Desialylated Transferrin and Mitochondrial Aspartate Aminotransferase Compared as Laboratory Markers of Excessive Alcohol Consumption

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Concentrations of both desialylated transferrin (dTf) and the mitochondrial isoenzyme of aspartate aminotransferase (EC 2.6.1.1, mAST) have been claimed to be increased in sera of alcoholic subjects. To investigate the diagnostic usefulness of these new biochemical markers of alcoholism and to compare them with more conventional markers, we measured dTf and mAST in the sera of controls, alcoholic subjects, and patients with nonalcoholic liver diseases (NALD). Alcoholic subjects had significantly (P < 0.001) higher ratios of dTf to total transferrin than did either healthy controls or patients with NALD (sensitivity 81%, specificity 97%). The mAST was increased in 92% of alcoholic subjects but also in 48% of patients with NALD. The mAST/dTf ratio differentiated the alcoholic subjects from those with NALD (P < 0.001) with a sensitivity of 92%, but the specificity was only 70%. In contrast, the conventional markers were less sensitive and less specific. We conclude that the best available single laboratory marker for current heavy alcohol consumption is the ratio dTf/dTf transferrin.

Additional Keyphrases: liver disease · alcoholism

Many biochemical and hematological abnormalities are associated with chronic excessive alcohol consumption. However, conventional laboratory tests such as γ-glutamyltransferase (GGT; EC 2.3.2.2), mean corpuscular volume (MCV), aspartate aminotransferase (AST; EC 2.6.1.1), and alanine aminotransferase (ALT; EC 2.6.1.2) lack diagnostic sensitivity and specificity (1, 2). Recently, desialylated transferrin (dTf) (also known as carbohydrate-deficient transferrin) (3–6) and the mitochondrial isoenzyme of AST (mAST) (7–9) have been proposed as being more sensitive and specific biochemical markers of alcohol abuse than are conventional laboratory tests. Using isoelectric focusing, Stibler et al. (10) described the presence of abnormal transferrin variants (with less sialic acid content) in the serum of alcoholic subjects. These abnormal transferrin variants in alcoholic sera have also been detected in our laboratory, by chromatofocusing followed by RIA (11, 12). Our aim in this present study was to compare the diagnostic usefulness of dTf and mAST and to compare their sensitivity and specificity with those of the more conventional laboratory markers.

Materials and Methods

Study Population
The alcohol consumption of all subjects, whether alcoholic or nonalcoholic, was assessed in detail. A questionnaire, developed in collaboration with the Department of Psychiatry to detail information on current and past drinking history, was completed by each subject and checked by a member of the investigating team. The amount of alcohol consumed daily for the previous fortnight was recorded, as was information on health status, smoking, and drug intake. We then collected 20 mL of blood from each subject. These procedures were approved by the Hospital and the University Ethics Committees.

Control subjects. The control groups comprised both healthy volunteers and hospital patients who had had an average ethanol intake of less than 40 g per day in the preceding two weeks and who had no previous history of regular heavy alcohol intake. We studied 16 nonalcoholic healthy volunteers (eight men, eight women; mean age 35 years) and 21 hospital controls (11 men, 10 women; mean age 49 years), with various nonalcohol-related liver diseases (NALD), including hepatitis (4), storage diseases (4), hepatocellular carcinoma (2), cirrhosis (8), congenital fibrosis (1), and nodular transformation (2), all confirmed by liver biopsy or appropriate clinical and laboratory assessment. Seventeen of the patients were total abstainers; four were social drinkers with a mean ethanol consumption of 14 g per week.

Alcoholic subjects. We studied 26 alcoholic subjects (23 men, three women, mean age 43 years), who had consumed >80 g of ethanol per day for the previous two weeks, indeed for the previous six months. Serum samples were collected within 48 h of the most recent drink. These patients, recruited from the Hospital Drug and Alcohol Services Unit and a Drug and Alcohol Dependence Clinic, were self-admitted alcoholics primarily admitted for detoxification. Their status of liver disease was based on clinical and biochemical abnormalities, not (for ethical reasons) liver biopsy.

Laboratory Tests
Laboratory investigations of samples from each subject included a multiple biochemical analysis on a SMAC II analyzer (Technicon Instruments Corp., Tarrytown, NY), full blood count on a Coulter counter, coagulation profiles (in alcoholic subjects only), and quantification of ethanol, total Tf, dTf, total AST, and mAST in serum.

Total and desialylated transferrin. Desialylated transferrin was isolated from normal transferrin by isocratic anion-exchange chromatography, in a modification of the method of Stibler et al. (13). Disposable plastic microcolumns were packed with 200 μL of diethylaminoethyl-Sepharose anion exchanger (Pharmacia Fine Chemicals, Uppsala, Sweden) and equilibrated with 2-N-morpholinoethanesulfonic acid (MES; Sigma Chemical Co., St. Louis, MO) buffer (pH 5.65, 20 mmol/L). Serum samples (40 μL) were saturated with 10

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3 Nonstandard abbreviations: GGT, γ-glutamyltransferase; MCV, mean corpuscular volume; AST, aspartate aminotransferase; ALT, alanine aminotransferase; Tf, transferrin; dTf, desialylated transferrin; mAST, mitochondrial aspartate aminotransferase; NALD, nonalcoholic liver diseases; and MES, 2-N-morpholinoethanesulfonic acid.
μL of ferric nitritetriacetate (25 mmol/L, pH 6.5), diluted 25-fold with 850 μL of MES buffer, and dialyzed in MES buffer at room temperature for 2 h. We then passed 400 μL of the dialysate through the microcolumns and eluted with 1 mL of MES buffer. The eluent was collected and analyzed for transferrin content with a radioimmunoassay (14). To compare two methods for isolating and quantifying dTF, we also assayed most samples4 by chromatofocusing with an ion-exchange column (Mono P), as previously described (11). Two main fractions were collected, one of pH >5.65 (desialylated transferrin) and the other with a pH between 5.6 and 5.2 (normal transferrin), and the transferrin content was measured by radioimmunoassay, as above.

Total and mitochondrial aspartate aminotransferase. mAST was measured in 300 μL of serum with a mAST kit supplied by Eiken Chemical Co., Tokyo, Japan, and an AST kit supplied by Trace Scientific, Sydney, Australia.

Statistical Analysis

The Mann–Whitney test for quantitative data between groups and linear-regression analysis for correlations and associations between variables were used. Sensitivities, specificities, and positive and negative predictive values were calculated according to standard methods (15).

Results

Desialylated Transferrin

Figure 1 (left) shows the distribution of dTF in the three subject groups. A small amount of dTF, ranging from 9.5 to 34.3 mg/L (median 20.1 mg/L), was present in healthy controls whose average alcohol consumption was >40 g of ethanol per day. The dTF values were not significantly different from normal in 21 patients with NALD (median 23.7 mg/L, range 14 to 48 mg/L). The dTF concentrations exceeded 40 mg/L in only four of these patients. However, these four subjects also had relatively high concentrations of total Tf (3.43 to 4.53 g/L, mean 4.06 g/L), and two had iron-deficiency anemia.

Figure 1 (right) shows the ratio of dTF to total Tf, expressed as a percentage. In healthy control subjects the ratio ranged from 0.33% to 1.19% (median 0.95%). A small but significant (P <0.01) increase in the ratio was observed in patients with NALD as compared with that in healthy controls. A ratio of dTF to total Tf of 2.16%, seen in one nonalcoholic cirrhotic patient, was due to a low concentration of total Tf (1.52 g/L) rather than increased dTF (32.8 mg/L).

In alcoholic subjects the ratio of dTF to total Tf ranged from 0.39% to 9.06% (median 2.41%), which was significantly higher (P <0.001) than in the healthy controls and patients with NALD.

Because none of the 37 control subjects had a dTF/total Tf ratio exceeding 1.3%, we chose this as the arbitrary upper reference point. Twenty-one of the 26 alcoholic subjects had values greater than this upper reference point, which gave a sensitivity of 81%, a specificity of 97%, and a diagnostic efficiency value (i.e., sensitivity + specificity) of 178%. The positive and negative predictive values were 96% and 88%, respectively.

There were no differences in dTF concentrations or in the dTF/total Tf ratio between alcoholics with and without biochemical evidence of liver disease. Five alcoholic subjects whose dTF concentrations were monitored during abstinence all had initially high values (mean 122 mg/L, range 63.1 to 188.5 mg/L). However, their dTF declined by

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4 These subjects included seven healthy controls, 26 alcoholics, and five NALD patients.
an average of 28% (range 6% to 56%) after abstention for seven days, and in one patient the value fell to within the normal range.

There was no significant correlation between dTf concentration and age and no sex-related differences in any of the study groups. No difference was found between total abstainers and normal consumers (<40 g ethanol/day), and there was no significant correlation between dTf concentration and average daily ethanol consumption.

The results obtained by RIA after micro-anion-exchange chromatography correlated significantly ($P < 0.001$) with the results obtained by RIA after chromatofocusing. The coefficient of correlation was 0.71 for the 38 pairs of data, although the latter method appeared to be more sensitive (the dTf/total TF ratio determined by chromatofocusing ranged from 0.15% to 25% compared with a range of 0.40% to 9.06% for micro-anion-exchange chromatography).

Mitochondrial AST

Mitochondrial AST activities in the three subject groups are shown in Figure 2 (left). In 16 healthy controls, the median activity was 1.27 U/L (range 0.39 to 3.97 U/L). The mAST activity was significantly higher in patients with NALD (median 2.41 U/L, range 0.10 to 22.94 U/L) than in healthy controls ($P < 0.05$). In alcoholic subjects the mAST activity (median 6.07 U/L, range 2.10 to 37.49 U/L) was significantly higher than in healthy controls ($P < 0.001$) or in subjects with NALD ($P < 0.01$). With mAST activity of 2.5 U/L as an arbitrary upper cutoff point, 24 of the 26 (92%) alcoholic subjects had an above-normal mAST activity. In addition, 10 (48%) patients with NALD had mAST activity exceeding the cutoff value. Increases of mAST activity in patients with NALD were always associated with increases in total AST; however, in alcoholic subjects, mAST was elevated even in those with normal total AST activity.

In Figure 2 (right) the ratio of mAST to total AST is expressed as a percentage. All alcoholic subjects who had a mAST activity >2.5 U/L (the upper reference value just mentioned) had a ratio of mAST to total AST of >6%. The mAST/total AST ratio was significantly higher in alcoholic subjects (median 11.52%, $P < 0.001$) than in patients with NALD (median 5.52%). The sensitivity and specificity for the mAST/total AST ratio >6% were 92% and 70%, respectively, giving a diagnostic efficiency value of 162%. Positive and negative predictive values of the test were 69% and 93%, respectively. There were no significant correlations between mAST activity and age, no sex-related differences in any of the study groups, and no differences in mAST activity between total abstainers and normal consumers of ethanol. In alcoholic subjects, the increases of mAST activity did not correlate with alcohol consumption, and there was no correlation between mAST activity and dTf concentration.

Diagnostic Efficiencies of Conventional Tests

Table 1 shows the results of efficiency tests calculated for the four conventional laboratory markers for alcohol abuse (GGT, MCV, AST, and ALT) in the groups studied. All four variables in the alcoholic group were significantly higher than in the healthy control group ($P < 0.001$). GGT was increased in 69%, MCV in 73%, AST in 69%, and ALT in 58% of the alcoholic patients. Detectable concentrations of ethanol in serum were found in only four alcoholic patients. The results of these laboratory tests were within the normal reference range in all healthy controls except one

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**Fig. 2.** Distributions of (left) mitochondrial aspartate aminotransferase activity and (right) the ratio of mitochondrial aspartate aminotransferase to total aspartate aminotransferase activity in sera of healthy controls, alcoholic subjects, and patients with NALD.

The horizontal bars represent the median values and the dashed line represents the arbitrary cutoff point.
Table 1. Diagnostic Efficiencies of Conventional Markers for Alcoholism*

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<th>Sensitivity</th>
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* At cutoff limits of GGT > 50 U/L; AST > 40 U/L; ALT > 35 U/L; ALT/AST > 1.

PPV, positive predictive value; NPV, negative predictive value.

(who had an above-normal MCV), but all four markers were increased in many of the patients with liver disease unrelated to alcohol. The least sensitive and specific test for alcohol consumption was ALT, followed by GGT, which was increased in 71% of patients with NALD. The highest predictive values, both positive and negative, were obtained for the MCV (67% and 80%, respectively). We found no significant correlations between the average daily alcohol intake by alcoholic subjects during the preceding two weeks and their values for the conventional markers studied.

In the 26 alcoholic subjects, the AST/ALT ratio (mean 1.66, SD 1.49, median 1.24, range 0.46–8.00) was not significantly different from ratios observed in the 21 patients with NALD (mean 1.25, SD 0.89, median 0.97, range 0.50–4.43). AST/ALT ratios > 1 were found in 69% of the alcoholic subjects and in 52% of the patients with NALD. Ratios > 2 were found in only 15% of the alcohols and in 19% of the patients with NALD.

Discussion

In this study we have shown that both the ratio of dTF to total TF and the ratio of mAST to total AST were more sensitive biochemical markers of chronic alcohol abuse than are the commonly used biochemical tests. The dTF/total TF ratio showed the best diagnostic efficiency value, 17.5%, compared with 162% for the mAST/total AST ratio and 149% for the best of the conventional markers (MCV). Both dTF and mAST had higher negative predictive values than the conventional markers; i.e., they detected more alcoholics. Moreover, the probability that alcoholism was not present when the test result was normal was higher in the new markers; however, mAST was less specific. We found no differences in either dTF or the dTF/total TF ratio between alcoholic subjects with and without biochemical evidence of liver disease. Thus this test did not appear to depend on the presence of liver injury associated with heavy drinking. This supports the findings of Behrens et al. (5), who found that in patients with biopsy-confirmed alcoholic liver disease, dTF concentrations were similar to those found in alcoholic subjects without liver disease; they also found no correlation between dTF concentrations and the severity of liver disease.

The results obtained in this study for the dTF/total TF ratio are in agreement with the results of Behrens et al. (5), who found a sensitivity of 81%, a specificity of 91%, and a positive predictive value of 89%. Some (19%) of our alcoholic patients did not show a positive result (ratio > 1.3%), for reasons we do not yet understand. A rare genetic variant of transferrin other than the C-variant has been postulated to be responsible for such results; however, this was investigated by Behrens et al. (5), who could not confirm this in their studies.

Four patients had slightly increased dTF values that were associated with relatively high concentrations of total serum transferrin. We therefore recommend the use of the ratio of dTF to total TF rather than dTF concentration for discriminating alcoholic subjects from nonalcoholic patients. Our results show that dTF as a percentage of total TF was increased (up to 9%) among alcoholic subjects, whereas no value exceeded 1.3% in the controls. This is in contrast to the report by Behrens et al. (5), who showed that dTF was more sensitive than the dTF/total TF ratio as an indicator of ethanol consumption. It is important to note that the majority of our hospital patients had, in addition to liver disease, various other nonalcohol-related conditions and were being treated with a variety of drugs. Therefore, our test was not only specific in the presence of liver disease unrelated to alcohol, but also appeared to be unaffected by various other conditions such as diabetes (12) and by therapeutic drug ingestion, whereas mAST is reportedly increased in some diseases, e.g., acute myocardial infarction (16).

The increase of mAST in alcoholics appears to be a direct result of alcohol ingestion, but there was no direct correlation with the actual quantity of alcohol consumed. Similarly, Nalpas et al. (7) found that, when increased, the mAST/total AST ratio is not influenced by the presence of biopsy-proven alcoholic liver disease. Factors associated with heavy alcohol consumption, apart from alcohol itself, may also influence the amount of mAST activity. mAST was increased in 92% of alcoholic subjects and in 48% of patients with NALD (P < 0.001), for a sensitivity of 92%, but the specificity was only 70%. Total AST and ALT activities were significantly lower in alcohols without biochemical and clinical evidence of liver disease than in those with such evidence. mAST activity apparently is also related to the extent of general liver cell and mitochondrial injury caused by alcohol.

All currently available methods for detecting excessive alcohol consumption are limited in diagnostic usefulness. The results of our study show that the conventional laboratory markers lack sensitivity and specificity when used singly—each test failed to detect approximately 30% of alcoholic subjects. Several reports in the literature indicate the AST/ALT ratio is helpful in discriminating alcoholic liver disease from liver diseases due to other causes. Cohen and Kaplan (17) found an AST/ALT ratio > 2 in 70% of their alcoholic patients, compared with 25% of patients with post-necrotic cirrhosis, 8% of patients with chronic active hepatitis, and 4% with viral hepatitis. Correia et al. (18) found ratios > 2 in 56% of their alcoholic patients but in only one patient among 115 with NALD, and Diehl et al. (19) found an AST/ALT ratio > 3 in 86% of their alcoholic patients and in only 32% of the NALD patients. In the present study, we report an AST/ALT ratio > 2 in only 15% of alcoholic patients and in 19% of NALD patients. This ratio therefore did not appear to be useful in the diagnosis of alcoholism.

Chromatofocusing by using a Mono P column followed by radioimmunoassay correlated well with micro-anion-exchange chromatography and provided a more sensitive assay; however, the former technique is more time consuming, requiring an additional purification of the serum, and as yet is not suitable for routine use. The micro-anion-exchange method requires only a small volume of serum.
(40 μL), and multiple samples can be analyzed at the same time; this seems, therefore, to be the simplest method for measuring dTF in serum.

In conclusion, both dTF and mAST were more sensitive than conventional tests for chronic alcohol consumption and also had a greater specificity. Unlike conventional tests, dTF appears to be useful as a diagnostic test in all relevant clinical situations, including screening for heavy alcohol intake in patients with liver disease. Similarly, the ratio of mAST to total AST in patients with NALD is more specific than any of the conventional markers of alcoholism. Because strong elements of denial complicate history-taking in patients who abuse alcohol and because of the apparent specificity of the dTF/total Tf ratio, a positive test in such situations should alert the physicians to a high probability of excessive alcohol intake.

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References

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