SHOULD WE USE CARBOHYDRATE DEFICIENT TRANSFERRIN AS A MARKER FOR ALCOHOL ABUSERS?

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ABSTRACT

Carbohydrate deficient transferrin (CDT) is one of the conventional markers for chronic alcohol consumption, is used by researchers and clinicians. A number of enzymes are affected by ethanol intake. The induction or inhibition of sialyl transferase and plasma sialidase may be involved in the CDT level elevation. An alteration of protein transport during post-translational modification could be a primary mechanism in the impairment of protein metabolism associated with chronic alcohol abuse. Transferrin being a steroid responsive protein, sex-based hormonal variations might contribute to the lower sensitivity of CDT. Varying hormonal statuses such as pregnancy, use of contraceptives, menopause/ menstrual cycle can alter iron homeostasis in women. CDT levels are markedly affected by iron homeostasis. Several CDT assay methods appeared promising, but it is not readily apparent which technique is the most accurate. Moreover, false-positive results of CDT have been reported in non-alcohol related hepatic failure and in rare conditions. Therefore clinical interpretation of CDT needs careful assessment in patients with alcohol-related or non-alcohol-related health disorders.

KEY WORDS

Carbohydrate deficient transferrin (CDT), Transferrin, Alcohol, Marker, Iron homeostasis.

INTRODUCTION

Stibler and Kjellin (1) first reported the presence of transferrin (Tf) isoforms with pls >5.7 in cerebrospinal fluid and serum from alcoholics. Increased amounts of these Tf isoforms appeared with high prevalence in serum from alcoholics and disappeared after abstinence, with a half-life of ~14 days. Carbohydrate deficient transferrin (CDT) is a biochemical marker of chronic alcohol misusers, used by researchers and clinicians. CDT is a collective term referring to isoforms of transferrin, which are deficient in sialic acid residues, corresponding to asialo-Fe₂-Tf, monosialo-Fe₂-Tf, and disialo-Fe₂-Tf (2). The pathomechanisms for the increase in CDT isoforms during chronic alcohol abuse are not completely understood. Experimental findings indicate that the ethanol-induced effect on glycoprotein metabolism is a multistep process in which protein transport and changes of enzyme activity may play an important role (3).

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TRANSFERRIN

Transferrin (Tf) (siderophilin) is a globular protein, which is responsible for iron transport in plasma (4, 5). It has MW 79.5 kDa, and consists of a single polypeptide chain of 679 amino acids arranged in two independent metal ion-binding globular domains, the N-terminal (amino acids 1-336) and the C-terminal (amino acids 337-679), and two N-linked complex glycan chains located in positions 413 and 611 (6). The two Tf N-glycan chains differ in their degree of branching, showing bi-, tri-, and tetra antennary structures. Each antenna of the Tf N-glycan chains terminates with a negatively charged sialic acid molecule. Because of this, asialo-Tf and the sialylated forms monosialo-through octasialo-Tf can occur in serum (7). The relative amounts of these Tf isoforms as a percentage of total serum Tf are <1.5% heptasialo-Tf, 1-3% hexasialo-Tf, 12-18% pentasialo-Tf, 64-80% tetrasialo-Tf, 4.5-9% trisialo-Tf, and <2.5% disialo-Tf in healthy persons (8, 9). Asialo-, monosialo-, and octasialo-Tf are not detectable (8) or represent <0.5% (asialo-Tf) and <0.9% (monosialo-Tf), respectively, of total Tf under nonpathological conditions (9). The pl of the Tf molecule decreases by ~0.1 pH units with each

sialic acid residue bound to the N-glycan chains (7) and the pH range is varying in between 5.5 and 5.9.

The carbohydrate represents about 6% of the transferrin molecule. At least 38 genetic variants of transferrin have been identified, resulting from variation in the polypeptide chain, differences in iron content, and differences in the structure of N-linked glycan chains (10). However, only 4 of these show a prevalence of >1%. The biological significance of the variants is not known except for the rare congenital defect, attransferrinemia. In healthy subjects, the tetrasialo variant is most prevalent. Differing amounts of sialic acid residues influence the electrophoretic mobility of the Tf molecule (11,12) and thus permit analytic separation of the different isoforms.

BIOCHEMISTRY OF TRANSFERRIN

Tf reversibly binds numerous polycations- iron, copper, zinc, cobalt and calcium- although only iron and copper binding appear to have physiological significance. Each transferrin molecule has the capacity to combine with two atoms of ferric ions and an associated anion, which is, in vivo, usually bicarbonate ion (13), this binding is a pH-dependent process (14). The iron-Tf has absorbance maxima at 470nm. The two carbohydrate (Tf N-glycan) structures bind a maximum of six sialic acid residues.

Transferrin is synthesized in the liver and to a small extent in the reticuloendothelial system and in endocrine glands such as testes and ovaries. It has a half life of approx. 7 days. Plasma levels are regulated primarily by availability of iron, because in iron deficiency states plasma levels rise and, on successful treatment with iron, return to normal levels. Much of normal catabolism is through loss of sialic acid and removal of the desialylated protein by the asialoglycoprotein receptors of the hepatic parenchymal cell (15).

TRANSFERRIN METABOLISM

Before the secretion of plasma glycoproteins, these macromolecules undergo a complex posttranslational process. After incorporation of the mannose-rich oligosaccharide core to the polypeptide in the rough endoplasmic reticulum followed by trimming, the final glycosylation occurs in the golgi complex (16). Protein glycosylation is involved in important functions such as intra- and intercellular signaling, compartmental translocation, antigenicity, protein solubility, biological half-life, and enzyme activity (17). This process includes sequential addition of N-acetylglucosamine, galactose, and sialic acid to

the two carbohydrate units of the protein (18, 19) and involves at least five different glycosyltransferase: N-acetylglucosaminyl transferase I, II, and IV, and galactosyl- and sialyltransferase. This process is regulated by specific sialyltransferases. Its carbohydrate units are added posttranslationally from a dolichol-oligosaccharide precursor by the action of a multiglycosyl transferase system (16). Hepatic plasma membrane sialidase removes the carbohydrate moieties from the Tf molecule (20). After glycosylation, transferrin is secreted by exocytosis (21).

CDT AS A MARKER OF ALCOHOL ABUSE

Most patients with liver disease have a normal CDT result (2, 22). Advanced chronic liver disease (Primary biliary cirrhosis, chronic active hepatitis and drug induced hepatic insufficiency) causes false positive results (2). CDT tends to have high sensitivity and specificity in distinguishing chronic, heavy drinking subjects from abstainers or very light social drinkers (22, 23, 24). The accuracy for this marker is generally poorer among drinkers with a low level of alcohol consumption (25), younger alcoholics (26, 27), and women (28, 29, 30). Interestingly, the sexes seem to differ in which isoform types are increased by alcohol. There is no consensus about the pattern of drinking needed to elevate serum CDT values, at least in patients who are alcohol dependent (24). Anton *et al.* (31) reported that in males CDT seems to respond mainly to frequency of drinking, whereas in females CDT is related primarily to drinking intensity. In men, heavy alcohol consumption mainly increases the levels of asialo and disialo Tf. In women, the increase in asialo and monosialo Tf is higher (9). It has been reported that the response of CDT to a certain alcohol intake varies considerably between individual (32). Furthermore, some alcoholic patients even with very heavy daily intake fail to manifest elevated CDT level (22).

Alcohol interferes with a number of glycoconjugation reactions, perhaps as the result of acetaldehyde inhibition of hepatic glycotransferases. The isoforms of transferrin have been studied as markers of alcohol consumption, as excessive ethanol consumption results in the appearance in serum of isoforms that are carbohydrate deficient (33). Many enzymes have been reported to be affected by ethanol intake. Some of them also are implicated directly in the protein glycosylation process. More specifically, the change (induction/inhibition) of two enzymes sialyl transferase and plasma sialidase activities involved in the protein sialylation/desialylation pathway. After chronic alcohol intake, liver membrane sialyl transferase decreases and plasma sialidase increases (20, 34). However, the

enzymatic change activities could, of course, be the consequence of an alteration of the membrane microenvironment that results from ethanol-induced modifications in membrane fluidity. The effect on glycosyltransferase could be mediated at least in part, by acetaldehyde (35). Studies of ethanol's effect on the Golgi system indicate that an alteration of protein transport during posttranslational modifications could be a primary mechanism in the impairment of protein metabolism associated with chronic alcohol abuse. Because of the higher reactivity of acetaldehyde than its precursor, ethanol, one may assume that it plays an important role in the process. Future studies on the effect of ethanol on specific enzymes are needed to further identify different ethanol-sensitive components. Once these components are identified, structural studies on their biological active centers could clarify the role of acetaldehyde in this process (36, 37).

Moreover, the response of CDT in normal subjects to alcohol consumption has been reported to differ from the response of alcohol-dependent individuals (22, 38, 39), which suggests a sensitization of the liver in regular drinkers. This means that subjects can react differently to alcohol, depending on their level of consumption.

CDT, GLUCOSE UPTAKE AND MALNUTRITION

A positive association between serum CDT concentration and the rate of insulin-mediated glucose uptake has been reported (40). High serum CDT levels have been related to increase insulin sensitivity (41). Several studies have shown transferrin to be a sensitive protein-energy balance marker as well as a prognostic indicator (42). Tf has a small extravascular pool, making plasma concentration more sensitive than albumin to decrease in hepatic synthetic rates. The use of Tf as a nutritional marker is complicated. In response to iron deficiency (the most common nutrient deficiency), the liver increases transferrin synthesis and secretion.

CDT AND FEMALE HORMONE STATUS

Tf is a steroid responsive protein (43). Absolute serum CDT concentrations from healthy women typically are higher than those of healthy men, but the exact reason is unclear (28, 44). In fact, disturbances of Tf isoforms have been observed in women as a function of hormonal status. Mean CDT values are significantly higher in the third trimester of pregnancy compared with the first and second trimesters. Similarly, week of pregnancy correlates with total Tf levels (45). Use of contraceptives (46) and hormone replacement

therapy (47) also has been associated with alterations of the normal values of CDT. Interestingly, women who are entering menopause produce significantly lower levels of CDT than do premenopausal women (46, 48). Such sex-based hormonal variations might contribute to the lower sensitivity of CDT as a marker of alcohol abuse among women. Leusink et al (48) reported that the amount and frequency of bleeding during the menstrual period are correlated positively with CDT levels in serum, which suggests that the CDT values are higher in premenopausal women due to an increase in transferrin synthesis that results from increased erythropoiesis.

TRANSFERRIN AND IRON HOMEOSTASIS

Each Tf molecule can bind a maximum of two metal ions, preferably Fe^{3+} . Depending on the Fe^{3+} supply of the organism, Tf molecules are iron free ($\text{Fe}_0\text{-Tf}$ or apo-Tf) or loaded with one ($\text{Fe}_{1\text{N}}\text{-}$ and $\text{Fe}_{1\text{C}}\text{-Tf}$, where N and C indicate the N- and C-terminal regions, respectively) or two ($\text{Fe}_2\text{-Tf}$) Fe^{3+} ions. The isoelectric point (pI) of the Tf molecule decreases by ~0.2 pH units with each Fe^{3+} ion bound (7). In healthy controls, Tf iron saturation is ~30%. Because of the iron transport function of transferrin in the body, any physiological factor that affects iron metabolism also may influence transferrin isoforms. Varying hormonal statuses such as pregnancy, use of contraceptives, and the menopause/ menstrual cycle, can alter iron homeostasis in woman. Experimental data seem to indicate that changes in microheterogeneity of Tf are determined independently of the rate of Tf synthesis and that the changes observed in glycosylation may modulate internal iron fluxes (49).

Cesarini *et al.* (50) reported that 20% to 30% of alcohol abusers have increased liver iron stores. Iron accumulation occurs among alcoholics as a result of increased intestinal mucosal permeability (51). It is well known that alcohol promotes the absorption of ferric iron from the gastrointestinal tract, possibly by inducing gastric secretion of HCl (52). Tf plays an important role in iron homeostasis by attaching to the receptors on the cell surface before iron is released into the cell. After alcohol intake, an increase of iron level, and secondarily, a decrease of transferrin synthesis occur. De Feo *et al.* (53) demonstrated that serum CDT levels are affected markedly by iron status. Subjects with iron overload had reduced CDT values, whereas CDT was increased in subjects who had iron deficiency. Moreover, iron supplementation significantly decreased CDT values in nonabusing patients with iron deficiency anemia. In hemochromatosis (increased serum ferritin levels), Tf iron saturation increases and the isoforms found

in serum are almost exclusively Fe_2 -Tfs. Subjects with hemochromatosis have shown increased CDT values (54). Iron deficiency caused either by a normal response to pregnancy or by chronic inflammation (rheumatoid arthritis) increases the highly sialylated transferrins (49), and higher amounts of Fe_0 - and Fe_1 -Tfs occur in serum (55). Enhanced transferrin production in iron deficiency satisfies the higher iron demands and is linked to higher CDT concentration with its concomitantly greater ability to deliver iron to tissues versus that of normal transferrin (56). It seems that the intensity of iron mobilization from the liver is the factor that most influences biochemical processes which increase CDT in serum (54).

CDT ASSAY METHODS

Several CDT assay methods appeared promising, in particular, liquid chromatography (chromatofocussing, HPLC, fast protein liquid chromatography) and isoelectric focusing, but there were insufficient paired studies from which to draw firm conclusions. The isoelectric point of a transferrin isoform varies according to the number of sialic acid group present. Several methods for separations of isoforms have been developed, based on the difference in charge (34). Attempts at quantification of desialylated transferrin have made use of immunofixation and computerised densitometry after isoelectric focusing. However, this procedure is long, cumbersome, and expensive for small numbers of sample (57), and is therefore unsatisfactory as a routine diagnostic test for chronic alcoholism. Moreover, quantitative evaluation of transferrin fractions is complicated (58).

Tf isoforms are also analysed by capillary electrophoresis (59) and capillary zone electrophoresis (60). The main problem with these techniques is coating of the capillary surface to prevent protein adsorption and finding a coating-compatible, highly ultraviolet-transparent buffer (60). Lectin affinity electrophoresis has been described for assessing Tf microheterogeneity in patients with alcoholic liver disease (61). Whether this method can gain wide acceptance for routine CDT analysis is still unclear. Compared with IEF, chromatographic CDT methods are less sensitive and less selective. Analysis time, column regeneration time and higher cost reduced the applicability of HPLC for large CDT analysis series. Whatever the method used for CDT detection, a huge requisite is its precision and accuracy.

RESULT REPORTING

There is also inconsistency in the method of reporting results: some investigators presenting

aggregate data for all isoforms with a PI of 5.7 or more, or in some cases >5.7 , and others are reporting only the concentration of the major carbohydrate deficient isoform. CDT has most often been reported as the quantity of the transferring isoform at or above pl 5.7 (62). Some researchers have preferred to use the transferrin ratio (the ratio of the isoform at pl 5.7 to total transferrin) or the transferrin index (the ratio of the chief abnormal isoform at pl 5.7, to the major normal isoform at pl 5.4 i.e. disialo- Fe_2 -Tf/tetrasialo- Fe_2 -Tf) as a diagnostic test for chronic alcoholism. It is not clear that the transferrin ratio or index offer any advantage over simple quantification of the carbohydrate-deficient isoforms except perhaps in situations where the total transferrin is likely to be abnormal. However, it is difficult to summarize the benefit CDT offers over the conventional less expensive measurement of γ -glutamyl transferase. Furthermore, it is not readily apparent which technique for the measurement of CDT is the most accurate (63).

Because of different analytical specificities, normal ranges for absolute and relative serum CDT are method-dependent. CDT values must always be interpreted with regard to the test-specific decision criteria. Therefore, the laboratory should report the CDT value, the cutoff value, and the method of analysis. Changing the CDT test can cause a sharp increase or decrease of CDT values, which can lead to a misinterpretation of the actual drinking status. Taking into account the social consequences of high CDT values, it is advisable to use borderline values (95th percentile plus analytical imprecision) instead of cutoffs (64).

PREANALYTICAL VARIATIONS

Several preanalytical conditions have been found to affect serum CDT concentrations. EDTA and heparin may disturb in vitro Fe^{3+} -Tf saturation and/or anion-exchange microcolumn non-CDT and CDT isoform fractionation (65). Sample storage for 3 days at room temperature produced a 25% increase in CDT (55). In addition, lipemia can interfere with turbidimetric CDT assays. Delipidation reduces the serum CDT concentration by ~22% (65). Strong hemolysis can also lead to false-positive results. Preanalytical conditions that do not affect the serum CDT concentration include collection of blood into containers with kaolin (coagulation accelerator) and/or polyacrylamide-gel separator (65); serum storage for ~30 h at room temperature, 7 days at 4°C , and several months at -22°C ; repeated freezing and thawing, diet, and common drugs taken by patients (55).

FALSE CDT RESULTS

In humans there are three genetic variants of transferrin- types B, C, and D- of which C is the most common. False positive results of CDT have been reported with rare conditions, such as genetic D-variants of transferrin (30) or an inborn error of glycoprotein metabolism (CDG syndrome) (30, 66).

One reason for high CDT concentrations despite normal alcohol consumption is the carbohydrate-deficient glycoprotein (CDG) syndrome. CDG syndromes are a group of autosomal recessive diseases, the defining feature of which is a carbohydrate defect in serum Tf. In affected subjects, one half of the Tf molecules lack two or all four of their terminal trisaccharides (N-acetylglucosamine, galactose, and sialic acid) (67). Studies of a number of enzymes involved in the protein glycosylation pathway have not revealed any significant difference in CDG as compared with healthy subjects. However, a metabolic error may occur in the early steps of protein glycosylation (68). On the other hand, the increase of CDT among subjects affected by carbohydrate-deficient glycoprotein syndrome could be related to an unidentified abnormality in the synthesis, processing, or transport of glycoproteins (69). Yamashita *et al.* (70) speculated that carbohydrate-deficient glycoprotein syndrome type I could be associated with an asparagine-N-linked oligosaccharide transfer deficiency. Though, the enzyme involved in the process has not been characterized, in some types of the CDG syndrome are attributable to deficiencies of phosphomannomutase or phosphomannose isomerase, it might be interesting to measure the corresponding enzyme activities in patients with chronic alcohol abuse.

Tf-B ("busy") and Tf-D variants can interfere with CDT analysis. The D-variant of Tf has been related to variations in the molecule structure as a consequence of amino acid substitution (11), which would induce differences in the pI of the protein. The genetic D-variant of Tf has a higher isoelectric point (pI) than Tf C, and the phenotype is very rare in the white population, accounting for less than 1% of the false positive results. However, the phenotype is more frequent among Asian, black, and South Americans populations (71). Eight Tf D variants have been reported to date. It has been suggested that the D-variant of Tf lack an oligosaccharide chain because of an inhibition of glycosylation caused by amino acid substitution at one of the carbohydrate-attachment site (11). False-negative results also have been found in cases of a very rare Tf B-variant genetic condition (2) and this genetic variant of Tf is characterized by an abnormally low pI as compared with Tf C.

Although CDT is usually unaffected by the presence of liver disease, false- positive results have been reported in primary biliary cirrhosis and in patients with severe non-alcohol-related hepatic failure (72, 73, 74). Complementary methodologies have been employed to reduce the number of CDT false-positives. For example, patients with primary biliary cirrhosis (PBC) have high levels of circulating antibodies against mitochondrial components. Those with suspected PBC who are positive for CDT thus can be evaluated further by using mitochondrial autoantibodies to pyruvate dehydrogenase (75).

DIAGNOSTIC EFFICIENCY OF CDT

For calculating parameters of diagnostic efficiency, reliable data regarding individual alcohol consumption is a prerequisite. Thus, for classifying false positives and true negatives, an objective "gold standard" is needed that determines the individual alcohol consumption without any error. Clinical background or personal reviews are customarily used for this purpose. However, if both were faultless, we would not need laboratory markers of chronic alcohol abuse. Because underestimating alcohol consumption is common, false true positives or false negatives will occur and markedly affect the criteria of diagnostic efficiency of CDT obtained in a clinical study (55).

CONCLUSION

Certain types of medical illness and conditions may affect levels of CDT. Because CDT is synthesized, glycosylated, and secreted by the liver, the use of CDT values in patients with liver disease has been an area of focussed interest. However, an elevated CDT value may not accurately represent alcohol consumption in patients with advanced liver disease. Thus the interpretation of CDT results may need to be carefully examined.

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