### ASSESSMENT AND DETECTION

**Clinical Characteristics of Carbohydrate-Deficient Transferrin (％Disialotransferrin) Measured by HPLC:**

**Sensitivity, Specificity, Gender Effects, and Relationship with other Alcohol Biomarkers**

**Jonas P. Bergström and Anders Helander**

Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden

*Author to whom correspondence should be addressed: Anders Helander, Alcohol Laboratory, L7:03, Karolinska University Hospital Solna, SE-171 76 Stockholm, Sweden. Tel.: +46-8-51771531; Fax: +46-8-51771532; E-mail: anders.helander@ki.se

1On Behalf of the WHO/ISBRA Study on State and Trait Markers of Alcohol use and Dependence Investigators.

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**Abstract** — **Aims:** The sensitivity and specificity of the relative disialotransferrin amount (％DST), considered the primary single target for measurement of the alcohol biomarker carbohydrate-deficient transferrin (CDT), were compared with the absolute CDT amount determined by the CDTect assay and with GGT and AST. **Methods:** Serum samples (n = 1387) were collected within the WHO/ISBRA Study on State and Trait Markers of Alcohol Use and Dependence. The subjects had been classified as “non-drinkers” (26%), “light/moderate drinkers” (50%), or “heavy drinkers” (24%) by use of the WHO/ISBRA Interview Schedule. An HPLC candidate reference method for CDT was used to quantify individual transferrin glycoforms. **Results:** No gender difference in％DST was noted for nondrinkers, but light/moderate and heavy drinking males had significantly higher levels than females. Of the alcohol biomarkers examined,％DST showed the strongest correlation with self-reported alcohol intake, except for female heavy drinkers. The area under the％DST ROC curve for male (0.83) and female (0.82) heavy drinkers was significantly higher compared with CDT by CDTect (0.68) and GGT (0.69). At the 40, 60, or 80 g ethanol/day thresholds,％DST showed lower test sensitivity in women but there was no significant gender difference in overall accuracy according to ROC curve analysis. **Conclusions:**％DST measured by HPLC showed overall higher sensitivity for “heavy drinking” and better correlation with recent high alcohol intake, compared with the absolute CDT amount, and GGT and AST. The observation that several “light/moderate drinkers” had elevated％DST levels and some also a measurable asialotransferrin indicated misclassification with the WHO/ISBRA Interview Schedule and emphasize the limitations of self-reports of drinking.

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**INTRODUCTION**

The iron-transportglycoprotein transferrin exists in different genetic variants owing to variations in the amino acid sequence (Kamboh and Ferrell, 1987; de Jong et al., 1990). Several glycoforms differing in the number of and/or structure of the maximum two N-linked oligosaccharide chains (N-glycans) are always present (Arndt et al., 2001; Helander et al., 2001). The major glycoform under normal conditions is tetrasialotransferrin that contains two disialylated biantennary glycans (i.e., four terminal sialic acids). Tetrasialotransferrin typically makes up 75–80% of serum transferrin (Helander et al., 2003). Other less abundant glycoforms normally found in blood are disialo-, trisialo-, pentasialo-, and hexasialotransferrin. Abnormal transferrin profiles are seen and used for preliminary diagnosis of rare congenital disorders of glycosylation (CGD) (Helander et al., 2004; Jaeken and Matthys, 2007).

A more common cause of an abnormal serum transferrin profile is sustained heavy alcohol consumption. Excessive drinking causes a transient change in the glycoform pattern by increasing the relative amounts of disialo- and asialotransferrin at the expense of tetrasialotransferrin (Bergström and Helander, 2008). This change is named carbohydrate-deficient transferrin (CDT; originally defined as the sum of asialo-, monosialo-, and disialotransferrin) and is used as an alcohol biomarker for heavy drinking (Stibler, 1991). When drinking is discontinued, the transferrin glycoform pattern normalizes with a half-life of ~1.5 weeks (Jeppsson et al., 1993), and reaching the baseline level usually takes 3–5 weeks (Helander and Carlsson, 1996).

The alcohol-induced change in transferrin glycosylation was identified more than 30 years ago (Stibler, 1991). Today CDT is routinely applied in many countries mainly for detection and follow-up of alcohol-related problems in health care and forensic (traffic medicine) settings (Helander, 2003). Over the years, several analytical techniques have been used for the measurement of CDT (Arndt, 2001; Bortolotti et al., 2006). In the large international WHO/ISBRA Study on State and Trait Markers of Alcohol Use and Dependence (Tabakoff et al., 2001; Conigrave et al., 2002), CDT quantification was achieved using the CDTect™ immunoassay (Pharmacia, Sweden), which was the standard routine method at that time but is no longer in use. A number of limitations of CDTect have hampered the interpretation and generalization of the CDT data arising from the WHO/ISBRA Study. With this assay, a CDT fraction consisting of asialo-, monosialo-, a minor part of disialo-, and traces of trisialotransferrin (Arndt et al., 1998) was separated from the non-CDT glycoforms using small ion-exchange columns followed by a transferrin immunnoassay (Stibler et al., 1991), but the individual glycoforms were not recognized. Furthermore, the CDT content was reported in an absolute amount (units/L), and not normalized to the total serum transferrin concentration (％CDT) which is the standard way today (Jeppsson et al., 2007). Because of this, gender-specific reference intervals had to be applied and the CDTect result was influenced by many causes of an elevated or a reduced total serum transferrin concentration (e.g., anemia, estrogen, pregnancy, and inflammation). Lastly, genetic transferrin variants were not detected but represented a cause of incorrect CDT values with the CDTect assay (Helander et al., 2001). These method-dependent short-comings are likely to have underrated the diagnostic accuracy...
and clinical value of the CDT biomarker (Salaspuro, 1999; Scouller et al., 2000; Koch et al., 2004; Fleming et al., 2004).

The present study used a high-performance liquid chromatography (HPLC) method, recently proposed as a candidate CDT reference method (Jeppsson et al., 2007), to identify and quantify individual transferrin glycoforms in serum samples collected within the WHO/ISBRA Study. The sensitivity and specificity of the relative disialotransferrin amount to total transferrin, considered the primary single target for the CDT measurement (Jeppsson et al., 2007), was compared with the absolute CDT results obtained by CDTect and with the conventional alcohol biomarkers gamma-glutamyltransferase (GGT) and aspartate aminotransferase (AST).

MATERIALS AND METHODS

Subjects and samples

The blood samples of the WHO/ISBRA Study were collected by Clinical Centres in Australia, Brazil, Canada, Finland, and Japan (Tabakoff et al., 2001). The present investigation comprised samples from 1387 subjects (68% men and 32% women) aged 18–65 years (mean 36.9, median 35), of whom 76% were white, ∼14% Asian/Indian, and ∼5% black (Bergström and Helander, 2008).

All participants were originally interviewed about their drinking habits using the structured WHO/ISBRA Interview Schedule. Based on this information, each subject was classified as being either a “nondrinker” (n = 360; 26%), indicated to be totally abstinent, or one who drinks alcohol on no more than six special occasions per year (e.g., birthdays), and no more than 15 g ethanol on each occasion; “light/moderate drinker” (n = 689; 50%), one who drinks at least once per month but <210 g ethanol/weak for men and <140 g ethanol/weak for women, and no past treatment for alcohol-related problems; or “heavy drinker” (n = 338; 24%), one who drinks >210 g ethanol/week for men and >140 g ethanol/week for women, but with no past treatment for alcohol-related problems. Detailed demographic data of the country in which patients were recruited, their ethnicity, age, gender, and drinking status are given elsewhere (Bergström and Helander, 2008).

Blood samples were collected at the time of the interview. After local separation of serum and plasma, the specimens were shipped in dry ice and stored at −80°C, a condition under which the serum transferrin glycoform pattern is stable for a very long time (Märtensson et al., 1998; Helander et al., 2003). Before taken for analysis, samples were thawed overnight at 4°C and centrifuged at 3500 g for 5 min.

All subjects had to provide written consent to enter the WHO/ISBRA Study. The Study was approved by the local Ethics Committees of each Clinical Centre.

Measurement of CDT, GGT, and AST

The HPLC candidate reference method for CDT provides reproducible separation and relative quantification of transferrin glycoforms (Helander et al., 2003). Serum transferrin was first iron-saturated by mixing 100-µL serum (for a few samples, only 10–50 µL was available) 5:1 (v/v) with ferric nitrolotrionic acid (FeNTA; final concentration 1.7 mmol/L). Lipoproteins were then precipitated by mixing the sample 6:1 (v/v) with Dextran sulfate and CaCl₂ (1.4 mg/L and 70 mmol/L, respectively). The samples were left at 5°C for 30–60 min and then centrifuged at 3500 g for 5 min. The clear supernatant was diluted fivefold with HPLC grade water, transferred to glass vials, and a 100-µL aliquot was injected into the HPLC system. Separation of transferrin glycoforms was performed on a SOURCE® 15Q 4.6/100 PE anion-exchange chromatography column (Amersham Biosciences, Sweden) by linear salt gradient elution using an Agilent 1100 HPLC system equipped with a G1365B multiple wavelength detector. Quantification relied on the selective absorbance of the iron-transferrin complex at 470 nm. The relative amount of each glycoform was calculated as a percentage of total transferrin (peak areas for all glycoforms), using the baseline integration mode. The lower limits of determination (LOD) and quantification (LOQ) of the HPLC method are ∼0.05 and 0.1%, respectively, of total serum transferrin in the normal transferrin concentration range (reference interval 1.9–3.3 g/L). The intra- and interassay coefficients of variation (CV) of the method for serum samples containing 1.0–5.6% disialotransferrin are <5%. The upper limit of the reference interval (97.5th percentile) for %disialotransferrin by HPLC was reported to be ∼1.7% (Turpeinen et al., 2001; Helander et al., 2003).

Serum CDT by the CDTect assay (Pharmacia, Sweden) and plasma GGT and AST were assayed as described elsewhere (Conigrave et al., 2002).

Statistics

Statistical calculations were performed using the Student–Newman–Keuls test for pairwise comparisons, a T-test (parametric) when the examined groups showed a Gaussian distribution or a Wilcoxon test (nonparametric) if not. For statistical analysis of correlations, Pearson’s correlation coefficient (parametric) or Spearman’s coefficient of rank correlation (nonparametric) was used (MedCalc software).

RESULTS

The %disialotransferrin levels in the three categories were compared separately for men and women. No gender difference in %disialotransferrin was noted for those classified as “nondrinkers,” while men showed significantly higher levels than women in both “light/moderate drinkers” (mean 1.40% vs. 1.23%; P < 0.01) and “heavy drinkers” (2.40% vs. 1.77%; P < 0.01) (Fig. 1). However, it should be pointed out that ∼8% of the subjects (48 men and 9 women) classified as “light/moderate drinkers” had %disialotransferrin levels in the range of 1.82–5.87% (mean 2.73%) which is above the recommended upper reference limit at ∼1.7%. When these results were excluded from the statistical calculation, there was no longer a significant difference in %disialotransferrin levels between light/moderate drinking men (mean ± SD 1.22 ± 0.23%) and women (1.19 ± 0.21%; P = 0.11). The 97.5th percentile for %disialotransferrin for male and female nondrinkers and light/heavy drinkers combined (outliers excluded) was ∼1.7%.

In all drinkers, the %disialotransferrin level was negatively correlated with the number of abstinence days (Fig. 2). Among those who had been drinking any amount of alcohol within 7 days prior to blood sampling, the frequency of elevated serum %disialotransferrin levels was 24.1%, compared with
the self-reported average daily alcohol consumption in the last month was not highest for the heavy drinkers aged 41–50 years but rather similar for all age groups (range for means 74–95 g/day). The only significant \( P < 0.01 \) difference in age-related alcohol consumption was a higher level for the subjects aged <31 years compared with those >50 years.

When the sample was instead grouped based on body mass index (BMI <20, 20–25, >25–30, and >30), the only significant \( P < 0.05 \) difference was that heavy drinkers with a normal BMI of 20–25 showed higher %disialotransferrin values (mean 2.66%, median 2.08%). As for the different age groups, the alcohol consumption level was comparable for all BMI subgroups (range for means 60–95 g/day). The only significant \( P < 0.01 \) difference was a higher alcohol intake in those with a BMI >30 compared with a BMI of 25–30.

The correlations of %disialotransferrin by HPLC, CDT by CDTect, and GGT and AST with self-reported mean daily alcohol consumption in the month prior to blood sampling are given in Table 1. For all drinkers combined, and for light/moderate drinkers of both genders, and also for male heavy drinkers, the strongest correlation with self-reported alcohol intake was found for %disialotransferrin. In female heavy drinkers, on the other hand, GGT was the strongest correlate with alcohol intake (Table 1).

The strongest correlation between the different alcohol biomarkers was obtained for %disialotransferrin and CDT by CDTect in both men (\( r = 0.56 \)) and women (\( r = 0.31 \)), while %disialotransferrin showed lower agreement with GGT (\( r = 0.31 \) for men, \( r = 0.24 \) for women) and AST (0.23 and 0.08). Given the large number of samples included, the correlations reached statistical significance \( P < 0.0001 \) in all cases except for %disialotransferrin and AST in women.

For evaluation of the overall test accuracy of %disialotransferrin as alcohol biomarker and for comparison with CDT by CDTect, and GGT and AST, receiver-operating characteristic (ROC) analysis was performed (Zweig and Campbell, 1993). The area under the ROC curves (AUC) for the combination of nondrinkers and light/moderate drinkers in comparison with heavy drinkers was not significantly different between men (AUC 0.83) and women (0.82) (Fig. 3A). The AUC only 1.6% positive results when the last intake had occurred more than 1 week back. It should be pointed out that even though the time between last alcohol intake and conducting the interview and sampling of blood ranged from <1 day to 30 days, most of them had been drinking quite recently (mean 3.9 days back).

When the whole sample was divided into four age groups (<31, 31–40, 41–50, and >50 years), the only statistically significant \( P < 0.05 \) difference in %disialotransferrin was that heavy drinkers aged 41–50 years showed higher levels (mean 2.80%, median 2.01%) compared with those aged <31 years (2.08%, 1.66%) and >50 years (2.02%, 1.64%). However,
Sensitivity and Specificity of %DST Measured by HPLC

Fig. 3. Receiver-operating characteristic (ROC) analysis was used to distinguish (A) female and male “heavy drinkers” from the combination of “nondrinkers” and “light/moderate drinkers” (classification was made according to the WHO/ISBRA Interview Schedule) by measurement of serum %disialotransferrin, CDTect and plasma GGT, and (B–D) different self-reported average daily alcohol consumption thresholds by %disialotransferrin for men and women separately.

Table 2. Comparison of sensitivities and specificities of serum %disialotransferrin (%DST) for “heavy drinking” at different cutoff limits for all subjects combined and for men and women separately

<table>
<thead>
<tr>
<th>%DST cutoff</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
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<td>68.8</td>
<td>84.5</td>
<td>57.1</td>
<td>91.6</td>
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<td>95.0</td>
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<td>48.8</td>
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<td>40.8</td>
<td>94.4</td>
<td>20.8</td>
<td>98.9</td>
</tr>
</tbody>
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*aClassification into the different drinking categories was done using the WHO/ISBRA Interview Schedule.

*bThe two categories: “nondrinkers” and “light/moderate drinkers” were combined and compared with those classified as “heavy drinkers.”

for %disialotransferrin was significantly \((P < 0.001)\) higher than that for CDT by CDTect (0.68) and GGT (0.69) (Fig. 3A). The sensitivity and specificity of serum %disialotransferrin for “heavy drinking,” according to the WHO/ISBRA Interview Schedule classification, at different threshold limits are shown in Table 2 for all subjects combined and for men and women separately. At any potential cutoff, women showed lower sensitivity but higher specificity compared with men. However, according to ROC curve analysis, the overall test accuracy for %disialotransferrin did not differ significantly between men and women at any of the alcohol consumption thresholds examined (≥40 g, 60 g, or 80 g ethanol/day) (Fig. 3B–D).

Only 75 of the 1387 serum samples analyzed within this study contained a detectable amount (>0.1%) of %asialotransferrin, 60 of those originally classified as “heavy drinkers” and the remaining 15 (13 men and 2 women) as “light/moderate drinkers.” This corresponded to a sensitivity of ~18% for detection of “heavy drinking” by the presence of asialotransferrin. The average %asialotransferrin level was 0.50 ± 0.45% (mean ± SD; range 0.10–2.43%) with corresponding %disialotransferrin levels of 4.52 ± 2.05% (range 1.70–10.7%).
DISCUSSION

CDT was originally defined as the sum of the asialo-, monosialo-, and disialotransferrin glycoforms, largely due to analytical reasons related to the early methods (Stibler, 1991). Subsequent studies revealed that only disialo- and asialotransferrin are clearly related to continuous high alcohol consumption (Landberg et al., 1995; Helander et al., 2001, 2003), with disialotransferrin being the more sensitive single indicator of the two (Helander et al., 2003; Bergström and Helander, 2008). Despite this many subsequent CDT methods in routine or research use have continued to measure a CDT fraction containing variable proportions of the original CDT glycoforms, but sometimes also including part of trisialotransferrin (Arndt, 2001; Bortolotti et al., 2006). Because this made the assays sensitive to factors besides heavy drinking and, in that way, more vulnerable to analytical interference, the clinical performance of “CDT” as alcohol biomarker has sometimes been far from optimal. The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) working group on CDT standardization recently concluded that disialotransferrin should be the primary single target molecule for CDT measurement, with HPLC considered as the analytical principle for an interim reference method until a mass spectrometric method is established (Jeppsson et al., 2007). It was further recommended that for clinical use CDT should be expressed in a relative amount to compensate for variations in the total transferrin concentration.

It was previously concluded that the self-reported alcohol consumption data used as reference standard in the WHO/ISBRA study were sometimes incorrect (Helander and Eriksson, 2002). This is likely to have influenced the overall accuracy of the calculated sensitivity and specificity figures for all alcohol biomarkers included. The present observations further emphasized the limitations of self-reports of drinking as the basis of classification, in that several samples from “light/moderate drinkers” contained markedly elevated %disialotransferrin levels and some also a measurable asialotransferrin. This clearly indicated that misclassification of patients had occurred with the WHO/ISBRA Interview Schedule.

Regardless of this interference, the present study established that determination of %disialotransferrin by the HPLC candidate reference method was superior to the absolute CDT amount measured by CDTect. This is also in line with previous observations (Simonsson et al., 1996; Helander et al., 2001; Turpeinen et al., 2001). The present study further demonstrated a much higher test sensitivity of %disialotransferrin for high alcohol consumption and also a better correlation with recent (~1 week) high intake. This indicates that the diagnostic accuracy and clinical performance of the CDT biomarker was previously often underrated (Scouller et al., 2000; Koch et al., 2004). In contrast to the CDT by CDTect results originally used in the WHO/ISBRA study (Conigrave et al., 2002), %disialotransferrin measured by HPLC also performed markedly better than the conventional alcohol biomarkers (liver function tests) GGT and AST.

Earlier CDT studies based on the CDTect method pointed out possible nonalcohol-related risks for analytical interferences such as age, BMI, and smoking (Whitfield et al., 1998; Conigrave et al., 2002). When the serum samples of the WHO/ISBRA study were taken for analysis of %disialotransferrin by HPLC (Bergström and Helander, 2008), these results indicated that previous findings of false-positive CDT values (Fleming et al., 2004) were largely method dependent, and did not reflect true baseline differences or alcohol-induced changes in the transferrin glycoprotein complex. With respect to CDT testing by HPLC, it was proposed that an adjustment of reference intervals for %disialotransferrin in relation to either of ethnicity, age, BMI, or smoking is not required (Bergström and Helander, 2008). Still, the present investigation identified a few statistically significant but minor (and possibly clinically non-relevant) differences in %disialotransferrin levels in relation to age and BMI among the heavy drinkers that were not reflected in correspondingly higher alcohol consumption levels.

Over the years, one controversial issue related to CDT testing has been a possible gender influence on the test accuracy, with many studies reporting higher diagnostic sensitivity for males (Anton and Moak, 1994; Conigrave et al., 2002). In general, the present study obtained higher correlations between the different alcohol biomarkers for men, and men classified as “light/moderate” and “heavy drinkers” also showed higher %disialotransferrin levels on average than women. However, for “light/moderate drinkers” the gender difference was indicated to be due to misclassification of subjects. Accordingly, when the “light/moderate drinkers” with elevated %disialotransferrin levels, of which several also showed a detectable asialotransferrin, were excluded from the statistical calculations, the former significant gender difference in %disialotransferrin levels for this category disappeared. Furthermore, despite a trend toward higher sensitivity figures for men at any given alcohol consumption threshold limit, ROC curve analysis did not confirm a statistically significant overall difference for %disialotransferrin between men and women. Likewise, ROC curve analysis did not reveal any gender difference for detection of “heavy drinking.” The overall %disialotransferrin showed higher test sensitivity and better correlation with recent high alcohol intake according to self-report but there was no major gender difference.

REFERENCES


