics committee whenever required. To evaluate any relationship between total transferrin concentrations and %CDT values produced by N Latex CDT, we included serum samples from 113 patients with a wide range of transferrin concentrations (0.43–4.22 g/L; reference interval, 20–640 mg/L or 2%–25% CDT). The measurement range is ~20–640 mg/L or 0.77%–25% CDT.

REFERENCE INTERVAL
We collected serum samples from 561 apparently healthy adults (255 men, ages 20–70 years; 306 women, ages 19–79 years). We included samples only from individuals who had no clinical indications or biochemical indications of metabolic diseases or of alcohol consumption. To assess alcohol consumption, we used both the section in the Kiddie–Sads–Present and Lifetime Version that is related to alcohol abuse and the Alcohol Use Disorders Identification Test.

INTERFERENCE TESTING
We evaluated potential interfering factors, such as transferrin saturation, iron deficiency, lipemia, transferrin genetic variants, and CDG. We obtained serum samples from healthy white individuals who carried the BC (n = 3) and CD (n = 4) transferrin variants and from 1 CDG type Ic patient.

Method Comparison
We compared %CDT results obtained with the N Latex CDT assay with those obtained by turbidimetric chromatographic separation followed by immunoaassay (the Axis-Shield %CDT or the Bio-Rad %CDT TIA assay) (27). We also compared the N Latex CDT assay with an HPLC candidate reference method (10) with 100 serum samples with percent disialotransferrin values within the reference interval of the HPLC method (97.5th percentile, <1.7%) and 100 samples with increased values (>2% disialotransferrin). Samples were selected to cover a wide range of percent disialotransferrin values (0.9%–22.2%).

STATISTICS
Results are expressed as the mean (SD). Differences between samples that were saturated with iron before analysis and unaltered samples were evaluated by means of a paired t-test. Passing–Bablok regression and the Wilcoxon test were used to compare methods. The Student t-test and ANOVA were used to evaluate sex and age differences in the control population. We defined reference values as the 2.5%–97.5% interval in the distribution of values in the reference population.

RESULTS
EVALUATION OF THE CDT mAB
Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting analysis with the CDT mAb showed only transferrin molecules lacking one or both N-glycans in serum samples obtained from alcoholic individuals; no reaction with transferrin was obtained with control samples (Fig. 1). The CDT mAb also detected transferrin in control samples after N-glycosidase F treatment (Fig. 1), which yields transferrin molecules lacking entire N-glycans. Control serum samples incubated with neuraminidase, which depletes only terminal sialic acid residues, were not detected by the CDT mAb (Fig. 1).

Epitope mapping of the CDT mAb with overlapping peptides corresponding to the human transferrin sequence identified 4 major binding sites (data not shown): 1 site in the N-terminal domain and 3 sites in the C-terminal domain. Because peptide sequences at or near the 2 N-glycan–binding sites (Asn413 and Asn611) were not detected, we concluded that the antibody is directed against a discontinuous structural epitope. This result suggests differences in 3-dimensional structure between transferrin molecules containing 2 N-glycans and those lacking 1 or both N-glycans (i.e., the CDT glycoforms). Apparently, this structural change and the formation of the CDT-specific structural epitope(s) occur when 1 N-glycan is missing, and no major additional changes occur when the second N-glycan is also missing.

INTERFERENCE TESTING
We evaluated potential interfering factors, such as transferrin saturation, iron deficiency, lipemia, transferrin genetic variants, and CDG. We obtained serum samples from healthy white individuals who carried the BC (n = 3) and CD (n = 4) transferrin variants and from 1 CDG type Ic patient.
and the R2 pool (mean, 3.08%; range, 3.0%–3.2%) were provided by Dade Behring, whereas the low pools (mean %CDT, 1.95%; range, 1.8%–2.1%) and high pools (mean, 5.33%; range, 3.0%–8.7%) were produced at each site. The intraassay CVs were 1.9%–7.0% (mean, 4.3%), and total CVs were 3.4%–10.4% (mean, 6.8%; Table 1). BN II systems showed higher analytical imprecision (mean total CV, 8.2%) than BN ProSpec systems (mean total CV, 5.3%). Imprecision results for the reagent set controls were similar to those for the serum pools, with total CVs of 3.8%–9.7% for Dade Behring N CDT Control SL/1 (mean concentration, 55.7 mg/L) and 3.7%–5.4% for N CDT Control SL/2 (mean concentration, 163 mg/L).

REFERENCE INTERVAL FOR %CDT VALUES
We studied the distribution of N Latex CDT values with 561 serum samples from healthy nonalcoholic individuals. Transferrin concentrations were 1.7–4.4 g/L (2.5th percentile, 2.0 g/L; 97.5th percentile, 3.8 g/L). The overall mean %CDT value was 1.76% (0.26%), and the range was 1.01%–2.85%. The %CDT results for men [1.78% (0.27%)] and women [1.77 (0.25%)] were not significantly different (P = 0.538), and no significant age-related differences were found (data not shown). We proposed an upper reference limit of 2.35% (97.5th percentile) for %CDT values obtained with the N Latex CDT assay. The 141 serum samples collected from children and adolescents showed similar results, with a median %CDT of 1.91% and 2.5th and 97.5th percentiles of 1.45% and 2.40%, respectively. On the basis of these results, we proposed the same upper reference limit (2.35%, 97.5th percentile) for %CDT obtained with the N Latex CDT assay (Table 2).

INTERFERENCE TESTING
For the 113 serum samples with a wide range of transferrin values, we found no marked effect of transferrin concentration on N Latex CDT results within the reference interval (2.0–3.6 g/L). At abnormally low concentrations,

---

**Table 1. Multicenter analytical performance of the N Latex CDT assay.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Imprecision (CV), %</th>
<th>Total Mean %CDT</th>
<th>Imprecision (CV), %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Within-run</td>
<td>Site A: BN II</td>
<td>Site B: BN ProSpec</td>
</tr>
<tr>
<td>R1b</td>
<td>5.0</td>
<td>8.3</td>
<td>4.0</td>
</tr>
<tr>
<td>R2b</td>
<td>5.8</td>
<td>8.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Low pool c</td>
<td>6.5</td>
<td>8.4</td>
<td>1.8</td>
</tr>
<tr>
<td>High pool c</td>
<td>5.8</td>
<td>8.1</td>
<td>5.8</td>
</tr>
<tr>
<td>R1c</td>
<td>1.9</td>
<td>4.9</td>
<td>3.9</td>
</tr>
<tr>
<td>R2c</td>
<td>3.2</td>
<td>6.4</td>
<td>3.0</td>
</tr>
<tr>
<td>Low pool c</td>
<td>2.9</td>
<td>6.0</td>
<td>2.1</td>
</tr>
<tr>
<td>High pool c</td>
<td>2.5</td>
<td>5.5</td>
<td>4.8</td>
</tr>
<tr>
<td>R1c</td>
<td>2.9</td>
<td>4.7</td>
<td>3.8</td>
</tr>
<tr>
<td>R2c</td>
<td>5.2</td>
<td>7.3</td>
<td>3.0</td>
</tr>
<tr>
<td>Low pool c</td>
<td>3.3</td>
<td>6.4</td>
<td>1.9</td>
</tr>
<tr>
<td>High pool c</td>
<td>2.8</td>
<td>5.3</td>
<td>4.7</td>
</tr>
</tbody>
</table>

* The study sites used either the BN II or the BN ProSpec system (Dade Behring).
* R1 and R2 were serum sample pools provided by Dade Behring.
* The low and high pools were individual serum pools produced at each study site.
Table 2. Distribution of %CDT values produced by the N Latex CDT assay for serum samples collected from healthy individuals at 2 study sites.\(^a\)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>2.5th percentile</th>
<th>Median</th>
<th>97.5th percentile</th>
<th>99th percentile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>561</td>
<td>1.29</td>
<td>1.76</td>
<td>2.35</td>
<td>2.47</td>
</tr>
<tr>
<td>Men</td>
<td>255</td>
<td>1.27</td>
<td>1.77</td>
<td>2.36</td>
<td>2.47</td>
</tr>
<tr>
<td>Women</td>
<td>306</td>
<td>1.37</td>
<td>1.76</td>
<td>2.35</td>
<td>2.44</td>
</tr>
</tbody>
</table>

\(^a\) The samples were collected in the Netherlands and France.

Method Comparison

%CDT values obtained with N Latex CDT were correlated with those obtained with the Bio-Rad %CDT TIA (\(n = 132; y = 0.838x + 0.354; r^2 = 0.862; S_{xy} = 0.58\)). Passing–Bablok regression analysis revealed the N Latex CDT results to be generally lower in the low %CDT range than with the Bio-Rad %CDT TIA (see the Figure in the online Data Supplement).

%CDT values obtained with the N Latex CDT assay were also correlated with percent disialotransferrin values obtained by HPLC in an analysis of 100 serum samples with typical values and 100 samples with increased percent disialotransferrin values (range, 0.9%–22.2%; Fig. 2; \(n = 200; y = 0.700x + 0.970; r^2 = 0.978; S_{xy} = 0.49\)). We used ROC curve analysis and the 97.5th percentile for percent disialotransferrin as determined with the HPLC method as a reference, along with these 200 samples to calculate the agreement of N Latex CDT results with reference method results. With the upper reference limit of 2.35% for %CDT obtained with N Latex CDT as a cutoff point, 97% of the results that showed increased %CDT in the HPLC analysis were increased in N Latex CDT, and 94% of the results that were below the cutoff point according to the HPLC method were also below the cutoff point in the N Latex CDT assay (Table 3).

Discussion

The important component of N Latex CDT is the mAb, which specifically recognizes the transferrin glycoforms that lack one or both entire N-glycans (corresponding to asialo-, monosialo- and disialotransferrins) (4). Disialo-
and asialotransferrins are associated with sustained heavy drinking, whereas monosialotransferrin is correlated with the amount of trisialotransferrin (28). Monosialotransferrin probably represents no obstacle to %CDT testing with N Latex CDT because it is usually present in very low concentrations (10). Trisialotransferrin has caused some confusion in CDT testing (7, 28, 32), because this glycoform is always present at higher concentrations and was (26, 33)—and still is (34)—included in the CDT fraction of some methods. Immunoblotting analysis, however, has demonstrated that the CDT mAb does not detect trisialotransferrin in the N Latex CDT assay.

Another advantage of N Latex CDT over the indirect column-based immunoassays is that the CDT mAb is not influenced by transferrin genetic variants. Genetic variants, which are rare in Caucasians but more common in other populations (8, 35), may cause falsely low and high CDT values with the column-based immunoassays (28). For example, trisialotransferrin D in samples from individuals with C and D genetic variants will coelute with disialotransferrin C and thereby cause overestimation of CDT.

Use of the N Latex CDT assay may therefore decrease the need for confirmatory CDT testing by HPLC or capillary electrophoresis (36). Additional studies are needed to confirm that other transferrin genetic variants and samples with divergent N-glycan structures, such as those occurring in the CDG subtypes, do not interfere with the N Latex CDT assay (22, 37, 38).

Besides variations in amino acid sequence and carbohydrate content, the degree of transferrin microheterogeneity also depends on the number of bound iron molecules (7). Under physiological conditions, serum transferrin is ~30% saturated with iron. Four transferrin glycoforms can be distinguished with respect to iron content: apotransferrin, N-terminal and C-terminal monosialotransferrins, and disialotransferrin. To exclude analytical interference due to variation in iron saturation, many CDT methods completely saturate the transferrin in the sample with iron before analysis. The degree of iron saturation also influences the binding affinity of the CDT mAb in the N Latex CDT assay, but this assay uses a chelating agent to completely deplete the iron from transferrin before analysis. The reproducibility results indicate that iron depletion is complete and stable during the immunonephelometric analysis.

The %CDT results obtained with the N Latex CDT assay correlated well with those of a column-based %CDT immunoassay (27) and with the percent disialotransferrin values obtained with an HPLC candidate reference method (10); however, because these methods measure different transferrin glycoforms as CDT, the values obtained with the different methods are not interchangeable. This fact highlights the need for standardization of CDT measurements.

Grant/funding support: The N Latex CDT assay and control materials used for the Multicenter evaluation, as described in Materials and Methods, were provided by Dade Behring.

Financial disclosures: The authors had complete independence in the interpretation of data and writing of the report.

References


Table 3. Sensitivity and specificity for the N Latex CDT assay calculated at different %CDT thresholds.

<table>
<thead>
<tr>
<th>%CDT threshold</th>
<th>Specificity, %a</th>
<th>Sensitivity, %a</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2</td>
<td>90</td>
<td>99</td>
</tr>
<tr>
<td>2.3</td>
<td>93</td>
<td>96</td>
</tr>
<tr>
<td>2.4</td>
<td>97</td>
<td>94</td>
</tr>
<tr>
<td>2.5</td>
<td>97</td>
<td>93</td>
</tr>
<tr>
<td>2.6</td>
<td>97</td>
<td>86</td>
</tr>
</tbody>
</table>

a The percent disialotransferrin results obtained with the HPLC method (10) were used as a reference (threshold, 1.7%).