Use of Capillary Zone Electrophoresis for Differentiating Excessive from Moderate Alcohol Consumption

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Background: The poorly sialylated transferrin isoforms in serum were analyzed by capillary zone electrophoresis (CZE) to differentiate moderate from heavy alcohol consumption.

Methods: We enrolled 614 volunteers, classified after interviews, self-reported drinking habits, and AUDIT scores as alcohol abusers (consuming >50 g/day ethanol for the previous 3 months or longer; n = 413) or moderate drinkers (<30 g/day ethanol; n = 201). Serum transferrin isoforms were separated at 28 kV and monitored at 214 nm on a P/ACE 5500 CZE with use of fused-silica capillaries and the related CEofix CDT reagent set. Immunosubtraction by anti-human transferrin and electrophoretic migration times identified the isoforms. Previous markers of alcohol abuse and an assay combining anion-exchange minicolumn chromatography with immunoturbidimetry (%CDT) were included in the study. Sensitivities and specificities were compared by ROC analysis.

Results: The asialylated isoform was missing in 95% of moderate drinkers but present in 92% of alcohol misusers. Disialotransferrin had a specificity and sensitivity of 0.75 at a cutoff of 0.7% of total transferrin, whereas the sum (asialo- + disialotransferrin) at a threshold of 1.2% had a sensitivity of 0.73 and a specificity of 0.92. Trisialotransferrin values did not distinguish between the two populations. Sensitivities and specificities of %CDT averaged 0.77 and 0.74, respectively, at a 2.6% cutoff; 0.67 and 0.83 at 2.8%; and 0.63 and 0.90 at 3%. CDT data were more sensitive and specific for males. Conventional biomarkers appeared less discriminating.

Conclusions: Asialotransferrin detected by CZE in sera of alcohol abusers offers the highest discrimination between excessive and moderate drinking.

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Carbohydrate-deficient transferrin (CDT) in serum has emerged as a useful biomarker for identifying alcohol misuse (1, 2). Whatever the method used for CDT detection, a huge requisite is its precision and accuracy because test results for alcohol abuse can lead to serious health, social, and forensic consequences.

In a previous report, we described the potential convenience of one asialylated transferrin (Tf) isoform to distinguish between two highly contrasting groups, namely, alcohol abusers entering a withdrawal treatment program and teetotalers (3). Analysis by capillary zone electrophoresis (CZE) and by ROC curves clearly indicated that the absence of this isoform was associated with abstinence. On the other hand, asialo-Tf was found in 89% of alcohol abusers in withdrawal treatment centers. It is unlikely that such increased sensitivities and specificities would be obtained for the clinical application of this CZE method to broad populations in general hospitals. Clinical usefulness thus had to be confirmed by testing this potential biomarker in patients with alcohol-related or non-alcohol-related health disorders, previously analyzed by clinical tools currently in the hands of practitioners, from interview to laboratory assays.

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5 Nonstandard abbreviations: CDT, carbohydrate-deficient transferrin; Tf, transferrin; CZE, capillary zone electrophoresis; GT, γ-glutamyltransferase; AST, aspartate aminotransferase; MCV, mean corpuscular volume; CRP, C-reactive protein; AUC, area under the curve; MT, migration time; and CI, confidence interval.
The present report aimed at evaluating alcohol consumption by the same method in two populations, matched for age and sex: (a) individuals who consumed moderate amounts of alcohol and (b) individuals suffering alcohol-related disease. Study participants included alcohol abusers, who claimed a consumption of >50 g/day ethanol for at least the previous 3 months, and occasional (social, moderate) drinkers self-reporting a mean daily ethanol intake <30 g. Healthy teetotalers were excluded because they had been tested in a previous study (3). Asialo- and disialo-Tf concentrations measured by CZE were compared with the conventional biomarkers of alcohol abuse (4) and with separation of CDT by ion-exchange minicolumn chromatography (5). The sensitivities and specificities at the cutoffs for Tf isoforms obtained by CZE were estimated for men and women.

Materials and Methods

CZE was conducted with a Analis reagent set (CEofix CDT Kit for P/ACE 5000 series) on a Beckman Coulter P/ACE System 5500 equipped with an ultraviolet detector and an interference filter at 214 nm. Uncoated fused-silica capillaries [57 cm × 50 μm (i.d.)] were obtained from Analis. CDT was detected by anion-exchange chromatography–immunoturbidimetry using the Axis-Shield %CDT reagent set. Human Tf polyclonal antiserum was purchased from Dako. Reagent sets for γ-glutamyltransferase (γGT) and aspartate aminotransferase (AST) activities were provided by Beckman Coulter. Mean corpuscular volume (MCV) was determined on a Cell-Dyn 4000 from Abbott. A Beckman Coulter Synchron LX20 was used for colorimetric reactions. Immunoturbidimetry was performed with a Immage immunonephelometer (Beckman Coulter). ROC analyses were performed with Analyze-it Software, Ver. 1.6.

Patient Selection

After informed consent, we enrolled 614 individuals who self-reported alcohol consumption. Participants were consecutively recruited by the medical staff from January to December 2001 among in- and outpatients (ratio 1:1) of the five public general hospitals of the Intercommunale de Santé Publique du Pays de Charleroi (Table 1). This study was approved by the Institutional Ethical Committee.

Alcohol intake was monitored by structured interviews, self-reported drinking habits, and the AUDIT questionnaire (6). Patients claiming an alcohol consumption >50 g/day for at least the previous 3 months and with an AUDIT score >11 were enrolled as alcohol abusers (n = 413). All members of this group expressed alcohol-related complaints.

Individuals with a self-reported alcohol consumption <30 g/day during the same period and an AUDIT score <7 were enrolled as moderate drinkers (n = 201). Metabolic, gastrointestinal, cardiovascular, and pulmonary problems were not excluded in this group, provided they were not alcohol-related or severely worsened by alcohol consumption.

Data collection on alcoholism diagnosis was blinded to the results of the analyses, and vice versa.

Serum Sampling

Blood samples were collected by venipuncture in Vacutainer serum tubes. Serum was obtained by centrifugation within 6 h of sampling and stored at −30 °C. All samples were analyzed by CZE within 1 week. MCV was measured after <4 h.

Conventional Biomarkers

Enzyme markers (γGT and AST), MCV, and C-reactive protein (CRP) were measured according to IFCC methods (7).

Detection of %CDT in Human Serum

After serum was filtered through anion-exchange minicolumns, the ratio of desialylated (0–2 sialic acid residues/molecule) isoforms to total Tf was determined by immunoturbidimetric assay with the %CDT reagent set according to the manufacturer’s instructions.

CZE

CZE was performed according to a recently described method (3). A reagent set for detection of Tf isoforms in diagnosis of alcoholism (CEofix CDT Kit) was used with

<table>
<thead>
<tr>
<th>Population</th>
<th>AUDIT score</th>
<th>n</th>
<th>Males</th>
<th>Females</th>
<th>Age, years</th>
<th>Alcohol, g/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol abusers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>27 (6)*</td>
<td>413</td>
<td>283</td>
<td>130</td>
<td>51 (22)*</td>
<td>166 (55)*</td>
</tr>
<tr>
<td>95% CI</td>
<td>22–32</td>
<td></td>
<td></td>
<td></td>
<td>46–56</td>
<td>158–174</td>
</tr>
<tr>
<td>Range</td>
<td>11–39</td>
<td></td>
<td></td>
<td></td>
<td>18–72</td>
<td>70–310</td>
</tr>
<tr>
<td>Moderate drinkers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>4 (3)</td>
<td>201</td>
<td>126</td>
<td>75</td>
<td>41 (20)</td>
<td>13 (7)</td>
</tr>
<tr>
<td>95% CI</td>
<td>2–6</td>
<td></td>
<td></td>
<td></td>
<td>36–46</td>
<td>12–14</td>
</tr>
<tr>
<td>Range</td>
<td>1–8</td>
<td></td>
<td></td>
<td></td>
<td>18–65</td>
<td>10–30</td>
</tr>
</tbody>
</table>

*p <0.05.  
*P >0.05.
the same modifications as described in our previous report (3).

Serum samples were diluted 1:1 (by volume) with a 1 g/L FeCl₃ solution for at least 3 min to saturate Tf iron-binding sites. The capillary was coated by “dynamic double coating” described elsewhere (8,9), to obtain stable electro-endosmosis and to avoid partial protein denaturation at the capillary surface. Double coating steps were as follows: (a) Malic acid buffer, pH 4.8, was injected under high pressure (20 psi) for 1 min. (b) The separation buffer, Tris-borate (pH 8.5), was injected for 1.5 s under high pressure (20 psi) and then under low pressure (0.5 psi) for 0.5 min. This latter procedure was omitted when the reagent set recommended by the manufacturer was used. (c) Before serum sample injection, a 10 g/L solution of sodium dodecyl sulfate was injected for 2 s at low pressure (0.5 psi) to keep β-lipoprotein peaks out of the peak domain of interest. This operation was not mentioned in the manufacturer’s guide because sodium dodecyl sulfate is included in the FeCl₃ solution of the reagent set. (d) Sera were then injected for 2 s at low pressure (0.5 psi).

A voltage of 28 kV was applied for 7 min. The Tf isoforms were detected by absorbance at 214 nm, as described by Blessum et al. (10), rather than at 200 nm, as recommended by the manufacturer. This modification was important because at 200 nm the ratio of noise to signal was higher, as reported by the same authors (10).

The peaks representing the different Tf isoforms, identified in our previous report (3), were quantified as a percentage of the total Tf content in terms of valley-to-valley area under the curve (AUC) because all were well separated. Results were printed on an electropherogram after treatment by integration software from Beckman Coulter. The ratios of the asialo-, disialo-, and trisialo-Tf peak areas to the cumulative area of the peaks of all Tf isoforms were calculated by this software. The migration times (MTs) of the isoforms were compared.

It has previously been demonstrated that the detection limit for asialo-Tf was 0.03% of total Tf (3). The mean interrun CVs for the “low” asialo-Tf (<1% total Tf AUC) and “high” (>1%) data were 7.4% and 4.6%, respectively. CVs were also <8% for disialo-Tf and for (asialo- + disialo-Tf). The within-run CVs were 2.5% for disialo-Tf and 4.5% for asialo-Tf.

VALIDATION OF TF ISOFORMS
Anti-human Tf rabbit antiserum was diluted 1:3 in serum samples after a first CZE analysis of undiluted serum. The electropherograms obtained before and after immunosubtraction were compared. The 1:3 dilution of anti-Tf was selected to maintain a remnant of P4, which helped in the comparison of electropherograms of native and immunoprecipitated sera (Fig. 1).

IDENTIFICATION OF THE ISOFORMS
Isoforms were further identified by comparing their MTs with those previously described for alcohol abusers entering a withdrawal treatment and teetotalers (3).

STATISTICS
Values for all biomarkers were available for all patients involved in the study. The asymmetry of the ranges of all biomarker values (γGT, AST, MCV, %CDT, and percentage of desialylated isoforms detected by CZE) measured in these broad and unfit populations was normalized with use of a natural logarithmic scale, as recommended (3,11) when diagnostic methods for linear statistical models are used (12). Sample values were compared by the nonparametric Wilcoxon test. Geometric means and SDs were calculated, leading to asymmetrical 95% confidence intervals (CIs). The range of each biomarker was also shown.

Results from the %CDT assay were compared using the 2.6% cutoff, as recommended by the manufacturer and according to Helander and coworkers (5,13). Three cutoff values were taken into account for this minicolumn anion-exchange chromatography–immunoturbidimetry method: 2.6%, 2.8%, and 3% of total Tf.

ROC curves (14) for 413 alcohol abusers and 201 moderate drinkers, matched for age and sex, were constructed for comparing the sensitivity and specificity of the markers. Results were expressed as the mean area under the ROC curve (AUCarea) and its 95% CI (14,15). Cutoffs for asialo-Tf, disialo-Tf, and of the sum (asialo- + disialo-Tf) were selected from the curves. Gender variations were compared by ROC curve analysis of female (n = 205) and male (n = 409) participants, using the cutoffs defined on the whole population.

RESULTS

CONVENTIONAL BIOMARKERS
Mean values for the enzyme biomarkers were above the reference intervals in the sera of alcohol heavy drinkers.

Table 2. Values measured in alcohol abusers and moderate drinkers.

<table>
<thead>
<tr>
<th>Assay</th>
<th>γGT, U/L (cutoff, 50 U/L)a</th>
<th>AST, U/L (cutoff, 37 U/L)</th>
<th>MCV, fL (cutoff, 100 fL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol abusers (n = 413)</td>
<td>Mean (SD)</td>
<td>71 (3)b</td>
<td>39 (2)c</td>
</tr>
<tr>
<td>95% CI</td>
<td>8–618</td>
<td>9–157</td>
<td>77–1118</td>
</tr>
<tr>
<td>Range</td>
<td>6–1191</td>
<td>10–674</td>
<td>65–122</td>
</tr>
<tr>
<td>Moderate drinkers (n = 201)</td>
<td>Mean (SD)</td>
<td>45 (3)</td>
<td>32 (2)</td>
</tr>
<tr>
<td>95% CI</td>
<td>5–417</td>
<td>7–139</td>
<td>72–101</td>
</tr>
<tr>
<td>Range</td>
<td>9–1145</td>
<td>10–384</td>
<td>61–103</td>
</tr>
</tbody>
</table>

a Cutoffs correspond to the upper limits of the reference intervals used in the Laboratory of Clinical Chemistry, CHU André Vésale.

b P ≤0.0001.
c P <0.001.
d P = 0.004.
A significant enhancement of mean γGT activity was induced by an ethanol intake >50 g/day, compared with an ethanol intake <30 g/day. The mean transaminase activities were also significantly different. Mean MCVs were both <100 fL but were statistically different, and the range was wider for alcoholics (Table 2).

**ANALYSIS OF THE ELECTROPHERGRAMS**

Typical electrophoretic profiles obtained from sera of a social drinker (AUDIT score = 3) and an alcohol abuser (AUDIT score = 24) are shown in Fig. 1. The electropherogram baselines were horizontal and smooth, and the peaks were well separated. In Fig. 1, the typical scale of the y-axis was 0.00. When a serum containing 2.5 g/L Tf was subjected to CZE with a 2 s injection (2.2 μL), the absorbance of the 80% predominant middle peak was 0.014 arbitrary units.

Six peaks, previously identified as asialo- to hexasialo-Tf (3), were observed in the serum from the alcohol abuser. A predominant middle peak with a MT averaging 6 min was present in the sera from both individuals (Fig. 1). The serum from the alcohol abuser contained three earlier migrating peaks. Two peaks were present only in the serum from the occasional drinker (Fig. 1). Those peaks have previously been differentiated as asialo-, disialo-, trisialo-, pentasialo-, and hexasialo-Tf and termed P0, P2, P3, P4, P5, and P6 (3). The peak between asialo- and disialo-Tf, which was immunologically confirmed to represent comigration of a Tf isoform and CRP (3), was not present in the sera of the two patients in Fig. 1. The CRP concentrations were low in these two individuals: 1 mg/L in the alcohol abuser and 0.3 mg/L in the moderate drinker (upper limit of the reference interval is 10 mg/L). This P1 peak was found in other patients who were moderate or heavy drinkers with circulating CRP concentrations >10 mg/L.

No peak migrating as P0 was observed in the electropherogram of the social drinker. Peak P2 was higher in the serum from the alcohol abuser than in serum from the moderate drinker. All peaks disappeared after the addition of anti-Tf except a remnant of tetrasialo-Tf (Fig. 1). Electropherograms obtained after addition of the polyclonal antiserum exhibited a baseline drift (Fig. 1). When the electropherogram was recorded continuously for more than 7 min, this drift was observed only after the addition of anti-Tf and was preceded by a broad bell-shaped increase in the baseline value (Fig. 2).

**MTs of the Isoforms in the Two Populations**

Each peak could be statistically differentiated from the others, based on retention times. The MTs of the same peaks were statistically identical in the two populations, averaging 5.5 min for P0, 5.8 min for P2, and 5.9 min for P3. Their CVs averaged 1–1.5%. The MTs of each peak were statistically similar to those for P0 to P6 observed in teetotalers and alcohol abusers entering a withdrawal treatment (3).

**Quantification of Tf Isoforms by Capillary Electrophoresis**

Alcohol abusers were almost the sole individuals exhibiting asialo-Tf. Disialo-Tf was significantly (P <0.001) more increased in the sera of alcohol abusers. The same result was obtained for the sum (asialo + disialo-Tf) (Table 3). The AUC for trisialo-Tf was statistically identical in alcohol abusers and social drinkers, and the ratio disialo-/trisialo-Tf was higher in alcohol abusers.

Genetic polymorphisms involving a trisialo-Tf value almost equivalent to the tetrasialo-Tf value were limited to nine individuals (Fig. 1), and concentrations of this isoform >9% (mean ± 2 SD) of total Tf were observed in 39 patients. The electropherogram for serum from a moderate alcohol consumer, designated as “Variant”, exhibited disialo- and tetrasialo- to hexasialo-Tf retention times similar to the ones obtained for sera from teetotalers or alcohol users. The high trisialo-Tf peak of the variant,
immunosubtractable by anti-human Tf polyclonal antibody, had a longer MT than in the other series. All isoforms were as clearly separated as in the sera of other participants regardless of their alcohol habits (Fig. 1).

%CDT MINICOLUMN IMMUNOASSAY
The %CDT values were significantly increased in alcohol misusers compared with occasional drinkers. The ranges and 95% CI were narrow for moderate drinkers and wider for alcohol misusers (Table 3).

ROC CURVE ANALYSIS
ROC curves obtained for γGT, AST, and MCV of 614 patients are shown in Fig. 3. The low discriminating power of these markers is apparent (Table 4) at the cutoffs used in the Laboratory of Clinical Chemistry of the ISPPC general hospitals (Table 2).

The ROC analysis based on CZE data for the greater ROC curve area obtained with relative asialo-Tf percentages compared with disialo-Tf and the sum (asialo- + disialo-Tf) is shown in Fig. 4. The absence of asialo-Tf in 95% of moderate drinkers and its presence in 92% of alcohol abusers are illustrated in Table 4. Sensitivity and specificity were both limited to 0.75 for a disialo-Tf cutoff of 0.7% of total Tf. Better results were obtained for a 1.2% cutoff of the sum (asialo- + disialo-Tf). Trisialo-Tf only poorly distinguished the groups (Table 4).

As shown in Fig. 5, the ROC curve area for %CDT was clearly lower than that for asialo-Tf values obtained by CZE. At the 2.6% cutoff recommended by Axis-Shield, sensitivity and specificity of the %CDT assay averaged 0.75 (Table 4). At the threshold values of 2.8% and 3%, the specificity of the assay was 0.90, but its sensitivity decreased to 0.6, as predicted by Whitfield (16) and by previous odds ratios estimated from metaanalysis of CDT studies (17).

CDT GENDER VARIATIONS
Areas under ROC curves were greater in men than in women (Table 5). Sensitivity and specificity were higher in men for %CDT as well as for CZE isoforms. Gender areas under ROC curves for %CDT assay were similar to those observed by Sillanaukee and Olsson (11).

Discussion
Individuals enrolled in the present study were hospitalized or visited the hospital for different health complaints.

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### Table 3. Relative percentages of CDT and the CZE Tf isoforms.

<table>
<thead>
<tr>
<th>Population</th>
<th>CDT (%)</th>
<th>Asialo-Tf (%)</th>
<th>Disialo-Tf (%)</th>
<th>Asialo- + Disialo-Tf (%)</th>
<th>Trisialo-Tf (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol abusers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>3.9 (1.8)</td>
<td>1.8 (1.8)</td>
<td>1.4 (2.7)</td>
<td>2.9 (2.9)</td>
<td>4.5 (2)</td>
</tr>
<tr>
<td>95% CI</td>
<td>1.2–12.2</td>
<td>0.5–3.5</td>
<td>0.2–10.1</td>
<td>0.3–17</td>
<td>1–19.8</td>
</tr>
<tr>
<td>Range</td>
<td>0.7–18.2</td>
<td>0–4.3</td>
<td>0–17.4</td>
<td>0–22</td>
<td>0.03–41</td>
</tr>
<tr>
<td>Moderate drinkers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>2.3 (1.3)</td>
<td>0.003 (0.03)</td>
<td>0.5 (1.8)</td>
<td>0.6 (1.8)</td>
<td>4.3 (1.5)</td>
</tr>
<tr>
<td>95% CI</td>
<td>1.4–3.8</td>
<td>0.001–0.02</td>
<td>0.2–1.7</td>
<td>1.9–3.4</td>
<td>1.9–19.4</td>
</tr>
<tr>
<td>Range</td>
<td>1.1–6</td>
<td>0–0.34</td>
<td>0–4.3</td>
<td>0–4.3</td>
<td>1.4–13.6</td>
</tr>
</tbody>
</table>

* a P < 0.00001.  
* b P = 0.20.
They all consumed alcohol, either moderately or in excess (Table 1). Interviews, self-reported alcohol habits, and AUDIT questionnaires determined their alcohol consumption and allowed definition of the range of their ethanol intake. Mean γGT and AST activities were widely dispersed in both populations (Table 2), confirming health problems.

Characterization and identification of the various Tf isoforms visualized in the electropherograms have recently been performed in alcohol abusers entering a withdrawal program and in teetotalers (3). Use of an anti-human Tf polyclonal antibody confirmed that six peaks obtained from sera of alcoholics were immunoreactive Tf isoforms (Fig. 1). Five peaks were observed in moderate drinkers, as shown previously in teetotalers (3).

The predominant human iso-Tf is known to be tetrasialylated (1–3). The corresponding signal (tetrasialo-Tf) was found in all electropherograms (Fig. 1). On the basis of the physicochemical features governing CZE (10), less sialylated isoforms migrated earlier and more sialylated ones later.

A high disialo-Tf concentration has been associated with the presence of asialo-Tf in excessive drinking, as measured by HPLC (18). The peaks migrating at 5.5 and 5.8 min in the sera of alcohol abusers (Fig. 1) found in our

**Table 4. Analysis of ROC curves for Tf isoforms, %CDT, and conventional markers in 614 sex-matched patients in general hospitals.**

<table>
<thead>
<tr>
<th>Marker</th>
<th>ROCarea</th>
<th>95% CI</th>
<th>Cutoff</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asialo-Tf</td>
<td>0.96</td>
<td>0.94–0.97</td>
<td>0%</td>
<td>0.92</td>
<td>0.95</td>
</tr>
<tr>
<td>Disialo-Tf</td>
<td>0.80</td>
<td>0.76–0.83</td>
<td>0.7%</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>(Asialo+ Disialo-Tf)</td>
<td>0.91</td>
<td>0.88–0.93</td>
<td>1.2%</td>
<td>0.73</td>
<td>0.92</td>
</tr>
<tr>
<td>Trisialo-Tf</td>
<td>0.59</td>
<td>0.55–0.64</td>
<td>4.76%</td>
<td>0.51</td>
<td>0.62</td>
</tr>
<tr>
<td>%CDT</td>
<td>0.82</td>
<td>0.75–0.85</td>
<td>2.6%</td>
<td>0.77</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.8%</td>
<td>0.67</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3%</td>
<td>0.63</td>
<td>0.90</td>
</tr>
<tr>
<td>MCV</td>
<td>0.61</td>
<td>0.56–0.66</td>
<td>100 fL</td>
<td>0.29</td>
<td>0.85</td>
</tr>
<tr>
<td>γGT</td>
<td>0.63</td>
<td>0.58–0.68</td>
<td>50 U/L</td>
<td>0.53</td>
<td>0.66</td>
</tr>
<tr>
<td>AST</td>
<td>0.59</td>
<td>0.54–0.64</td>
<td>37 U/L</td>
<td>0.45</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Fig. 3. ROC curve analysis for AST, γ GT, and MCV distributions. The y axis is the sensitivity, and the x axis is (1 – specificity). ○, γ GT; □, AST; △, MCV. Arrows indicate the cutoffs for the different biomarkers on their respective curves.

Fig. 4. ROC curve analysis of the relative percentages of the Tf asialylated isoform (0-sialo; ○), disialylated Tf (2-sialo; □), and the sum asialo+ disialo-Tf (0+2-sialo; △) obtained by CZE. The y axis is the sensitivity, and the x axis is (1 – specificity). Arrows indicate the cutoffs for the different biomarkers on their respective curves.
study should represent asialylated and disialylated Tf isoforms, respectively. The P0 peak (MT, 5.5 min) was almost solely encountered in the sera of alcohol abusers. Peak P2 (MT, 5.8 min) was higher (Fig. 1), and the AUC percentage was higher (Table 3) in alcohol abusers than in moderate drinkers. When present, monosialo-Tf was not taken into account because it represented comigrating monosialo-Tf and CRP (3). Relative trisialo-Tf percentages were similar in the two populations (Table 3), as they were in our study comparing alcohol abusers with teetotalers (3). Only 6% of the 614 individuals under study had a trisialo-Tf concentration (H11022) 9% of total Tf, and genetic polymorphisms involving similar concentrations of trisialo- and tetrasialo-Tf accounted for 2%.

The identification of the isoforms was supported by their immunoprecipitation with anti-Tf (Figs. 1 and 2), and by retention times identical to those of alcoholics and teetotalers (3). The baselines of the native serum electropherograms were smooth and horizontal, whereas those obtained after addition of the polyclonal antibody had a drift that may be attributable to the addition of immunoglobulins migrating just before the Tf isoforms and seen as a broad increase in the CZE baseline before the drifting (Fig. 2). All isoforms were clearly separated in undiluted sera [Figs. 1 and 2 and Ref. (3)]. This feature allowed us to calculate the relative percentages of the isoforms in the valley-to-valley mode.

ROC curves are known to provide a graphic illustration of the association between specificity and sensitivity of any diagnostic test over all possible cutoff values (14). Conventional markers appeared to have low sensitivities and specificities (Fig. 3 and Table 4). In the present study, γGT appeared to be a poor marker for distinguishing the groups (Table 4), which is in accordance with results from previous studies (19). AST provided almost no discriminating power. MCV had a high specificity but a low sensitivity (Table 4), as described previously (20).

We found asialo-Tf in serum from 10 of the 201 moderate drinkers in the present study (5% false positives), whereas this isoform was present in serum from 370 of 413 alcohol abusers (92% true positives). The sensitivities and specificities of CZE for disialo-Tf and of the sum (asialo- + disialo-Tf) were lower (Fig. 4 and Table 4).

Asialo-Tf has been defined as the native Tf amino acid sequence (2, 13, 21). Lectin binding to glycan chains devoid of sialic acid helped to identify an asialylated isoform in sera of alcoholics (22, 23). We have defined asialo-Tf as the sole CZE form kinetically unaffected by enzymatic treatment with neuraminidase (3). These results allowed us to presume that the earliest migrating form in our electropherograms did not contain any sialic acid on the N-glycan chain(s), but it did not permit us to claim that it was devoid of glycans chain(s). Disialo-Tf has been depicted as a disialylated isoform containing one N-glycan chain (13, 21). A more detailed analysis would be needed, using neuraminidase and N-glycosidase and

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\text{Table 5. Gender variations of ROC curves for men and women.}^{a}
\]

<table>
<thead>
<tr>
<th></th>
<th>Men (n = 409)</th>
<th>Women (n = 205)</th>
</tr>
</thead>
<tbody>
<tr>
<td>%CDT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROCarea</td>
<td>0.80</td>
<td>0.76</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.72–0.87</td>
<td>0.67–0.84</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.77;* 0.66&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.64;* 0.68&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.67;* 0.90&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.64;* 0.84&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Asialo-Tf</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROCarea</td>
<td>0.96</td>
<td>0.87</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.92–1</td>
<td>0.81–0.94</td>
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<tr>
<td>Sensitivity</td>
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</tr>
<tr>
<td>Specificity</td>
<td>0.97</td>
<td>0.86</td>
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<tr>
<td>Disialo-Tf</td>
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<tr>
<td>ROCarea</td>
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<tr>
<td>95% CI</td>
<td>0.74–0.88</td>
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<tr>
<td>Sensitivity</td>
<td>0.81</td>
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<tr>
<td>Specificity</td>
<td>0.75</td>
<td>0.76</td>
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<td>Asialo- + Disialo-Tf</td>
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<td>95% CI</td>
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<tr>
<td>Sensitivity</td>
<td>0.63</td>
<td>0.33</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.95</td>
<td>0.93</td>
</tr>
</tbody>
</table>

*Sensitivities and specificities of %CDT are given for the cutoffs of 2.6% and 3%. Cutoffs of 0%, 0.7%, and 1.2% of total Tf defined in the sex-matched populations have been applied to the CZE desialylated makers.

<sup>a</sup>%CDT cutoff: 2.6%; 3%.
applying mass spectrometry (24), to highlight glycosyla-
tion of the CDT isoforms.

Our CZE method using asialo-Tf as a biomarker of 
 alcohol abuse favorably compared with separation of the 
 CDT fraction by ion-exchange chromatography on mini-
columns and quantification by immunoassay (Fig. 5 and 
 Tables 4 and 5). The %CDT measured immunologically 
after separation through minicolumns was higher than 
the sum (asialo- + disialo-Tf) obtained by CZE. This 
discrepancy might be explained by a more accurate iso-
lation of the desialylated isoforms by CZE. Partial coelu-
tion of trisialo-Fe₂-transferrin in the desialylated fraction 
harvested on minicolumns and partial retention of di-
sialo-Fe₂-transferrin have been described (25, 26). We 
agree with the previous claim that isolation of the indi-
vidual Tf isoforms is more accurate than en masse sepa-
ration of desialylated forms using minicolumns 
(3, 13, 18, 27).

Concerning the controversy surrounding involvement 
of trisialo-Tf in CDT isoforms (15, 28–35), the low area 
under the ROC curve for trisialo-Tf (Table 4) confirmed 
that it would not be convenient for the determination of 
the desialylated isoforms. Our data demonstrated that 
increased relative concentrations of disialo- and asialo-Tf 
attributable to excessive consumption of alcohol were not 
associated with increased trisialo-Tf (Table 3).

When results obtained by analyzing sera of teetotalers 
and alcoholics entering a withdrawal program (3) were 
compared with the present data involving inpatients and 
outpatients from general hospitals, all of whom con-
sumed ethanol moderately or excessively, asialo-Tf sen-
sitivity increased from 0.89 to 0.92, whereas the specific-
ity decreased from 1 to 0.95. The discriminating power of 
disialo-Tf was dramatically decreased. For the sum 
(asialo- + disialo-Tf), the specificity of 0.9 came at the 
expense of a sensitivity decrease from 0.84 to 0.75. 
Asialo-Tf appeared the most powerful marker for differ-
etiating heavy from moderate alcohol consumption 
(Figs. 4 and 5 and Tables 4 and 5). Thus our preliminary 
(3) and the present study gave experimental confirmation 
of the use of asialo-Tf as a marker of alcohol abuse, as 
proposed by Arndt (36). However, more data are needed 
before CDT can be replaced by asialo-Tf.

The patients recruited in the present study did not fill 
the 30–50 g/day gap separating moderate from heavy 
alcohol consumption. It has been claimed that CDT may 
have a place in monitoring alcohol consumption, even in 
men whose alcohol intake is in the 20–60 g/day range 
(37). Combining the results for asialo-Tf and the sum 
(asialo- + disialo-Tf) obtained by CZE with self-reported 
alcohol habits and assessment of alcohol intake by accu-
rate questionnaires (15) and validated structured inter-
views (27) might reduce confusion and errors in the 
evaluation of excessive drinking.

The present results confirmed observations concerning 
hospital setting conditions, in which CDT appeared mark-
edly more specific than conventional markers, especially 
γGT (38). The abundance of individuals with health 
complaints in the present study reinforced this assess-
ment.

Sensitivity and specificity were lower in the female 
groups of excessive and moderate drinkers for both 
%CDT and CZE at cutoffs defined on sex-matched pop-
ulations (Table 5). This gender variation has been recently 
demonstrated for %CDT (5, 39) and agrees with the most 
recent WHO/ISBRA assessment that CDT is a slightly but 
significantly better marker of high-risk consumption in 
men (40).

Turpeinen et al. (27) recently reported a 1.8% cutoff for 
HPLC analysis of disialo-Tf. At this threshold, the authors 
reported a sensitivity of 52% and a specificity of 98% for 
differentiation between moderate and heavy drinkers, 
and the area under the ROC curve was 0.87 (95% CI, 
0.81–0.93). In our CZE study, the area under the ROC 
curve for disialo-Tf was 0.80 (95% CI, 0.76–0.83), and 
the sensitivity and specificity for discriminating exces-
.sive from moderate alcohol consumers were both 75% 
(Table 4).

This discrepancy may be attributed in part to the 
partial overlapping of disialo- and trisialo-Tf observed 
in HPLC methods when asialo-Tf is present (27, 41), 
whereas these isoforms were well separated by our CZE 
method (Fig. 1). This discrepancy can also be attributable 
to incomplete recovery of CDT after sample pretreatment. 
Integration in the horizontal baseline mode was rather 
risky when two peaks, one decisive in the determination 
of CDT (disialo-Tf) and one not useful for the diagnosis of 
alcoholism (trisialo-Tf), overlapped. The reliability of CZE 
for quantification of CDT isoforms likely rests on the 
complete isolation of disialo-Tf [Fig. 1 and Ref. (3)]. This 
hypothesis is supported by the high, probably overesti-
mated, HPLC cutoff for the disialo-Tf percentage [1.8% 
(27)] compared with the 0.7% obtained by our CZE 
method.

The separation and identification of the Tf isoforms by 
HPLC and CZE raise the question: what do we respec-
tively measure? Are the molecules behind the peaks we 
observe different? Characterization of the isoforms sepa-
rated by HPLC and CZE, using lectins (22, 23), neuramin-
idase (3), or N- and O-glycosidases alone (3, 21) or added 
in succession should allow us to progress in the definition 
of standards requested for CDT (2).

In conclusion, concerning the use of the CZE Tf elec-
tropherograms in clinical conditions, we suggest a rapid 
(<10 min) analysis of the asialo-Tf isoform, which will be 
present in 92% of alcohol abusers and absent in 95% of 
moderate alcohol consumers (Table 4). Some questions 
and possibilities behind using asialo-Tf as a clearly de-
defined analyte for laboratory diagnosis of chronic alcohol 
abuse have been discussed earlier (36). They remain open, 
as does establishing reliable cutoffs for men and women. 
Some progress has to be made concerning the precision of 
qualitative and quantitative evaluations of desialylated Tf 
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isoforms. Comigration of CRP and the monosialo-Tf form (3) forced us to discard this desialylated form from CDT evaluation because of the frequency of inflammatory reactions that could induce CRP. It has been shown that there was no correlation between CDT and free hemoglobin as a measure of hemolysis (42). We are now conducting a study on possible interference between Tf sialylation and the chronic major pathologies designated by the World Health Organization as other main health challenges for the 21st century, namely cancer and cardiovascular diseases. Interferences in assays by the serum molecules involved in major diseases, such as bilirubin in liver dysfunction or carcinoembryonic cancer markers, should be carefully studied.

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