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CARBOHYDRATE-DEFICIENT TRANSFERRIN – A CONTEMPORARY BIOMARKER IN COMPARISON WITH TRADITIONAL LABORATORY MARKERS OF CHRONIC ALCOHOL ABUSE

TRANSFERIN DEFICIJENTAN UGLJENIM HIDRATIMA – SAVREMENI BIOMARKER U POREĐENJU SA TRADICIONALNIM LABORATORIJSKIM MARKERIMA HRONIČNOG ALKOHOLIZMA

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Summary: Timely identification of excessive alcohol use and its potential complications is a prerequisite for successful treatment. Several routine tests have been used in laboratories that may help in diagnosing alcoholism, such as determination of MCV, AST, ALT, GGT, but it has been shown that they lack specificity and sensitivity. Contemporary biomarkers are increasingly being used today that may due to their unique characteristics help in discovering the onset of chronic alcohol abuse, as well as in abstinence and relapse monitoring. The term carbohydrate-deficient transferrin (CDT) stands for a small group of human transferrin isoforms (asialo, monosialo, and disialotransferrin) with a lower degree of glycosylation in comparison to the dominant transferrin isoform (tetrasialotransferrin). Persons consuming large quantities of alcohol (≥50–80 g daily) over a period of at least two weeks have increased concentrations of transferrin isoforms lacking one (disialotransferrin) or both (asialotransferrin) carbohydrate chains. In this paper the traditional markers of chronic alcohol abuse (GGT, AST, ALT, and MCV) were determined, as well as the new biomarker CDT, after which diagnostic evaluation was performed and their usability and clinical value in routine laboratory practice were estimated. These markers were also determined in heavy alcoholics on admission into hospital and after two weeks of therapy, with the aim of estimating their diagnostic value for abstinence and relapse monitoring.

Keywords: alcoholism, biomarkers, CDT, transferrin

Kratak sadržaj: Pravovremeno prepoznavanje prekomerne upotrebe alkohola i potencijalnih komplikacija preduslov je za uspešno lečenje. U laboratorijama se koristi nekoliko rutinskih testova koji mogu da pomognu u dijagnostici alkoholizma, kao npr. određivanje MCV, AST, ALT, GGT, ali su se pokazali kao nedovoljno specifični i osetljivi. Danas su sve više u upotrebi savremeni biomarkeri koji zahvaljujući svojim jedinstvenim osobinama mogu da pomognu kod otkrivanja pojave hroničnog alkoholizma, kao i pri praćenju apstinencije i relapsa. Naziv transferin deficijentan ugljenim hidratima (CDT) podrazumeva manju grupu izoformi humanog transferina (asialo-, monosialo- i disialo-transferin), koje imaju niži stepen glikozilacije u poređenju sa dominantnom izoformom transferina (tetrasialotransferin). Osobe koje unose velike količine alkohola (≥50–80 g dnevno) u periodu od najmanje 2 nedelje imaju povišene koncentracije izoformi transferina kojima nedostaje jedan (disialotransferin) ili oba (asialotransferin) ugljenohidratna lanca. U ovom radu obavljeno je određivanje tradicionalnih markera hronične alkoholne potrošnje (GGT, AST, ALT i MCV), kao i novog biomarkera CDT, a zatim je izvršena dijagnostička evaluacija i procena njihove upotrebljivosti i kliničke vrednosti u rutinskoj laboratorijskoj praksi. Takođe je izvršeno određivanje navedenih markera kod teških alkoholičara pri prijemu na bolničko odeljenje i nakon 2 nedelje terapije u cilju procene njihove dijagnostičke vrednosti za praćenje apstinencije i relapsa.

Ključne reči: alkoholizam, biomarkeri, CDT, transferin

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Introduction

According to the definition of the World Health Organization, alcoholics are persons who use alcohol excessively, slowly becoming dependent on alcohol and suffering either from open mental disturbances or interferences with bodily or mental health, interpersonal relations and social and economic functioning (1, 2). Timely detection of excessive alcohol consumption and its potential complications is a prerequisite for successful treatment. Many persons who abuse or are dependent on alcohol seek help, while at the same time denying the basic problem. In the assessment of alcohol dependence the sequential approach is applied which implies: a) screening, b) procedures for diagnosing alcohol dependence, and c) monitoring of the patient's state during treatment (3).

With the aim of developing more efficient strategies for decreasing alcohol consumption, it is important to review on a deeper level the pathogenetic mechanisms of disorders linked with alcohol and develop more effective diagnostic approaches for early detection of dangerous alcohol use. There is an obvious need for an independent laboratory indicator of increased alcohol consumption that would help clinicians suspect alcoholism as the real cause of symptoms. Biomarkers have proved themselves useful in diagnostics because of their advantage in relation to polls, whose results often fail to elicit admission from the patient about alcohol consumption (4). Several routine tests have been used in laboratories that may help in diagnosing alcoholism, such as MCV, AST, ALT, GGT, but they have been shown to lack specificity and sensitivity, since their values are also increased in hepatobiliary diseases, poisoning, obesity, use of certain medications, etc. (5).

New biomarkers are increasingly being used today that have high specificity and sensitivity. One of them is CDT, a glycoprotein synthesized in the hepatocytes from where it is secreted into circulation. Due to prolonged alcohol consumption, the microstructure on the transferrin glycoform is changed towards the creation of a proportionally larger concentration of the so-called carbohydrate-deficient transferrin (CDT), thus making it a very useful marker of alcohol abuse. This discovery was made in 1976 when Stibler and Kjellin (6) found increased concentrations of transferrin glycoforms in the cerebrospinal fluid of alcoholics.

Transferrin is composed of three different structure parts: a polypeptide chain with 679 amino acids, two Fe^{3+} binding sites, one inside the N-terminal and the other inside the C-terminal part, as well as two N-carbohydrate chains (7).

Details of the pathomechanisms of CDT increase during chronic alcohol consumption have not been elucidated, but there is evidence that ethanol or its metabolite acetaldehyde disturb the synthesis of carbohydrate chains in the Golgi apparatus (8, 9).

The main difference between CDT and the earlier traditional markers of alcohol abuse is its diagnostic accuracy. Namely, CDT has a big advantage due to its high specificity, meaning that a positive result almost always indicates suspicion of heavy alcoholism, and is not influenced by hypertension, bronchitis, asthma, diabetes mellitus, angina pectoris, obesity, metabolic disturbances, depression and digestive system diseases. Increased risk of false positive results in heavy liver damage, carbohydrate-deficient glycoprotein syndrome (CDGS), genetic D-variant of transferrin, pregnancy, use of estrogen, anemia, low ferritin, high total transferrin, or false negative results in the genetic B-variant transferrin and low total transferrin values, highly correlates with the method used for CDT quantification (10).

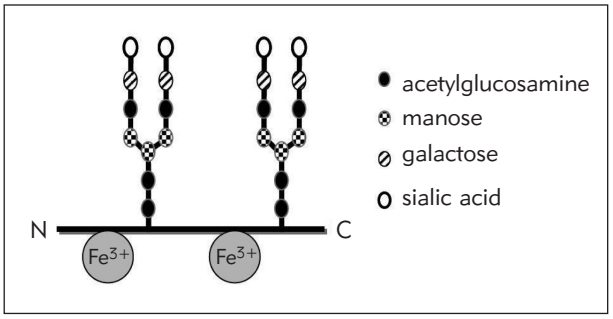

Figure 1. Structure of the main transferrin glycoform – tetrasialotransferrin that makes up 80% of total transferrin in the serum of healthy adults. Line between N and C presents an individual peptide chain.

Sensitivity of the CDT test, i.e. its ability to detect heavy forms of alcoholism depends on several factors, such as the amount of alcohol consumed, population, behaviour and habits of alcoholics, time of sampling after the end of alcohol abuse (11).

With the introduction of various analytic techniques and methods for CDT determination during the last 15 years, whose values were expressed as absolute (mg/L, or U/L) or relative units (%CDT) in relation to total transferrin or tetrasialotransferrin, and showed various sensitivity and specificity because of the involvement of various transferrin isoforms in »CDT«, problems emerged in many studies regarding the comparison of clinical and analytic results (12).

Problems with the standardization of CDT determination have led to the meeting of the Task Force for CDT Standardization (WG-CDT), following a recommendation from the IFCC, whose primary goal was to define the most suitable CDT analyte, choose a referent method for CDT determination, produce reference materials for the chosen analyte, and recommend it for clinical use. The definition of disialotransferrin as the primary, target molecule for CDT determination and individual analyte for CDT standardization, and the recommendation that HPLC be the temporary referent method, marked the first step toward the standardization of CDT determi-

nation. These actions would improve the diagnostic characteristics of CDT as a biomarker of chronic alcohol consumption and promote comparability between laboratories (13).

Material and Methods

In this paper the parameters indicating increased alcohol consumption were determined in the sera of 90 examined patients. During patient admission into the wards of the Special Hospital for Addictive Diseases, Institute of Psychiatry and Institute of Infectious and Tropical Diseases, interviews were done according to the standard protocol and data were obtained about patient age, profession, health condition, duration of alcohol use, quantity and type of liquor, as well as the date of last alcohol intake. On the basis of such »history of alcohol consumption«, the examinees were divided into two groups: group of patients whose daily alcohol intake was less than 60 g (abstainers/occasional drinkers) and group with daily alcohol intake of 60 g and more (heavy alcoholics). Standard drinks contain 12–14 g of alcohol, which is equal to a bottle of beer, glass of table wine or 4 cl of 40% hard liquor. Control group (n=26) was made up of examinees not consuming alcohol or doing so at most 6 times a year on special occasions (e.g. birthdays) and then consuming not more than 15 g of alcohol at once. Average age of patients in the group of abstainers and occasional drinkers was 44.0 years (22–65), in the heavy alcoholics group 45.5 (24–69), and in the control group 45.4 (31–62). Samples were collected into tubes without additives, centrifuged and frozen at –20 °C.

The principle of CDT determination

N Latex CDT Kit (Siemens, Germany) is a new commercial test based on direct competitive nephelometric determination. The method uses monoclonal antibodies (mAb) that identify with great specificity transferrin glycoforms lacking one or both complete N-glycans (e.g. disialo, monosialo and asialotransferrin as CDT glycoforms) in combination with the simultaneous determination of total transferrin. Polystyrene particles coated with CDT-mAb agglutinate with CDT-coated polystyrene particles. CDT, depending on its concentration, inhibits this reaction, enabling nephelometric determination within 18 minutes. No preparatory operation is needed. Considering the fact that the degree of iron saturation of transferrin affects the antibody binding affinity, in the first incubation step a chelating agent is used that separates iron from the complex with transferrin. Simultaneous determination of total transferrin enables automatic calculation of CDT quantity as a percentage in relation to total transferrin (% CDT). Range of measurement is 20–640 mg/L or 0.77–25 %CDT, with the cut-off according to the manufacturer's recommendation being 2.5%.

Determination of %CDT was done at the Institute of Medical Biochemistry of the Clinical Centre of Serbia on a nephelometer BN II (Dade Behring, Germany).

Methods for the determination of AST, ALT and GGT

Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT) were determined immediately after sample centrifugation by spectrophotometry, using the biochemical analyzer Dimension Xpand Plus, Dade Behring. Methods for the determination of AST and ALT were modified according to the recommendation by the International Federation for Clinical Biochemistry (IFCC), with pyridoxal phosphate used as coenzyme. The method for GGT determination uses gamma-glutamyl-3-carboxy-4-nitroanilide as substrate.

The reference values for AST are <37 U/L, ALT <41 U/L, and GGT <55 U/L.

The method for MCV determination

Mean corpuscular volume (MCV) was determined by the direct measurement of hematological parameters using the Coulter principle, from vein blood with anticoagulant, on a hematological counter Hmx, Coulter Beckman, USA. Reference values for MCV are 83.0–97.2 fL.

Results

A new and promising approach to the diagnosis of alcoholism is based on a combination of traditional laboratory markers, MCV or liver enzymes, with the new alcohol marker CDT, to increase the sensitivity of

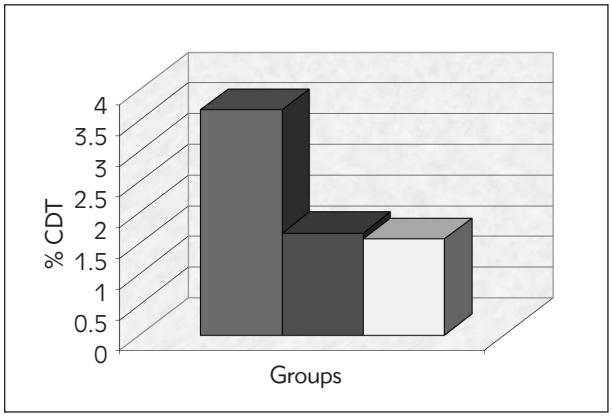

Figure 2. Values of the %CDT parameter in patients from Group A (heavy alcoholics), Group B (abstainers and occasional drinkers) and Group C (control group).

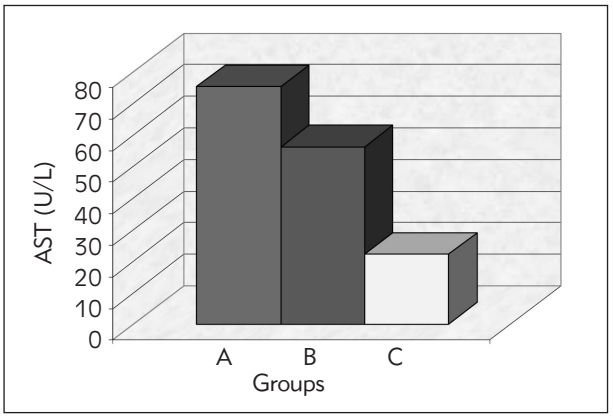

Figure 3. Values of the AST parameter in patients from Group A (heavy alcoholics), Group B (abstainers and occasional drinkers) and Group C (control group).

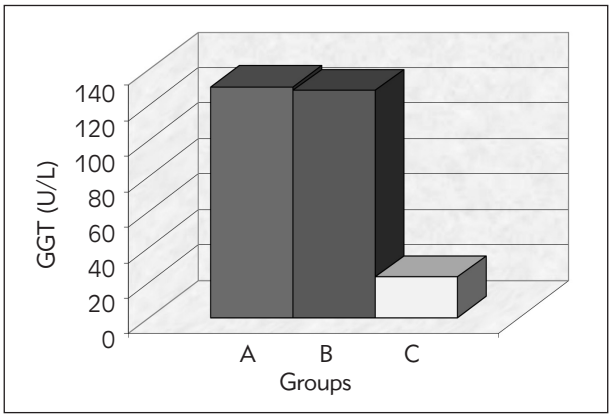

Figure 5. Values of the GGT parameter in patients from Group A (heavy alcoholics), Group B (abstainers and occasional drinkers) and Group C (control group).

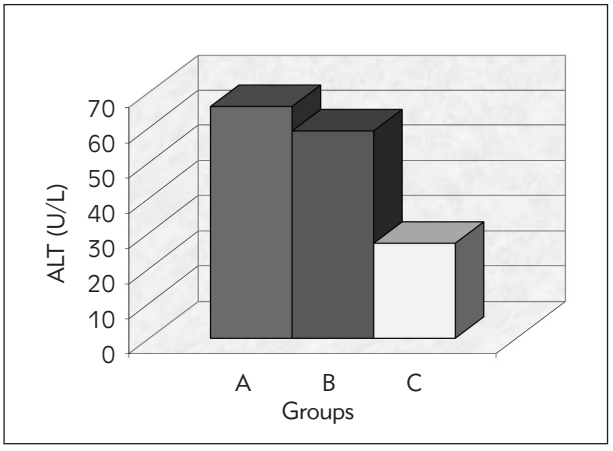

Figure 4. Values of the ALT parameter in patients from Group A (heavy alcoholics), Group B (abstainers and occasional drinkers) and Group C (control group).

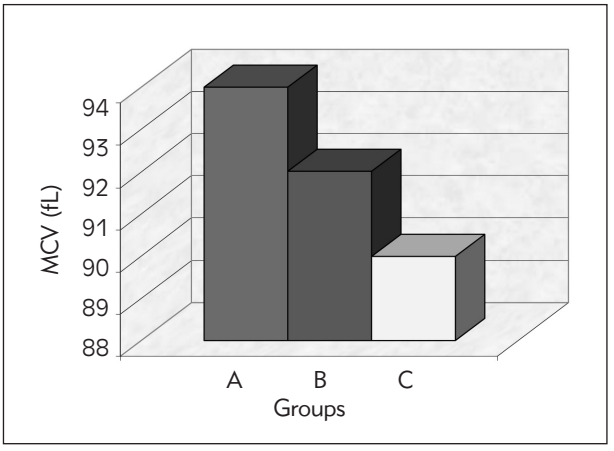

Figure 6. Values of the MCV parameter in patients from Group A (heavy alcoholics), Group B (abstainers and occasional drinkers) and Group C (control group).

determination and accuracy of alcohol detection. The measurement of GGT serum activity was the first marker of alcohol consumption, however, its specificity and sensitivity were reduced in the early stage of alcoholism, as well as due to some non-alcoholic liver diseases. Other laboratory tests used for indications of chronic and severe alcoholism, such as AST, ALT and MCV, have limitations regarding sensitivity and specificity, and should be interpreted with care. Considering the limits of the mentioned markers, and also the problems regarding patient admission of alcohol use, physicians are often unable to detect and monitor such patients. Due to its unique characteristics, CDT has shown itself to be practical in many clinical cases of chronic alcoholism detection, as well as in the monitoring of abstinence and relapse.

The new biomarker of chronic alcohol consumption, CDT, as well as routine laboratory markers GGT, AST, ALT and MCV were determined in three patient groups. The first group, Group A, was comprised of patients whose daily alcohol intake was 60 g and more (heavy alcoholics). Group B included patients with daily alcohol intake of less than 60 g (abstainers and occasional drinkers), while Group C was comprised of examinees not consuming alcohol or doing so rarely and without crossing the limit of 15 g of alcohol. The results of determination are presented in *Figures 2–6*.

Correlation of parameters

Correlation between the values of determined parameters was estimated by a non-parametric test, using the Spearman's correlation, since the obtained data did not have a normal distribution. The significance of the obtained values of Spearman's correlation coefficients (rs) for the probability of 0.05, in the comparison of parameter values, revealed where there is a significant correlation between them. Spearman's correlation coefficients calculated using the computer programme SPSS 11.0 are presented in *Table I*.

Table I Correlation of parameters – values of the Spearman correlation coefficients (rs).

Parameters	Group			
	A	B	C	All
CDT / AST	0.260	0.278	0.176	0.456
CDT /ALT	0.086	0.239	0.108	0.277
CDT / GGT	0.195	0.085	0.023	0.382
CDT / MCV	0.072	0.123	0.133	0.271

$p<0.05$, there is a correlation between parameters within the examined groups

Markers of alcoholism before and after abstinence

The markers of alcoholism were determined in patients admitted for hospital treatment immediately after admission and after two weeks of abstinence, thus enabling the estimation of applicability of the given parameters in routine work. The results of alcohol parameters before and after two weeks of abstinence are shown in *Table II*.

Discussion

None of the biomarkers of alcoholism widely accepted today in clinical use and thoroughly investigated in numerous research centres across the world shows the characteristics of an ideal parameter for reliable detection and monitoring of alcohol abuse. One of the most often used markers is GGT that exhibits a noted variation in the sensitivity and specificity of determination, because its activity rises except in the case of increased alcohol consumption and use of a large number of drugs, toxin action, infection, ischemia, hormones, obesity, fatty liver, etc. (14, 15). The activity of enzymes coming from the liver (AST, ALT) is often increased in patients who chronically use alcohol, although they have been found to be increased also in abstainers suffering from chronic liver diseases, viral hepatitis, obesity, fatty liver, drug use, etc. The situation is similar with MCV, whose sensitivity and specificity are lower in relation to other alcoholism biomarkers, because its values may also be increased in liver damage, vitamin B12 and folic acid, deficiency, hematological diseases, reticulocytosis, hypothyroidism, etc. (16).

Precisely because of the limited sensitivity of individual laboratory markers, the combinations of traditional markers MCV or liver enzymes with new alcoholic markers such as CDT and EDAC were introduced (*Early Detection of Alcohol Consumption*), thus improving the possibility of accurate alcoholism detection. New biomarkers that have high specificity and sensitivity are increasingly being used today. One of them is CDT, the first test to be approved in the USA by the FDA (*Food and Drug Administration*) as the biomarker for alcoholism (17–20).

Due to its unique characteristics, CDT has shown itself as a very useful and practical test during the screening of increased alcohol consumption in out-patients in primary practice, psychiatric clinics, emergency rooms, urgent care, as well as in the preoperative preparation of surgery patients. It also has an important application in occupational medicine, where it is used in systematic examinations, employment procedures, the issuing of driving licenses, for underage drinking, life insurance, workplace responsibility and forensic purposes. For diagnostic purposes it is used in gastroenterology/hepatology wards for distinguishing liver diseases caused by alcohol from liver diseases of different etiology, pancreatitis, esophagitis, gastrointestinal tract carcinoma, etc. (21).

In this paper the samples of patients whose daily alcohol intake was over 60 g (heavy alcoholics, N=64), samples from patients with daily alcohol intake of less than 60 g (abstainers/occasional drinkers, N=26), as well as samples from patients not consuming alcohol or doing so on rare occasions and so that their daily intake does not exceed 15 g (control group, N=26), were analyzed.

Routine laboratory markers of chronic alcohol consumption (GGT, AST, ALT and MCV) were estimated in all three groups as well as the new

Table II Values of examined parameters in patients before and after two weeks of abstinence.

Period	Statistical parameters	Parameters				
		CDT (%)	AST (U/L)	ALT (U/L)	GGT (U/L)	MCV (fL)
Before	min $\bar{x}$ max	1.66 3.52 5.81	27 81.2 322	10 67.9 201	18 121.3 913	89 95.6 105
After	min $\bar{x}$ max	1.31 2.17 * 4.40	19 51.1 116	18 48.4 186	13 99.17 413	84 94.5 106

* statistically significant difference ($p<0.05$) in relation to the period before abstinence

biomarker CDT, after which their diagnostic evaluation was performed. The mean value of CDT (3.66%) in the group of heavy alcoholics was statistically significantly higher ($p<0.05$) in comparison with the group of abstainers/occasional drinkers (1.66%), as well as in comparison with the control group (1.56%), indicating a good correlation with regard to the amount of alcohol introduced into the organism. Mean values of AST (75.33 U/L), ALT (65.63 U/L) and GGT (129.73 U/L) in the heavy alcoholics group, and in the group of abstainers/occasional drinkers AST (56.42 U/L), ALT (58.81 U/L) and GGT (128.04 U/L), were statistically significantly higher ($p<0.05$) in relation to the control group where the mean value of AST was 21.90 U/L, ALT 27.20 and GGT 23.40 U/L. Mean value of MCV (93.78 fL) in the group of heavy alcoholics was statistically significantly higher ($p<0.05$) in comparison with the mean value of patients in the control group (90.20 fL).

Using the computer programme SPSS 11.0, correlation was made between the values of determined parameters, and a significant correlation was established between the parameters CDT and AST in the group of patients marked as heavy alcoholics. Spearman's correlation coefficient for the probability of 0.05 was $r=0.260$. Comparison of parameters in all patient groups revealed significant correlations (CDT/AST, $r=0.456$, CDT/ALT, $r=0.277$, CDT/GGT, $r=0.382$, CDT/MCV, $r=0.271$).

With the aim of estimating the validity of biomarkers for abstinence or relapse monitoring in patients consuming alcohol chronically ($N=18$), they were determined on admission to hospital treatment as well as two weeks after the end of use. Statistical processing of obtained results by the Mann-Whitney test revealed a statistically significant difference ($p<0.05$) only for the parameter CDT, recommending it as a useful biomarker for abstinence and relapse monitoring in chronic alcoholics. Also, it is more important to determine the individual changes of CDT in relation to initial values, than to use cutoff values for estimating the duration of abstinence or relapse (22).

N LATEX CDT is the first direct immunochemical method based on monoclonal antibodies (mAb), that specifically recognize the structure of transferrin glycoforms lacking one or both complete carbohydrate chains. The method was established in June 2005.

Relative values (%CDT) are automatically recalculated in relation to total transferrin. This technique does not require sample pretreatment and is fully automatic in the routine nephelometric procedure.

The determination of CDT by the Latex N CDT method is not influenced by the presence of B or D phenotypes due to the fact that monoclonal antibodies do not react with the genetic variants of transferrin. This is a large advantage for the new automatic CDT test in comparison to many other methods. The second large advantage of the direct CDT test is seen in its optimal diagnostic characteristics in adolescents and adults, as well as in males and females (23, 24).

The introduction of various analytic techniques and methods for CDT determination in the last 15 years, whose values were expressed as absolute (mg/L or U/L) or relative units (%CDT) in relation to total transferrin or tetrasialotransferrin, that have shown different sensitivity and specificity due to the involvement of various transferrin isoforms in »CDT«, in many studies problems emerged regarding the distribution of clinical and analytic results.

With the definition of disialotransferrin as the primary, target molecule for CDT determination and individual analyte for CDT standardization, as well as the recommendation that HPLC be the temporary referent method until mass spectrometry becomes widely available, the first steps were made towards the standardization of CDT determination. In clinical practice, CDT values should be expressed as the relative ratio (%CDT) to compensate for false high and false low values in case of increased or decreased values of total transferrin. These actions would improve the diagnostic characteristics of CDT as a biomarker for chronic alcohol consumption and promote comparability between laboratories. As a result of these measures, the quality of cooperation between laboratory and court medicine would be improved, along with medical diagnostics and patient care (12, 13, 25, 26).

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