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Evaluation of Carbohydrate-deficient Transferrin

To the Editor:

Recently Bean et al. *(1)* compared isoelectric focusing/immunoblotting/laser densitometry (IEF/IB/LD), Axis %CDT TIA, and Axis %CDT HPLC for the determination of CDT. This study shows shortcomings that need further discussion.

1. The three methods, as presented in *Materials and Methods* in that paper, are not semiautomated. The statement that "similar results are obtained on an array of turbidimetric instruments . . . " on page 987 cannot be deduced from the data presented. None of the 10 analyzers mentioned was tested in this study, and appropriate references are not given. Thus, the title does not reflect the content of the paper.

2. On page 983, the authors state that "inclusion of the trisialotransferrin fraction increases the accuracy in the diagnosis of sustained alcohol usage." However, on page 988, the discussion ends with "whether the inclusion of the trisialotransferrins results in improved efficacy of the test awaits further analysis. . . "

3. Predictive values, diagnostic sensitivities, and specificities strongly depend on the definition of false positives and negatives and patients' sex and liver function *(2)*. However, no information is given on how daily alcohol consumption for groups 1–4 was verified. Furthermore, groups 2–4 combine men and women (with and without alcohol-induced liver disease). Although gender-specific upper reference limits of CDT are described in detail and generally accepted *(2)*, the authors establish gender-nonspecific cutoffs without giving any reasons for this procedure.

4. The study illustrates the need for a unified definition of CDT. On page 983, the IEF/IB/LD is said to summarize asialo-, mono-, and disialotransferrins as CDT; on pages 984, 987, and 988, only asialo- and disialotransferrin. %CDT TIA analyzes asialo-, mono-, di-, and 50% of trisialotransferrin; %CDT HPLC mono-, di-, and 50% of trisialotransferrin but not asialotransferrin (pages 984–985). IEF/IB/LD measures partially iron-loaded, %CDT TIA, and %CDT HPLC iron-saturated isotransferrins. Thus, CDT values obtained by three methods, measuring different analytes as CDT, are tested for equality in this study. How the inclusion of 50% trisialotransferrin in CDT determination by %CDT TIA and %CDT HPLC was verified and tested for reproducibility is not described or cited. On page 986, the authors discuss the "separation of CDT from fully sialylated transferrin isoforms" without defining the latter term, although asialo- to octasialotransferrins have been described. Thus it remains unclear which isotransferrins are meant.

5. Cofocusing or coelution of tetrasialotransferrin with CDT causes false positives *(3, 4)*. Therefore, tetrasialotransferrin must and not "should" be "avoided in the eluate" (page 984).

6. A similar correlation between IEF/IB/LD and %CDT TIA and between IEF/IB/LD and %CDT HPLC was obtained not "because all three tests measure relative CDT . . . rather than absolute CDT. . . ," as concluded by the authors, but because the %CDT TIA minicolumn performance was "calibrated until the correlation between HPLC and TIA methods was at maximum".

7. Lanes a and b and c and d have been exchanged in the legend of Fig. 1. In interpreting Fig. 1, monosialotransferrin is ignored, although its bands are clearly visible.

8. In contrast to present knowledge *(2, 5)*, genetic transferrin D variants did not cause false positives in this study. This finding should have been discussed by the authors.

9. Whether for %CDT TIA "each specimen requires two measurements" or only one because "its precision is low enough to allow for single determination of CDT. . . " (page 987) remains unclear.

10. Superficial errors, e.g., "Grøbæk" instead of "Grønbæk", "Sudrehagen" instead of "Sundrehagen" (one of the co-authors) further weaken the merit of the study.

Because of these shortcomings, the study does not really contribute to a better understanding of the diagnostic performance of CDT as a marker of chronic alcohol abuse.

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Dr. Bean responds:

To the Editor:

We appreciate the opportunity to address the challenges identified by Drs. Arndt and Hackler. The focus of this reply centers on questions 1–4 and 6–9 because they present a legitimate concern for a better understanding of the manuscript. Each question is answered individually.

Question 1. The carbohydrate-deficient transferrin turbidimetric immunoassay (%CDT TIA) testing described in this study used the microtiter plate version and was performed at Axis Biochemicals (Oslo, Norway). In a similar study, data obtained after testing 153 serum specimens by %CDT TIA in both microtiter plate and Cobas Mira S formats show similar %CDT values (Fig. 1). This parallel study was part of the %CDT TIA method validation experiments conducted at Axis Biochemicals and was not described in the CDT manuscript. As shown in Fig. 1, the correlation coefficient between the Cobas Mira S and the Microtiter plate version is better than $r^2 = 0.99$. In addition, at the reference value of 6% CDT, the cutoff for the %CDT TIA, the corresponding value for Cobas Mira S is also close to 6% CDT. The intercept of the correlation curve with the *y*-axis is small, altogether indicating a strong correlation between the two methods.

In addition, the %CDT TIA procedure recently introduced in the market by Specialty Laboratories uses a Behring BNII nephelometer. Serum specimens analyzed by IEF/IB/LD (n = 30) were also analyzed using the Behring nephelometer as part of the assay validation task list used at Specialty Laboratories. There was no significant difference between the CDT results obtained by both assay formats.

Thus, we believe that the title does reflect the content of the paper because the %CDT TIA is run successfully in semiautomated instruments.

Question 2. The two methods that included the trisialo transferrins in their quantification schemes provided better diagnostic accuracy than IEF/IB/LD, so we deduced im-

proved performance. Until more sophisticated analytical tools allow precise definition of the mixtures of glycans in the CDT generated by alcohol abuse, however, the conclusion remains a hypothesis.

Question 3. Alcohol consumption was evaluated in all individuals by a questionnaire administered at the time of blood sampling.

Because alcohol abusers under-report their alcohol consumption, individuals in group 4 were also interviewed by a physician with respect to previous and present diseases as well as previous and present drinking behavior (time of drinking, relapse, withdrawal syndrome).

Only patients diagnosed according to DSM-III-R as having "severe alcohol dependence" with at least seven symptoms present were included in this study. A semistructured questionnaire related to the topology of Lesch *(1)* was applied to classify drinking patterns as well as the severity of somatic, psychic, and social deterioration. The drinking pattern of the patient before admission was established with respect to amount, frequency, and drinking rhythm. For most patients, an extensive panel of laboratory procedures including liver enzyme tests, mean corpuscular volume, and blood alcohol concentrations was also available.

Total abstainers were active participants in an anti-alcohol organization, with no history of alcohol abuse. Social drinkers were healthy individuals reporting a moderate alcohol consumption of <40 g daily; there were no former alcoholics in this group. Only one individual in

this group occasionally drank >40 g daily, but on average he drank according to the social drinkers group.

Regarding gender-specific cutoffs, males and females showed a similar mean value (3.4 DU) when serum specimens derived from healthy males (Specialty employees, n = 50) and healthy females (Specialty employees, n = 50) were tested by IEF/IB/LD (Specialty Laboratories, IEF/IB/LD test validation records).

The mean values for the %CDT TIA and the %CDT HPLC in females were 3.3 ± 0.7% and 3.6 ± 0.8%, respectively (P. Bean, A. Husa, K. Liegmann, and E. Sundrehagen. Manuscript in preparation.) In groups 1–3 of this study, the mean values for the %CDT TIA and %CDT HPLC in males were 3.5% CDT and 3.7% CDT, respectively. By contrast, CDTect presents gender-specific cutoffs because it measures absolute values of CDT. Procedures that measure CDT in relation to total transferrin operate accurately with a single cutoff.

Question 4. The first part of this question refers to the need for a unified definition of CDT in view of the many CDT procedures available. We acknowledge the need but recognize that the proposition is challenging at present. Most current technologies for analysis of oligosaccharide composition lack the sophistication to provide enough detailed information of the array and kind of monosaccharides of CDT. The ones that do are too costly and require clean concentrated CDT, which is usually unavailable.
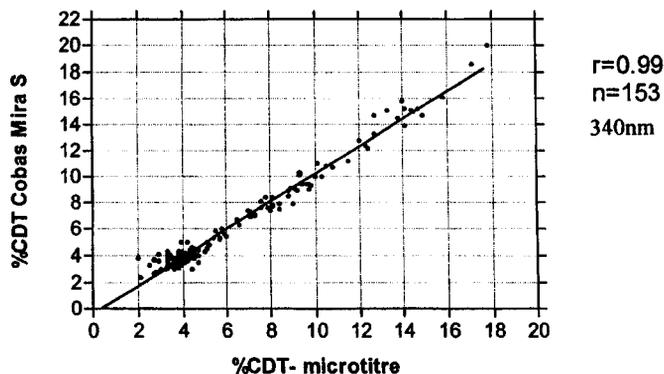
We invite the reader to keep in

Fig. 1. Correlation %CDT TIA microtiter plate version compared with %CDT TIA Cobas Mira.

mind that the main concern is diagnostic performance rather than carbohydrate composition. Knowledge of the precise oligosaccharide composition is secondary if CDT identifies the medical condition of harmful alcohol consumption with a clearly specified accuracy. Biochemical composition remains a primary concern for scientists and laboratory workers attempting to improve the performance and cost of CDT testing.

The second part of this question refers to verification of the inclusion of 50% of the trisialotransferrins in both the %CDT TIA and %CDT HPLC. The HPLC method used in this study identified almost all isoforms of transferrins (except asialo-) by distinct peaks separated at baseline. Evaluations of the transferrin peaks in the chromatogram were performed with a Nelson data module as specified in *Materials and Methods*. The %CDT concentration was expressed as a relative amount to total transferrin by peak integration of 50% of the trisialotransferrins and all disialo- and monosialotransferrins. Total transferrin concentration was determined by integration of all peaks in the chromatogram.

Inclusion of 50% of the trisialotransferrins in the %CDT TIA was achieved by adjusting the ionic strength of the separation buffer used in this procedure. The inclusion of 50% trisialo-transferrins was verified by testing a QC serum panel of 15–20 samples with approved %CDT HPLC values.

Reproducibility of these results is explained in detail in the answer to question 9.

The third part of this question asks for a definition of "fully sialylated transferrin isoforms". For the purposes of this study, fully sialylated transferrin isoforms were complex carbohydrates with at least two sialic acids in each branch: bi-, tri-, or tetra-antennary.

Question 6. It is likely that many factors contribute to the close agreement of the IEF/IB/LD with these two new procedures, a commonality for all three is that they measure relative CDT. CDTect shows a smaller correlation coefficient with IEF/IB/LD and measures absolute CDT.

Question 7. We thank the reviewers for bringing up the error in the legend of Fig. 1. The legend should read, "The banding pattern of the serum from a nondrinker (*lanes a and b*) and an alcohol abuser (*lanes c and d*) before (*lanes b and d*) and after (*lanes a and c*) separation by %CDT TIA minicolumns".

In interpreting Fig. 1 of the original manuscript, our intention was to simplify the description of the gel by focusing only on the dominant CDT bands. The disialotransferrins are the pillar of all CDT procedures, and the trisialotransferrins are new to both the %CDT TIA and the %CDT HPLC. The monosialo- and asialotransferrin isoforms are discriminated differently in the three procedures described in the CDT manuscript.

Question 8. The likelihood of finding a genetic D variant that generates a CDT false positive is rather small *(2)*. There are genetic variants of several kind: D_1, D_2, D_3, and $D[_{Chi}]$. Of these, only variant D_3 generates false positives for CDT when patient sera are tested by IEF/IB/LD. The two females with genetic variants in this study, both social drinkers, showed variants of the D_1 type. The %CDT TIA and %CDT HPLC render a negative CDT result with type D_1 sera.

The *Discussion* section of this study focused mainly on clinical utility. A more extensive analysis will describe the effect of genetic variants in the generation of false positives by these new procedures (Bean et al., in preparation).

Question 9. Studies regarding precision and reproducibility were conducted at two sites: Specialty Laboratories and Axis Biochemicals.

The validation of the %CDT TIA–Behring BNII conducted at Specialty Laboratories included determinations of intra- and interassay variations. The intraassay coefficient of variation was determined by running serum specimens 20 times in the same experiment. The interassay coefficient of variation was determined by running the serum specimens five times on five different occasions. The intraassay coefficient of variation determined with two specimens containing 2% CDT and 11% CDT showed results of 7% and 11%, re-

spectively. Interassay coefficients of variation for specimens containing 2% CDT, 3% CDT, 6% CDT, 11% CDT, and 85% CDT were 11%, 9%, 6%, 9%, and 6%, respectively.

The validation of the microtiter plate version of the %CDT TIA conducted at Axis Biochemicals included similar determinations of intra- and interassay variations. The intraassay coefficient of variation was determined by running serum specimens 20 times in the same experiment. The interassay coefficient of variation was determined by running three specimens on 10 different occasions. The intraassay coefficient of variation determined with three specimens containing 5% CDT, 7% CDT, and 12% CDT showed results of 2%, 2%, and 3%, respectively. The interassay coefficients of variation for these same three specimens were 3%, 4%, and 2%, respectively.

The results of these two independent studies indicate that reproducible results are obtained by single determinations when the %CDT TIA is used in both the Behring nephelometer and microtiter plate formats.

Finally, the aim of this study was to raise the awareness of new semiautomated procedures now available at reasonable cost with improved potential for identification of harmful alcohol consumption. A better understanding of the diagnostic performance of CDT as a marker of chronic alcohol abuse will be achieved when more questions like the ones posed by Drs. Arndt and Hackler find their proper answers and the answers are published.

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