Analyte Comigrating with Trisialotransferrin during Capillary Zone Electrophoresis of Sera from Patients with Cancer

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Background: Serum concentrations of monoglycosylated isoforms of transferrin are increased by chronic ethanol intake. We investigated transferrin glycosylation in patients with cancer, in which aberrant glycosylation is also induced.

Methods: We used a P/ACE 5000 series capillary zone electrophoresis (CZE) apparatus and a CZE carbohydrate-deficient transferrin reagent set to study 200 cancer patients who consumed alcohol moderately and 33 who were alcohol abusers; we then compared these patients with 56 healthy teetotalers, 89 moderate, and 112 excessive alcohol drinkers without known malignancies. Transferrin isoforms were identified by immunosubtraction with anti-human transferrin polyclonal antibody.

Results: Seven peaks, P0–P6, were visualized and completely or partly immunosubtracted when CZE separation was performed at pH 8.5. P0 was present in 95% of alcohol abusers with or without cancer. P3 was significantly higher in cancer patients and was only partly immunosubtracted as trisialotransferrin in all groups. The comigrating analyte was not altered by papain, precipitation by ethanol, or extraction by organic solvents, but was sensitive to acid hydrolysis, suggesting a polysaccharide structure. When isolated at pH 8.25, this analyte was higher in cancer patients. ROC curve analysis identified localized malignant neoplasia at P3 values above 5.8% of total transferrin (sensitivity, 0.78; specificity, 0.87), regardless of alcohol consumption. Disseminated cancers were better differentiated above 8% (sensitivity, 0.94; specificity, 0.96).

Conclusions: Malignant neoplasia, unlike excessive ethanol intake, did not alter the addition of two N-glycans to transferrin. A peak comigrating with trisialotransferrin had characteristics of a polysaccharide in all adults and was increased in sera of patients with cancer.

Carbohydrate-deficient transferrin (CDT)5 is considered a reliable marker of alcohol abuse (1, 2). CDT is not a single molecule but a group of isoforms: asialo-, monosialo-, and disialotransferrins (1, 2). These three isoforms lack one chain compared with the two N-glycan chains of the predominant tetrasialotransferrin (1–5). Deglycosylation/desialylation of transferrin (Tf) has been related to immoderate ethanol consumption compared with abstinence (6) or moderate consumption (7).

Paradoxically, little attention has been devoted to the possible interference of this glycosylation-related biomarker of alcoholism with tests for the major diseases designated by the WHO as other health challenges for the 21st century (8), i.e., cancer, AIDS, and cardiovascular disease. Because Tf, and consequently CDT, is synthesized, glycosylated, and secreted by the liver (3), the use of CDT values in patients with liver disease has been an area of interest. The results of studies using anion-exchange minicolumn chromatography followed by immunoassay indicated that serum CDT is increased in patients with hepatocellular carcinoma and in patients with end-stage liver disease who are waiting for transplantation (9, 10). Separation of Tf isoforms by lectin affinity chromatography.

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phory demonstrated higher concentrations of trisialotransferrin in patients with hepatocarcinoma than in healthy individuals (11).

The scarcity of data relating glycopathology and the diagnosis of malignant neoplasia may be related to the types of studies conducted to identify glycosylation changes in human cancer. Human tissue sections and immunohistochemical techniques with lectins or antibodies have generally been used (12). A few diagnostically relevant investigations have been published (12, 13), but the numbers of specimens evaluated were often small.

Biochemical methods allowing the study of large series of patients have been rare until recently. Johnson’s group reported that hepatocellular carcinoma-associated α-fetoprotein isoforms analyzed by electrospray ionization–mass spectrometry represented a group of glycoproteins whose carbohydrate structures were all characterized by being monosialylated, whereas those associated with benign liver disease and nonseminomatous germ-cell tumors were di- and asialo species, respectively (14–16). These authors concluded that both the serum concentration and percentage of monosialylated α-fetoprotein are potential diagnostic markers for hepatocellular carcinoma with nondiagnostic α-fetoprotein (15, 16).

We have developed a capillary zone electrophoresis (CZE) method that separates six isoforms immunosubtractable by anti-human Tf (6, 7). It appears that asialotransferrin is present in 90–95% of alcohol abusers (6, 7) and 5% of moderate drinkers (7) and is undetectable in abstainers (6). Disialotransferrin is higher in alcoholics, and trisialotransferrin is not affected by ethanol intake.

The aim of this study was to measure Tf isoforms in the serum of patients with and without cancer and to characterize a nontransferrin CZE peak that is increased in cancer.

Materials and Methods

Materials

For CZE, we used a reagent set from Analis (Ceofix-CDT reagent set for P/ACE 5000 Series) and a Beckman Coulter P/ACE System 5500 equipped with an ultraviolet detector and an interference filter at 214 nm. The uncoated fused-silica capillaries [57 cm × 50 μm (i.d.)] were from Analis. We also measured CDT by anion-exchange chromatography/immunoturbidimetry with the Axis-Shield %CDT reagent set. Serum Tf was measured by the Beckman Coulter IMMAGE TRF test. Rabbit anti-human Tf polyclonal antibody (anti-Tf) and anti-human C-reactive protein (anti-CRP) were purchased from Dako. Papain, hexane, diethyl ether, petroleum ether, sulfuric acid, and ethanol were from Merck.

Volunteers and Patients

Patients consecutively diagnosed with cancer (n = 233; 110 women and 123 men) in three Hospital University Centers of the Intercommunale de Santé Publique du Pays de Charleroi were recruited from February 2001 to March 2002. Different types of cancer (e.g., colon, rectum, breast, lung, and others) were included. Localized (n = 155) and disseminated (n = 78) tumors were studied. The mean (SD) age was 62 (10) years. Tumor dissemination was investigated by abdominal ultrasound computed tomography or by nuclear magnetic resonance imaging. Blood samples were analyzed immediately after diagnosis and before any medications or nutritional supplements were administered.

Alcohol consumption was evaluated by use of the Alcohol Use Disorders Identification Test (AUDIT) questionnaire (17) and self-reported alcohol habits. Patients with cancer (n = 33) who claimed a daily alcohol consumption >50 g were considered alcohol abusers (1).

The participants who did not have cancer (n = 257) were in- and outpatients from the Departments of Gastroenterology and Internal Medicine of Intercommunale de Santé Publique du Pays de Charleroi. They were examined by a physician and interviewed by trained staff, and their alcohol history was evaluated by AUDIT questionnaire and self-reported alcohol habits. Teetotalers, social drinkers, and alcohol abusers were continuously enrolled from February 2001 to March 2002. Healthy adult teetotalers [n = 56; 18 males and 38 females; mean (SD) age, 53 (11) years] were abstainers for philosophical reasons. Moderate alcohol drinkers [n = 89; 41 males and 48 females; mean age, 55 (15) years] did not suffer from cancer or from hepatic or cardiovascular disease. Their ethanol intake was <30 g/day (1). Alcohol abusers [n = 112; 61 males and 51 females; mean age, 55 (12) years] were included on the basis of the same criteria and consumed >50 g ethanol/day. Interviews and laboratory tests revealed no neoplastic pathologies in these populations.

All patients were informed and agreed with the aims and modalities of the study that have been approved by the institutional ethics committee. Data collection on cancer diagnosis, alcohol consumption, and hepatic diseases was blinded to the results of the analyses and vice versa. No adverse events were encountered.

Methods

Serum Tf. The Beckman Coulter IMMAGE TRF test measures the rate of increase in light scatter from particles suspended in solution as a result of complexes formed during an antigen (Tf)-antibody (goat polyclonal serum against human Tf) reaction, with a laser emitting at 670 nm. A 0.1-μL serum sample was added to 21 μL of antibody in a total of 340 μL of buffered medium. The imprecision (CV) of the assay, as provided by the manufacturer, was 4–6%. Calibration was performed automatically by the Beckman Coulter instrument.

Immunoturbidimetric assay for CDT (%CDT). The ratio of monoglycosylated (0–2 sialic residues per molecule) Tf isoforms to total Tf was determined by anion-exchange chromatography/immunoturbidimetry with the Axis %CDT reagent set, according to the manufacturer’s instructions. Serum Tf was saturated with Fe³⁺ before the...
low-sialic-acid Tf isoforms were separated by passage through an anion-exchange chromatography minicolumn. Whole Tf and the monoglycosylated forms were measured separately with use of the same anti-Tf antibody (6, 7). The imprecision (CV) of the assay, as provided by the manufacturer, was 3.9–6.7%.

CZE. Serum samples (0.5 mL) were diluted with 0.5 mL of an aqueous solution containing 1 g/L FeCl₃ for at least 3 min to saturate Tf iron binding sites. The capillary was coated by the dynamic double-coating method described elsewhere (7) to obtain stable electro-endosmosis and to avoid partial protein denaturation at the capillary surface. The 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 was Tris-borate, pH 8.5 or 8.25, injected for 1.5 min under high pressure (20 psi) and then under low pressure (0.5 psi) for 0.5 min. (c) Before serum sample injection, a 10 g/L sodium dodecyl sulfate solution was injected for 2 s at low pressure (0.5 psi). (d) Sera were injected for 2 s at low pressure (0.5 psi), and 28 kV were applied for 7 min. Tf isoforms were detected by absorbance at 214 nm (6, 7).

Results were recorded on a printed electropherogram. The clear separation of the peaks allowed quantification as percentages of the total Tf content, in terms of the valley-to-valley area under the curve (AUC). The peak areas and the retention times were calculated by integration software from Beckman. CZE quantification of each peak was based on the ratio of the AUC for that peak to the sum of the areas of the peaks for all isotransferrins. Each peak was thus expressed as a percentage of total Tf (% Tf). The imprecision of the assay in our hands has been published elsewhere (6).

Immunologic validation of Tf isoforms. Anti-human Tf polyclonal antiserum was diluted 1:3 in serum samples after a first CZE analysis. The electropherograms obtained before and after immunosubtraction were compared to identify immunoreactive Tf peaks (6). Anti-human CRP was injected into the capillary under pressure for 3 s before serum injection.

Chemical identification of molecules. Chemical tests were repeated on sera from 10 cancer patients. The protein nature of the molecule was checked by enzymatic treatment with papain and by precipitation with ethanol. Papain was added to 0.1 mL of serum at concentrations of 5 and 3.3 g/L. After a 1-h incubation at 37 °C, the serum electrophoretic profile was obtained. Serum proteins were precipitated by addition of 1 mL of ethanol to 0.3 mL of serum. After centrifugation at 3000g for 20 min, the supernatant was evaporated under reduced pressure for 10 min at 60 °C. The residue was diluted in 0.2 mL of phosphate-buffered saline, pH 7.4, and subjected to CZE.

Lipids were extracted by the addition of 0.2 mL of hexane, diethyl ether, or petroleum ether to 0.2 mL of serum. The procedure was repeated three times before CZE analysis.

After 0.5 mL of serum had been subjected to ethanol precipitation and centrifugation, 0.2 mL of the supernatant was hydrolyzed by 2 mol/L sulfuric acid (18). The same procedure was performed without preliminary treatment with ethanol. CZE analysis was performed on the supernatant obtained by a last centrifugation at 3500g.

Dissociation of comigrating analytes in peak P3. Sera from 20 teetotalers, alcohol abusers, moderate drinkers, and cancer patients, selected from the above series without conscious bias, were first analyzed by CZE with Tris-borate buffer, pH 8.5. Immunosubtraction by anti-Tf (1:3 dilution) was performed under the same conditions, and peaks were identified. In a second step, the same procedure was applied with the same buffer, the pH of which was decreased to 8.25. Chemical analyses with ethanol and sulfuric acid were performed at each pH. Sera from an additional 50 patients presenting with primary (n = 25) or metastatic (n = 25) tumors were subjected to the same analysis. All peaks separated at pH 8.5 or 8.25 were expressed as percentages of total AUC.

Statistics
Statistical analyses were performed with Analyze-it, Ver. 1.6, software. Results are expressed as the mean (SD) with the 95% confidence interval (CI) and range. The statistical significance of the differences in CDT and Tf isoforms among the clinical groups was evaluated using the Wilcoxon and Friedman nonparametric tests. P < 0.05 was considered statistically significant.

We constructed ROC curves (6, 7) for the relative percentages of peaks P0 and P3 in samples from 233 patients with neoplasia, 56 healthy volunteers, 89 moderate alcohol consumers, and 112 alcohol abusers. Using the Analyze-it 1.6 computer program, we calculated the sensitivity and specificity of the relative percentages of the AUCs for asialo- and trisialotransferrin based on the areas under the ROC curves (ROCAUC) and their 95% CIs (6, 7).

We calculated the cutoff percentages for the P3 peak related to the total AUCs for Tf as measured by CZE by comparing P3 values in the whole population with malignant neoplasia, in primary and disseminated tumors, respectively, with the values for the 257 individuals, alcohol abusers or not, not suffering from cancer. ROC curves focusing on asialotransferrin also compared teetotalers, social drinkers, and alcoholics with patients with neoplasia who were moderate drinkers or alcohol abusers.

Results and Discussion
Serum Tf concentrations
Neoplastic malignancy, ethanol abstinence, or excessive intake did not alter mean (SD) serum Tf [2.2 (0.7) g/L in healthy teetotalers, 2.3 (0.6) g/L in heavy alcohol consumers, and 2.2 (0.5) g/L in cancer patients; 95% CIs, 2–2.5 g/L]. Modifications of isoforms induced by alcohol abuse
or malignant neoplasia apparently reacted equally in the assay.

%CDT IMMUNONEPHELOMETRIC ASSAY USING ANION-
EXCHANGE MINICOLUMNS
Although slightly higher than the highest cutoffs currently applied (5, 19), the mean %CDT for 200 neoplastic patients not abusing alcohol was not significantly higher than that for teetotals or alcohol moderate consumers (Table 1). The highest %CDT was measured in alcohol abusers, with or without cancer. These results differed from the significant increase in %CDT observed previously in hepatocellular carcinoma (9, 10). This type of neoplastic malignancy was not evaluated in our study.

CZE VISUALIZATION OF TF ISOFORMS
Electropherograms from one individual from each population, obtained with Tris-borate separation buffer, pH 8.5, are shown in Fig. 1. The distribution and retention times of the peaks were similar to those observed previously (6, 7). A predominant tetrasialotransferrin isoform observed previously (1–7) and two forms, P5 and P6, with longer retention times were found in the sera of all patients (Fig. 1). Two to four earlier-eluting forms were seen in samples from alcohol abusers and cancer patients. The number of early peaks was two in teetotals, moderate drinkers [as reported in our earlier study (7)], and cancer patients; three or four in alcohol abusers; and four in alcoholic patients with cancer.

Samples from alcohol abusers, with or without cancer, had a P0 peak and a high P2 peak (Fig. 1). In the two cancer patients, peak P3 was higher than in the other individuals studied (Fig. 1) and was only partly removed by anti-Tf immunosubtraction (Fig. 2). A small P3 signal, similar to the P1 signal, also remained after anti-Tf treatment of the sample from the alcohol abuser. All other peaks were completely immunosubtracted (Fig. 2).

REVIEW OF ALL ELECTROPHEROGRAMS
Peak P0 was undetectable in 188 of 200 nonalcoholic cancer patients, in all of the teetotals, and in 84 of 89 moderate ethanol consumers, but was detectable in 28 of 33 cancer patients who misused alcohol and in 105 of 112 alcoholics.

Peak P1 was detected in all cancer patients, 21 of 89 moderate drinkers, and 65 of 112 alcohol abusers. It was not detected in healthy teetotals.

The mean P2 value did not differ significantly in teetotals, nonalcoholic cancer patients, and moderate drinkers, but was higher in alcohol abusers. The mean P3 value was not changed regardless of alcohol intake for individuals without neoplasia, but was higher in almost all cancer patients, alcoholic or not.

CZE CDT ISOFORMS ISOLATED AT pH 8.5
Quantitative values for the Tf isoforms are shown in Table 1. The mean P0 value was 0 in teetotals and moderate alcohol consumers, with or without cancer, and 0.5% in

<table>
<thead>
<tr>
<th>Population*</th>
<th>%CDT</th>
<th>P0</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTT (n = 56)</td>
<td>Mean (SD)</td>
<td>2.4 (0.9)</td>
<td>0</td>
<td>0</td>
<td>0.5 (0.2)</td>
<td>4.4 (1.4)</td>
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<tr>
<td>95% CI</td>
<td>2.2–2.6</td>
<td>0.4–0.6</td>
<td>4–4.8</td>
<td>75.1–83.4</td>
<td></td>
<td></td>
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<tr>
<td>Range</td>
<td>1.1–4.2</td>
<td>0–1.5</td>
<td>2.5–9.1</td>
<td>70–85</td>
<td></td>
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<td>AA (n = 112)</td>
<td>Mean (SD)</td>
<td>4.3 (2.8)</td>
<td>0.5 (0.4)</td>
<td>0.07 (0.07)</td>
<td>2.2 (2.5)</td>
<td>4.8 (2)</td>
</tr>
<tr>
<td>95% CI</td>
<td>3.7–4.8</td>
<td>0.1–1.4</td>
<td>0.06–0.08</td>
<td>1.7–2.6</td>
<td>4.4–5.2</td>
<td>75.9–77.3</td>
</tr>
<tr>
<td>Range</td>
<td>1.3–12</td>
<td>0–13.8</td>
<td>0–1.2</td>
<td>0.3–12.4</td>
<td>0.8–15.4</td>
<td>65–82</td>
</tr>
<tr>
<td>MD (n = 89)</td>
<td>Mean (SD)</td>
<td>2.3 (0.6)</td>
<td>0.03 (0.2)</td>
<td>0.03 (0.03)</td>
<td>0.6 (0.5)</td>
<td>4.5 (2.1)</td>
</tr>
<tr>
<td>95% CI</td>
<td>2.2–2.5</td>
<td>0.01–0.2</td>
<td>0.02–0.04</td>
<td>0.5–0.8</td>
<td>4.1–4.9</td>
<td>73–78.8</td>
</tr>
<tr>
<td>Range</td>
<td>1.3–4.9</td>
<td>0–1.2</td>
<td>0–0.7</td>
<td>0.2–2.6</td>
<td>3.5–13.7</td>
<td>69.4–83.5</td>
</tr>
<tr>
<td>Neo (n = 200)</td>
<td>Mean (SD)</td>
<td>3.1 (2.1)</td>
<td>0.04 (0.2)</td>
<td>0.6 (0.5)</td>
<td>0.6 (0.7)</td>
<td>10.3 (3.8)</td>
</tr>
<tr>
<td>95% CI</td>
<td>2.1–4.0</td>
<td>0.01–0.2</td>
<td>0–1.2</td>
<td>0.4–0.6</td>
<td>9.7–10.9</td>
<td>51.2–72.4</td>
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<td>Range</td>
<td>1.3–4.9</td>
<td>0–0.9</td>
<td>0.03–6.6</td>
<td>0.03–2.7</td>
<td>2.3–24.6</td>
<td>43.9–83.8</td>
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<td>NeoAA (n = 33)</td>
<td>Mean (SD)</td>
<td>4.2 (2)</td>
<td>0.4 (0.3)</td>
<td>0.6 (0.7)</td>
<td>1.4 (2.3)</td>
<td>8.9 (5)</td>
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<tr>
<td>95% CI</td>
<td>3.8–4.6</td>
<td>0.2–1.5</td>
<td>0.3–0.9</td>
<td>0.3–10.6</td>
<td>6.6–11.1</td>
<td>50.8–75</td>
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<tr>
<td>Range</td>
<td>1.4–11.7</td>
<td>0–10.3</td>
<td>0.1–6.9</td>
<td>0.1–11.7</td>
<td>3.3–24.9</td>
<td>45.2–77.4</td>
</tr>
</tbody>
</table>

* HTT, healthy teetotals; AA, alcohol abusers without cancer; MD, moderate drinkers without cancer; Neo, patients with neoplasias; NeoAA, patients with neoplasias who were abusing alcohol.
alcohol abusers. The mean P1 value was 0.6% of total Tf in cancer patients. P1 represented CRP, being immunosubtracted by anti-CRP (Fig. 3). This result agreed with the presence of CRP in cancer (20). The mean P2 value was 1.4–2.2% of total Tf in alcohol abusers (P > 0.05), whereas it was 0.5–0.6% in abstainers and moderate drinkers, with or without cancer.

Increases in the CDT isoforms P0 and P2 in all alcohol abusers led us to define the primary alteration produced by ethanol as a reduction in Tf glycosylation (21, 22), the extent of sialylation being dependent on the number of N-glycan chains. On the other hand, aberrant glycosylation associated with malignant neoplasia (23–26) would not involve the monoglycosylated isoforms, known as CDT, detected by CZE in nonalcoholic cancer patients, as indicated in Table 1.

PEAK P3 IN THE VARIOUS POPULATIONS
In teetotalers, social drinkers, and ethanol misusers, the P3 value was <5% of total Tf. Although ranging from 2.3% to 24.6% of total Tf, P3 was increased twofold in cancer, regardless the self-reported alcohol habits and AUDIT scores (Table 1). P4 represented a significantly higher relative percentage in individuals without cancer than in patients with cancer, as could be predicted from variations in the relative percentages of the earlier migrating peaks.

Two conclusions may be drawn from Table 1: (a) the presence of asialotransferrin and disialotransferrin >1% of total Tf is related to heavy ethanol intake, independent of the presence of malignant tumors; and (b) peak P3 is significantly higher in cancer patients than in other individuals, independent of alcohol consumption. This high P3 value and the peak heterogeneity revealed by anti-Tf immunosubtraction (Fig. 2) drew our attention to this comigration of trisialotransferrin with another analyte.
The chemical treatments performed on 20 sera from cancer patients are summarized in Table 2. Extraction by organic solvents did not modify the electropherograms. Hydrolysis by papain at two concentrations markedly decreased the Tf peaks, but a portion of the P3 peak remained. It might be presumed that the proteolytic activity of papain was restricted by allosteric hindrance from Tf N-glycan chain sialic acid residues (27), but ethanol similarly precipitated all peaks except the same portion of P3. We concluded that the analyte comigrating with trisialotransferrin was not a protein.

All peaks in sera from cancer patients disappeared after acid hydrolysis, because of removal of glycan chain oligosaccharide branchings. In neoplastic sera, the acid hydrolysis of the nontransferrin part of P3 also occurred after ethanol protein precipitation (Table 2). The analyte would thus likely be a polysaccharide (PS) (18).

SEPARATION OF PS FROM TRISIALOTRANSFERRIN
A comparison of electropherograms of the serum of a cancer patient, run in Tris-borate separation buffer at either pH 8.5 or pH 8.25, is shown in Fig. 4. Peaks P3 and PS comigrated at pH 8.5, averaging 10.5% of total Tf, but were separated into peaks P3 (2.5% of total Tf) and PS (7.3% of total Tf) when separation occurred at pH 8.25. Immunosubtraction by anti-Tf showed that peak P3 represented true trisialotransferrin, whereas the PS peak was the comigrating analyte. Sulfuric acid hydrolysis, without ethanol precipitation, and separation at pH 8.25 confirmed that the PS peak represented the unknown species comigrating with trisialotransferrin at pH 8.5 (Fig. 4).

Each isoform of Tf has an isoelectric point between pH 5.2 and 5.9, with that of trisialotransferrin being pH 5.6 (4). These isoelectric points are far from the CZE separation pH conditions, pH 8.5 or 8.25. A 0.25 variation in pH units would not modify the migrating properties of the Tf isoforms. By contrast, a slight pH variation would significantly affect (reduce) the electric charge of a polysaccharide, thereby altering its migration.

CHARACTERIZATION OF PS
The same study was performed on 20 sera from alcohol abusers and moderate users. At pH 8.25, the PS peak migrated very near the P0 peak in samples from alcoholics or even comigrated with this peak (Fig. 5). The close association of the P0 and PS peaks was confirmed by immunosubtraction of P0 by anti-Tf, whereas the PS peak was not altered. This last result shows that lowering the pH of the separating buffer to 8.25 to separate PS and trisialotransferrin can lead to interference with the diagnosis of alcohol abuse based on the presence or absence of peak P0 (6, 7). The PS peak might be identical to the early unidentified peak reported by Lanz et al. (28) after CZE separation of Tf isoforms at pH 8.3.

<table>
<thead>
<tr>
<th>Question</th>
<th>CZE analysis</th>
<th>Results</th>
<th>Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein?</td>
<td>Papain at 5 or 3.3 g/L for 1 h at 37 °C</td>
<td>P0–P6 disappeared except part of P3</td>
<td>Not protein</td>
</tr>
<tr>
<td>Lipid?</td>
<td>Extraction through hexane, diethyl ether, and benzene</td>
<td>No peak modified</td>
<td>Not lipid</td>
</tr>
<tr>
<td>Saccharide?</td>
<td>2 mol/L H₂SO₄</td>
<td>P0–P6 disappeared</td>
<td>Saccharicid</td>
</tr>
</tbody>
</table>

Shown in Table 3 is a comparison of the %Tf obtained for peak P0 at pH 8.5, peak P0/PS at pH 8.25, peak P3 at pH 8.5, and trisialotransferrin isolated at pH 8.25 in 20 sera randomly selected in all groups and in 25 samples from patients with nonmetastatic and metastatic disease. The immunosubtractable trisialotransferrin separated at
pH 8.25 was statistically similar (AUC, 3–4% of total Tf) in cancer patients with primary or metastatic tumors, teetotalers, and alcohol consumers. A substantial P0 peak was found only in alcohol abusers. Its presence, at low values, in cancer patients was attributable to the selection of alcohol abusers to that group. The P3 peak was significantly higher ($P < 0.0001$) in cancer patients than in the other groups, but was lower in patients with localized than disseminated tumors ($P = 0.02$). The high P3 value in cancer patients was related to a high concentration of the circulating PS. In contrast, the amount of PS was similar and <3% of the Tf AUC in teetotalers and moderate or excessive alcohol consumers.

The presumed PS might represent a glycosaminoglycan or a derived PS known to be increased in the neoplastic process, such hyaluronan (29, 30), dermatan sulfate (31), or heparan sulfate (32). Further CZE analysis that allows differentiation among glycosaminoglycans (33) or tandem capillary electrophoresis (34) could be used to test this hypothesis.

Because the PS peak comigrated with the P0 peak at pH 8.25, it appears problematic as a diagnostic analyte in alcohol abuse. The potential use of the P3 peak obtained at pH 8.5, which contains trisialotransferrin and comigrating PS, as a cancer diagnostic tool was investigated in more detail.

Fig. 4. Electropherograms of serum from a cancer patient, separated at pH 8.5 and pH 8.25, without or with addition of anti-Tf, and the same pH 8.25 electropherogram after protein precipitation by ethanol (Ethanol PH 8.25) followed by acid hydrolysis (H2SO4 PH 8.25).

The x axis is the CZE migration time (min). Peaks: P0, asialotransferrin; P1, CRP/monosialotransferrin; P2, disialotransferrin; P3/PS, comigration of trisialotransferrin and PS; P4, tetrasialotransferrin; P5, pentasialotransferrin; P6, hexasialotransferrin; P3, trisialotransferrin; P5, polysaccharide.

Fig. 5. Electropherograms of serum from an alcohol abuser (AA) separated at pH 8.5 and pH 8.25, without and with addition of anti-Tf.

The x axis is the CZE migration time (min). Peaks: P0, asialotransferrin; P2, disialotransferrin; P3/PS, comigration of trisialotransferrin and PS; P4, tetrasialotransferrin; P5, pentasialotransferrin; P6, hexasialotransferrin; P3, trisialotransferrin; P5, polysaccharide.
The ROC curve is the graphic representation of a trade-off between the false-negative and the false-positive rates for every possible cutoff \((35)\). ROC analysis of the global performance of P3 based on samples from 233 patients with neoplasia and 257 individuals without cancer is presented in Fig. 6. The ROC area was 0.94 (95% CI, 0.91–0.96), and the best trade-off was for a cutpoint for P3 of 6.4% of total Tf (sensitivity, 0.95; specificity, 0.84). In addition, when we compared alcohol-induced P0 values for nonalcoholic cancer patients and the subgroup of 112 alcohol abusers without cancer, we obtained a ROC area of 0.96 (95% CI, 0.94–0.98) with a sensitivity of 0.92 and a specificity of 0.95, as reported previously when alcohol abusers and moderate drinkers were investigated \((6)\). These results encouraged us to compare the P3 values for the 157 patients with primary tumors with those for the 43 patients with disseminated tumors. The 257 noncancer patients were included in the ROC analyses as the subgroups alcohol abusers, moderate drinkers, and teetotalers (Table 4).

The mean P3 value appeared significantly higher in patients with disseminated than in patients with localized tumors (Table 4). When P3 values for patients with localized and disseminated tumors, respectively, were compared with those for populations without cancer, regardless of self-reported alcohol consumption and AUDIT score, the ROC areas were 0.9 for the three populations for detection of localized tumors and 0.97 for detection of disseminated tumors (Table 4). This indicated that P3 values measured by CZE as a biomarker for...
neoplasia were better for diagnosing disseminated tumors than primary tumors, regardless of alcohol consumption. The P3 threshold that offered the best differentiation for localized tumors among healthy teetotalers and excessive and moderate drinkers was 5.8% of total Tf (sensitivity, 0.78). For disseminated tumors, the sensitivity was 0.94 for healthy abstainers and moderate drinkers at a cutpoint of 8% of total Tf. This cutpoint had to be lowered to 7.7% of total Tf to obtain the same sensitivity when the alcohol abusers were included in the control group.

The characteristics of different control groups may influence the specificity at a selected cutpoint (35). The ratio of false-positive individuals will likely vary from one noncancer population to another, and this may in turn modify the cutpoint chosen by ROC programs when different control groups are compared with diseased individuals. This was observed in ROC curves obtained for differentiation of metastases in alcoholics (Table 4). ROC curves must be used cautiously regarding definition of cutpoints, and sensitivity and specificity should be determined in each laboratory based on its own populations. In ROC curve analyses, diagnostic accuracy is best expressed by the areas under the ROC curves (ROC areas) (36–38), which were high in these studies.

Table 5 shows the numbers of patients with P3 >5.8% of total Tf, and with P3 >8% of total Tf for disseminated cancers (n = 43). In 73–90% of patients with localized colorectal, breast, and lung tumors, P3 was >5.8% of total Tf. Such a P3 percentage was found in only 3 of 56 healthy abstainers. When all cancer types were pooled, the sensitivity was 0.78 for primary tumors, fitting with the ROC curve trade-off results shown in Table 4. P3 was >8% of total Tf in 53 of 57 patients with metastases (94%; Table 4).

**GENDER VARIATION OF P3**

Outlined in Fig. 7 are the differences in ROC areas for men (Fig. 7A) and women (Fig. 7B) with malignant neoplasias compared with male and female individuals without

### Table 4. ROC curve differentiation of P3 in localized and disseminated cancers vs the noncancer groups studied.

<table>
<thead>
<tr>
<th>Alcohol*</th>
<th>Neoplasia</th>
<th>Mean AUC</th>
<th>95% CI</th>
<th>Cutoff, % of total Tf</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTT (n = 56)</td>
<td>Local</td>
<td>0.90</td>
<td>0.86–0.95</td>
<td>5.8</td>
<td>0.78</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>Metastasis</td>
<td>0.98</td>
<td>0.95–1.0</td>
<td>8</td>
<td>0.94</td>
<td>0.96</td>
</tr>
<tr>
<td>MD (n = 89)</td>
<td>Local</td>
<td>0.90</td>
<td>0.85–0.94</td>
<td>5.8</td>
<td>0.78</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>Metastasis</td>
<td>0.97</td>
<td>0.94–1.0</td>
<td>8</td>
<td>0.94</td>
<td>0.94</td>
</tr>
<tr>
<td>AA (n = 112)</td>
<td>Local</td>
<td>0.89</td>
<td>0.85–0.93</td>
<td>5.8</td>
<td>0.78</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>Metastasis</td>
<td>0.97</td>
<td>0.95–1.0</td>
<td>7.7</td>
<td>0.94</td>
<td>0.96</td>
</tr>
</tbody>
</table>

* HTT, healthy teetotalers; MD, moderate drinkers without cancer; AA, alcohol abusers without cancer.

### Table 5. Percentage of P3 AUC relative to total Tf (%P3) in patients with different malignant tumors and in healthy individuals.

<table>
<thead>
<tr>
<th>Localized cancers</th>
<th>n</th>
<th>%P3</th>
<th>Cutoff, % of total Tf</th>
<th>No. with P3 &gt; cutoff*</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal carcinoma</td>
<td>41</td>
<td>8.2 (3.3)</td>
<td>5.8</td>
<td>32</td>
<td>0.78</td>
</tr>
<tr>
<td>95% CI</td>
<td></td>
<td>7.3–9.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>30</td>
<td>9.9 (3.7)</td>
<td>5.8</td>
<td>27</td>
<td>0.9</td>
</tr>
<tr>
<td>95% CI</td>
<td></td>
<td>8.9–10.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung carcinoma</td>
<td>26</td>
<td>9.7 (3.1)</td>
<td>5.8</td>
<td>19</td>
<td>0.73</td>
</tr>
<tr>
<td>95% CI</td>
<td></td>
<td>8.0–11.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>46</td>
<td>7.9 (3.5)</td>
<td>5.8</td>
<td>35</td>
<td>0.76</td>
</tr>
<tr>
<td>Total</td>
<td>143</td>
<td>7.1 (5.1)</td>
<td>5.8</td>
<td>113</td>
<td>0.78</td>
</tr>
<tr>
<td>95% CI</td>
<td></td>
<td>6.3–7.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disseminated cancers</td>
<td>57</td>
<td>9.2 (2.8)</td>
<td>8</td>
<td>53</td>
<td>0.94</td>
</tr>
<tr>
<td>95% CI</td>
<td></td>
<td>8.6–9.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy individuals</td>
<td>56</td>
<td>4.5 (0.9)</td>
<td>5.8</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>95% CI</td>
<td></td>
<td>3.3–5.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Number of values >5.8% of total Tf in various localized cancers and in healthy volunteers, and >8% of total Tf in disseminated tumors.
cancer. The ROC areas were 0.94 (95% CI, 0.89–0.98) in men and 0.89 (95% CI, 0.84–0.95) in women. This better sensitivity of the P3 marker in men will need differential cutoffs determined from a larger series.

Can the comigrating peak be used as a diagnostic tool?

ROC curves outlined the relevant sensitivity and specificity of the comigrating P3 peak at pH 8.5. The power of P3 to differentiate cancer is attributable to the highly significant increase in the PS in malignant neoplasia. We could isolate the PS peak by performing CZE at pH 8.25, but under those conditions, it interfered with the P0 peak used in diagnosis of alcoholism. Peaks P0 and PS would thus be overestimated at pH 8.25, hampering diagnosis of alcohol abuse as well as of cancer.

In conclusion, malignancy did not change the concentration of monoglycosylated isoforms of Tf, whereas ethanol intake did. Malignancy did not decrease the hepatic Tf glycosylation of glycoproteins. Cancer did not hamper the detection of alcohol abuse based on the presence of asialotransferrin and increases in disialotransferrin, both detected by CZE. Sera from patients with cancer contained a saccharide comigrating with trisialotransferrin, whereas its concentration was not increased in individuals without cancer or in those who were teetotalers or moderate or heavy alcohol consumers. The significantly higher concentrations in cancer patients accounted for the higher P3 peak measured at pH 8.5. This circulating PS concentration was higher with disseminated than local cancers. Because of the poor sensitivities of most circulating tumor markers, an analysis of multiple markers is needed (39). A framework has been suggested by Johnson (40), starting with DNA, followed by RNA, and culminating with the glycan portion of glycoproteins. CZE seems particularly useful to study protein glycosylation (6, 7) and glycosaminoglycans (33), and to measure the PS comigrating with the P3 peak.

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References


Fig. 7. ROC curves for P3 in men (A) and women (B).

The y axis shows the sensitivity, and the x axis shows (1 − specificity). Arrows indicate the P3 cutoffs: 6.4% of total Tf in men (A) and 5.8% in women (B).


