Alcohol Abuse and Carbohydrate-deficient Transferrin Analysis: Are Screening and Confirmatory Analysis Required?

To the Editor:

Carbohydrate-deficient transferrin (CDT; asialo- plus monosialo- plus disialo-Fe_2-transferrin) is currently the most specific laboratory marker of chronic alcohol abuse *(1, 2)*. Because of the high prevalence of chronic alcohol abuse in many countries, CDT plays an important role in the areas of employment, traffic safety, and forensic medicine. The potentially strong social impact of an increased CDT value justifies the need for maximally reliable preanalysis, analysis, and interpretation of CDT. There is a highly developed quality-assurance system in toxicologic and forensic drug analysis, including standards and calibration materials, internal quality-control materials, external quality-control programs, and a system of screening and confirmatory analyses *(3–5)*. Although CDT quality-control materials and surveys have become available in recent years and have greatly contributed to the improvement of CDT analysis, an international CDT standard is still lacking. Furthermore, a system for CDT analysis comprising screening and confirmatory analysis, analogous to forensic analysis of cannabinoids, heroin, and cocaine, has not been established. This is surprising because the dependency potential (addictiveness) of ethanol is reported to be comparable to that of cocaine and heroin *(6, 7)* and much higher than that of cannabis *(6, 7)*. Many state laws regulate medico-legal diagnosis of "acute alcohol abuse", demanding ethanol analysis by two independent analysis methods: an enzymatic NADH method and headspace gas chromatography *(8)*. In contrast to this, the forensic laboratory diagnosis of "chronic alcohol abuse" by measurement of CDT is essentially not regulated and is usually based on only one (immunologic) analytical method. Here we summarize some arguments in favor of confirmatory methods for CDT.

CDT shows an overall diagnostic specificity that is distinctly higher when compared with other markers of chronic alcohol abuse, e.g., γ-glutamyl transferase and mean corpuscular volume of erythrocytes *(1, 2)*. In their systematic review and meta-analysis of CDT as a marker of chronic alcohol abuse, Scouller et al. *(9)* report "typical specificities of 75–100%". Diagnostic specificities of CDT in the range of 92–96% were found for alcoholics and hazardous drinkers, based on the Timeline Followback and Composite International Diagnostic Interview as reliable criteria for the diagnosis of alcoholism and alcohol intake *(10)*. In the worst case (75% diagnostic specificity), this would mean that 1 of every 4 CDT measurements would be a false positive and, under optimum conditions, 4–8 of every 100 CDT measurements would be false positives. This is not acceptable for medico-legal situations.

False-positive CDT reports indicating chronic alcohol abuse can be attributable to conditions such as transferrin D variants and primary biliary cirrhoses *(1, 2)* or to analytical problems. The latter can occur as a result of incomplete fractionation of the CDT and non-CDT isoforms by anion-exchange microcolumns *(11)*. Because transferrin but not CDT antibodies are used in the subsequent immunologic step for quantification of CDT in the column eluates, coelution of non-CDT isoforms causes false-positive increased CDT values. This has been shown previously *(11)* and can be applied to all commercial CDT tests based on microcolumn CDT and non-CDT fractionation followed by immunoassay.

Strategies to reduce analytical nonspecificity attributable to incomplete CDT separation are listed below:

- The simplest protocol to reduce false positives attributable to incomplete CDT and non-CDT fractionation should comprise duplicate analysis of CDT by the same assay using two microcolumns (but not a single column and duplicate immunoassay of the same eluate). This can prevent false positives attributable to a single faulty microcolumn.
- Reanalysis of borderline and increased CDT results can additionally improve the reliability of the final CDT analysis result by reducing the effects of interassay variance (e.g., by taking the mean of the two measurements). At the same time, reanalysis of borderline and increased CDT results, instead of duplicate analysis of each sample (normal, borderline, increased CDT values), distinctly reduces both the number and the costs of CDT analyses.
- Duplicate analysis or reanalysis does not allow the detection of entire lots of faulty microcolumns. This can be achieved by checking new assay lots before they are used in routine analysis against lots that are already in use (which is in accordance with Good Laboratory Practice and recommendations of the College of American Pathologists).
- Plotting the percentage of normal, borderline, and increased CDT results of each analysis series against the date of analysis (with the *x* axis as the analysis date and the *y* axis as the percentage of normal, borderline, and increased CDT results) has been used successfully as an additional tool for detection of faulty assay lots in our laboratory (100–150 CDT analyses/workday).

In any case, applying the same analytical method for duplicate analysis, reanalysis, or confirmatory analysis does not allow detection of false positives attributable to analytical interferences, e.g., by transferrin D variants or by unknown properties of a certain serum sample. Thus it is clear that a second method is needed for confirmation of increased CDT results when analyzing serum samples with forensic or potentially forensic consequences.

The requirements for screening and confirmatory CDT analytical methods can be derived from the general protocols for drug analysis *(3–5)*: The screening method should

be specific, sensitive, reproducible, fast, and ideally can be automated. It should distinguish negative samples from those with borderline or abnormal results to minimize the number of confirmatory analyses. The availability and use of sensitive and validated drug screen tests make false negatives unlikely. Therefore, negative drug screen results usually do not require a confirmatory analysis *(4)*. However, a positive drug screen result should always be confirmed by a second, independent method. There is consensus that reporting a positive drug screen result without confirmatory analysis is, in some cases, not Good Laboratory Practice *(4, 5)*.

The confirmatory method should be independent from the screening method, using a different separation and/or detection principle. The analytical specificity should be higher than that of the screening method, and the diagnostic sensitivity should be at least the same. This strategy markedly reduces the number of false positives and increases the legal position for the person tested. Confirmatory analysis allows the exclusion of analytical interferences by drugs or food constituents. The latter are often used as an excuse by persons who deny drug abuse. An example of this situation has been described recently in this journal *(12)*. The strategy of screening and confirmatory analysis is common sense for forensic drug analysis. Chromatographic methods such as thin-layer chromatography, HPLC, and gas chromatography (GC) have been successfully used for confirmation *(3–5)*. Most often, the screening/confirmatory methods comprised immunoassay/GC–mass spectrometry (GC-MS) or immunoassay/liquid chromatography–MS (LC-MS). Because of their excellent sensitivity and specificity, GC-MS and LC-MS are considered reference methods.

Possible biological and analytical causes for false-positive CDT results have been discussed above. Regarding a CDT screening/confirmatory analysis concept, false-negative CDT results also have to be considered. Currently there is insufficient information about the prevalence of false-negative CDT results when using CDT tests based on microcolumn CDT and non-CDT fractionation followed by immunoassay. Incomplete elution of the CDT isoforms from the anion-exchange microcolumn can cause severe undermeasurement of CDT. The interference of genetic transferrin variants with CDT quantification by assays based on anion-exchange microcolumn fractionation of CDT and non-CDT isoforms has recently been demonstrated *(13)*. Transferrin B variants cause false negatives and transferrin D variants false positives. As long as there is no proof that false negatives are rather rare, general rejection of a confirmatory analysis in the case of negative CDT screen results can not be made. This makes the need for a practicable and validated CDT confirmatory analysis method even greater. Currently there is no such method. However, there are several methods for physicochemical CDT analysis. These should be considered as confirmatory methods for CDT *(2)*. In contrast to "blind" extraction of CDT isoforms with anion-exchange microcolumns, electrophoretic and HPLC methods have the advantage of visualizing the transferrin isoform patterns in the serum sample. This allows both detection of transferrin variants and confirmation of complete transferrin Fe^{3+} load and stability during the analysis process, which is basic for reliable CDT measurement *(2)*.

In the absence of a reliable and practicable physicochemical confirmatory method, positive immunologic drug screen results may be confirmed by a second immunologic method *(4)*. Thus, a combination of two commercial CDT tests, based on CDT and non-CDT separation on ion-exchange microcolumns followed by a turbidimetric immunoassay, can be a first step toward improvement of CDT analysis. Of course, two immunoassays can not be considered independent methods and would be forensically liable. However, in the current situation, a nonoptimal confirmatory method is better than no confirmatory analysis at all. Such a combination can serve only as a minimal and interim solution until a CDT reference method has been established and made available.

Currently there are no GC-MS or LC-MS methods for routine CDT analysis. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, used mainly for investigation of protein structures, has been valuable for investigating the carbohydrate moieties of the different CDT isoforms *(14)*. However, this method is not practicable for routine quantitative CDT analysis. Application of GC-MS to CDT analysis is hampered mainly by the need for evaporation of the transferrin isoforms with high molecular masses, which is almost impossible without destruction of the protein molecules. This problem could be avoided by the use of LC-MS. However, the complex glycoprotein structure of the transferrin isoforms makes reproducible formation of specific ion fragments (compared with drugs with well-known ion fragment patterns) hard to obtain. This and the lack of CDT isoform standards, as are available for many drugs, further hamper the development of a MS method for quantitative CDT measurement.

Isoelectric focusing (IEF) is referred to as the reference method for serum transferrin isoform analysis. It is superior in detecting and phenotyping genetic transferrin variants with isoelectric points close to that of the main non-CDT isoform, tetrasialo-Fe_2-transferrin *(2)*. However, IEF is quite laborious, quantification of the transferrin bands is hard to obtain, and automation is difficult. IEF is, however, suitable as a qualitative confirmatory method for small sample volumes.

Capillary electrophoresis is considered a fast, sensitive, and specific method with the potential for automation. Several applications, including immunosubtraction techniques, have been described *(2, 15)*. Validation of electrophoretic procedures as CDT confirmatory methods should include examination of the potential effects of transferrin Fe^{3+} loss during

electrophoresis on the electropherogram and the final CDT result *(2)*.

HPLC has been successfully used for routine determination of serum CDT *(16)* and allows reliable detection of the most important genetic transferrin variants *(2)*. There seems to be potential for further improvement of the sensitivity and resolution of the current CDT HPLC applications, especially for detecting the minor CDT isoforms asialo-Fe_2-transferrin and monosialo-Fe_2-transferrin. Again, the potential effects of transferrin Fe^{3+} loss during the chromatographic process on the chromatogram and, thus, the final CDT result should be considered when validating HPLC as a confirmatory CDT method. HPLC seems to be the most suitable analytical method at present. Compared with capillary electrophoresis, HPLC as a method is more common in laboratories. Nevertheless, establishing an in-house HPLC method suitable for forensic CDT confirmatory analysis requires experience in developing HPLC methods. Thus, the use of HPLC as a CDT confirmatory method is hampered by the lack of a validated commercial application.

Who is responsible? At a more general level, several national and international organizations are involved in defining the criteria for Good Laboratory Practice. At a more specific level, professional societies for forensic and toxicologic medicine have established guidelines for drug analysis. Development, validation, and implementation of a CDT standard and a CDT reference method will take effort and expense. Therefore, we strongly recommend international cooperation between scientific societies and the diagnostic industry. From an analytical point of view, the availability of a CDT standard and CDT reference method can remedy the discrepancies among strategies in the laboratory diagnosis of acute and chronic alcohol abuse by establishing screening and confirmatory analysis, as is true for drugs. This would definitely improve the reliability of CDT measurements (e.g., detection of transferrin D variants as an important cause for false-positive CDT results). Such a strategy of CDT screening and confirmatory analysis by immunoassay/HPLC or immunoassay/capillary electrophoresis will improve patient outcomes and the legal position of the laboratory.

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Torsten Arndt[1*]
Jürgen Kropf[2]

[1] Bioscientia GmbH
Konrad Adenauer Strasse 17
D-55218 Ingelheim, Germany

[2] Abteilung Klinische Chemie
und Molekulare Diagnostik
Philipps-Universität Marburg
Baldingerstrasse
D-35033 Marburg, Germany

*Author for correspondence. Fax 49-6132-781-428, e-mail arndt@bioscientia.de.

Does Centrifugation Cause the ex Vivo Release of DNA from Blood Cells?

To the Editor:

Using a sex-mismatched bone marrow transplantation model, we have previously demonstrated that hematopoietic cells represent the predominant origin of cell-free plasma DNA *(1)*. In that study, plasma samples were obtained by high-speed centrifugation followed by high-speed microcentrifugation. The comparison of the total DNA concentrations in plasma obtained by centrifugation and microcentrifugation for different numbers of times revealed no significant difference. We therefore concluded that centrifugation is not associated with damage to blood cells and artificial increases in cell-free plasma DNA concentrations. However, there remains a possibility that the first spin might have already destroyed blood cells, leading to the ex vivo release of DNA. This theoretical possibility might produce an aberrantly high proportion of cell-free plasma DNA originating from hematopoietic cells. In fact, to prevent cell lysis, the use of low-speed centrifugation, at 500*g* or even lower, has been a usual practice in harvesting