Glycoconjugates in the detection of alcohol abuse

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Abstract

Up to 30% of all hospital admissions and health-care costs may be attributable to alcohol abuse. Ethanol, its oxidative metabolites, acetaldehyde and ROS (reactive oxygen species), non-oxidative metabolites of alcohol [e.g. FAEEs (fatty acid ethyl esters)] and the ethanol-water competition mechanism are all involved in the deregulation of glycoconjugate (glycoprotein, glycolipid and proteoglycan) metabolic processes including biosynthesis, modification, transport, secretion, elimination and catabolism. An increasing number of new alcohol biomarkers that are the result of alcohol-induced glycoconjugate metabolic errors have appeared in the literature. Glycoconjugate-related alcohol markers are involved in, or are a product of, altered glycoconjugate metabolism, e.g. CDT (carbohydrate-deficient transferrin), SA (sialic acid), plasma SIJ (SA index of apolipoprotein J), CETP (cholesteryl ester transfer protein), β-HEX (β-hexosaminidase), dolichol, EtG (ethyl glucuronide) etc. Laboratory tests based on changes in glycoconjugate metabolism are useful in settings where the co-operativeness of the patient is impaired (e.g. driving while intoxicated) or when a history of alcohol use is not available (e.g. after trauma). In clinical practice, glycoconjugate markers of alcohol use/abuse let us distinguish alcoholic from non-alcoholic tissue damage, having important implications for the treatment and management of diseases.

Introduction

There are approx. 2 billion people worldwide who consume alcoholic beverages and 76.3 million with diagnosable alcohol use disorders [1]. It is estimated that 3.8% of all global deaths and 4.6% of global burden of disease and injury can be attributed to the use of alcohol, e.g. alcohol-use psychiatric disorders, cancer, cardiovascular disease and liver cirrhosis [2]. Up to 30% of all hospital admissions and health-care costs may be attributable to alcohol abuse [3]. Even at the stage of alcohol-dependence, physicians are likely to identify only 20–50% of patients who are attending for medical care [4]. The main patterns of alcohol drinking include: social drinking, hazardous drinking, binge drinking, heavy drinking and alcohol-dependence (Table 1).

Alcohol abuse behaviours may be easily missed in clinical settings because of patients’ memory errors, under-reporting and stress at the time of the interview. Patients are often unlikely/unable to attend interview [5]. Therefore questionnaires such as CAGE (named after the key questions asked in the questionnaires), MAST (Michigan Alcohol Screening Test) or AUDIT (Alcohol Use Disorders Identification Test) that depend on self-reporting are often avoided in routine use. Biochemical markers provide objective information to screen patients for a possible problem with alcohol, e.g. in primary care settings, emergency rooms, or in psychiatric or gynaecological clinics. Biomarkers are particularly useful in settings where the co-operativeness of patients is impaired (e.g. driving while intoxicated) or when a history of alcohol use is not available (e.g. following trauma). Biochemical markers are also useful in differential diagnosis, to help motivate a change to positive drinking behaviour and to help the clinician in the early recognition of drinking relapse [3].

Glycoproteins, glycolipids and proteoglycans, are referred to as glycoconjugates or complex carbohydrates. Ethanol, its metabolites, e.g. acetaldehyde, ROS (reactive oxygen species) and FAEEs (fatty acid ethyl esters), and the ethanol–water competition mechanism, are all involved in the deregulation of glycoconjugate metabolic processes including biosynthesis, modification, transport, secretion, elimination and catabolism [6]. As the bulk of ingested ethanol and most serum glycoproteins are metabolized in the liver, both metabolic processes interfere with each other, resulting in glycoconjugate metabolic errors. The most obvious and specific test for alcohol abuse is the measurement of blood, breath, urine, saliva or sweat alcohol [3]. However, this simple test cannot distinguish between acute and chronic alcohol consumption. Moreover, due to the short elimination time of ethanol and the fact that alcohol drinking does not necessarily signify alcohol abuse, its value as a marker of alcohol abuse is limited [7]. Therefore an increasing number of glycoconjugate biomarkers of alcohol abuse with longer half-lives than ethanol appear in the literature.
Glycoconjugate biomarkers

CDT (carbohydrate-deficient transferrin) is a glycoprotein that has been widely investigated as the most specific of the currently available methods of alcohol abuse detection. CDT is a mixture of the asialo, monosialo or disialo isoforms of transferrin, the N-glycosylated protein [8,9]. It appears in serum after regular high alcohol intake of more than 50–80 g of alcohol per day for at least 1 week [10]. The sensitivity and the specificity in alcohol-dependent persons are 82% and 97% respectively [8]. Transferrin exists in normal serum in forms containing 0–9 sialic acid residues, predominantly tetrasialotransferrin (80%). Alcohol-induced dysfunction of the liver asialoglycoprotein receptor might be involved in increased CDT levels. The inhibition of sialyltransferase and the induction of plasma sialidase (two enzymes involved in the sialylation and desialylation of proteins respectively) have also been considered to increase serum CDT [11,12]. Destabilization of ST (sialyltransferase) mRNA (by accelerating its degradation) and decrease in the hepatic synthetic rate of ST results in a concomitant decrease in the steady-state level of ST mRNA [13]. It is highly likely that increased presence of asialoconjugates and free SA (sialic acid) after alcohol exposure are promoted by increased transcription rates of cytosolic and plasma membrane sialidase mRNA [14]. During abstinence, CDT normalizes with a half-life of 15 days, remaining elevated for several weeks. When drinking resumes, even low levels of ethanol can lead to a rapid re-elevation [4]. CDT is the only test approved by the Food and Drug Administration for the identification of heavy alcohol use [15]. Low-sensitivity CDT is apparent in women, and occasional and young drinkers, as well as the general population. False-positive results can occur with non-alcoholic liver diseases, carbohydrate-deficient glycoprotein syndrome, pregnancy, cystic fibrosis, and iron deficiency [10]. The expression of CDT levels as a percentage of total transferrin (%CDT) helps in the avoidance of falsely high or low levels of CDT in these situations.

Serum GGT (γ-glutamyl transferase) is a membrane-bound glycoprotein enzyme of hepatocytes and biliary epithelial cells which catalyses the transfer of the γ-glutamyl moiety of glutathione to various peptide acceptors [9]. In alcohol-dependent persons, a significant rise in serum GGT occurs after consumption of more than 40 g of alcohol per day, whereas in previous non-drinkers at least 60 g per day for a minimum of 5 weeks is required for this increase. Therefore GGT is an indicator of chronic heavy drinking, but not binge drinking. However, moderate (social) drinkers show significantly higher levels of GGT than abstainers [9,16]. An increased GGT activity in serum, after alcohol intoxication, may be due to its increased synthesis, release (result of ischaemia) or subsequent shedding of membrane-bound enzyme [17]. However, GGT is released, similarly to widely used markers of chronic alcohol abuse [AST (aspartate aminotransferase) and ALT (alanine aminotransferase)], in cases of inflammation, liver cell damage/necrosis and oxidative stress [18–20]. The half-life of GGT is 14–26 days with a return to the normal value in 4th–5th week of abstinence [20]. Although GGT is relatively sensitive (60–90%), its specificity is decreased by obesity, diabetes, non-alcoholic liver disease, pancreatitis, hyperlipidaemia, cardiac insufficiency, severe trauma, medications and nephrotic syndrome [9]. To increase GGT sensitivity without a loss of specificity, the combined use of GGT and CDT (abbreviated as γ-CDT) has been proposed [21,22].

Plasma SIJ [SA index of apoJ (apolipoprotein J)] is decreased by 50–57% in alcoholics (specificity ~100%) [23]. Plasma SIJ correlates with alcohol consumption and relapse in alcohol-dependent subjects (~90% sensitivity). By increasing the activity of sialidase and decreasing sialyltransferase, long-term ethanol intoxication decreases sialylation of plasma apoJ. ApoJ (or clusterin) is a highly sialylated glycoprotein that contains 28 mol of SA per mol of apoJ; this is highly significant when compared with the six sialic acid residues on transferrin. ApoJ is found in high-density lipoprotein complexes and is thought to be involved in the exchange of lipids, especially cholesterol, between lipoproteins [24]. The term SIJ expresses the ratio of mol of SA per mol of apoJ. Levels of SIJ return to normal over a period of weeks (4–5 week half-life) [15].

### Table 1 | The main patterns of alcohol drinking

<table>
<thead>
<tr>
<th>Pattern</th>
<th>Number of units/problems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Social drinkers</td>
<td>Usually drink not more than 2–3 units of alcohol per day.</td>
</tr>
<tr>
<td>Hazardous drinkers</td>
<td>Drink more than 1–2 units of alcohol/day (women) and more than 3–4 units/day (men). They are at risk of harm</td>
</tr>
<tr>
<td>Binge drinkers</td>
<td>Occasionally drink more than 5 units of alcohol/day. The pattern is also called single occasion drinking</td>
</tr>
<tr>
<td>Heavy drinkers</td>
<td>Drink regularly more than 6 units of alcohol/day. If immediate harm appears, they are called problem drinkers (also called harmful drinking or alcohol abuse)</td>
</tr>
<tr>
<td>Alcohol-dependent persons</td>
<td>People that chronically drink alcohol. The most severe stage of drinking, with physical and psychological dependence. At least three of the following criteria are met: tolerance, withdrawal symptoms after cessation of drinking, impaired control, preoccupation with acquisition and/or use, persistent desire or unsuccessful efforts to quit, sustains social, occupational or recreational disability, and use continues despite adverse consequences.</td>
</tr>
</tbody>
</table>

1 unit of alcohol contains 10 g of absolute ethanol [3].
IgA is a heavily glycosylated molecule [25]. The reactivity of IgA with acetaldehyde has been shown to be elevated in heavy drinkers and alcohol-dependent persons, but not in social drinkers or patients with non-alcoholic liver disease [26]. An increased ratio of IgA/IgG is highly indicative of alcoholic liver disease [9]. The sensitivity and specificity of circulating anti-IgA IgAs are 65–73% and 88–94% respectively [27]. Owing to a short half-life, free acetaldehyde has limited biomarker potential [28]. However, acetaldehyde readily forms Schiff bases with amines and forms irreversible acetaldehyde–protein adducts. Adducts (neo-antigens) can induce auto-antibodies toward themselves [10]. Proteins with detectable acetaldehyde adducts include haemoglobin, serum proteins, albumin, CYP450 2E1 or red blood cell membrane proteins; the half-life of these protein adducts depends on the carrier protein [28]. A single high-dose of alcohol (2 g/kg) increases blood haemoglobin-associated acetaldehyde and the level of salivary IgA when the conventional markers MCV (mean corpuscular volume) or GGT show no change [29–31]. As saliva is an easily and non-invasively obtained material, salivary IgAs seem to show promise in binge drinking detection [32,33].

CETP (cholesteryl ester transfer protein) is a plasma glycoprotein synthesized by the liver which transfers cholesteryl esters, triglycerides and phospholipids between lipoproteins [17]. In the circulation it is bound mainly to HDL (high-density lipoprotein) particles. Alcohol consumption reduces the concentration and activity of CETP and reverses the direction of transfer of cholesteryl esters and triglycerides, thus increasing the plasma HDL cholesterol concentration (common laboratory abnormality in alcohol-dependent persons) [17]. The sensitivity and specificity of plasma CETP is comparable with conventional alcohol drinking markers, MCV, GGT, AST and ALT [34]. Similarly to CDT and SIJ, CETP purified from alcohol abusers shows a glycosylation defect that may be due to the decreased activity of sialyltransferase and increased sialidase activity [35]. Clinical applicability of CETP as a marker of alcohol misuse is, however, limited due to several factors affecting plasma CETP levels (e.g. various diseases, differences in diet, drugs) or to complicated laborious chromatographic methods [17].

**Biomarkers involved in (or products of) glycoconjugate metabolism**

Dolichol is a long-chain polyprenol which acts as a glycosyl carrier in the biosynthesis of N-linked glycoproteins [36]. Dolichol is very sensitive to attack by free radicals, thus its function is easily influenced by excessive amounts of ROS generated during drinking [37–39]. Ethanol and dolichol are oxidized by alcohol dehydrogenase. Owing to this competition, elevated dolichol levels in the blood and urine have been suggested as markers of alcohol-dependence [36,40]. Moderate alcohol consumption (60 g/day) did not affect urinary dolichol levels, whereas chronic drinking in alcohol-dependent persons did [28]. The half-life of urinary dolichol is approx. 3 days, in serum over 7 days [17]. Despite the high specificity of urinary dolichol detection using HPLC (96%), the sensitivity is moderate (68%) or even low (9–19%), which limits the usefulness of urinary dolichol in alcohol-dependence detection [17].

β-HEX (N-acetyl-β-hexosaminidase, β-hexosaminidase) is a lysosomal exoglycosidase that releases N-acetylhexosamines from the non-reducing end of oligosaccharide chains of glycoconjugates [41]. β-HEX, particularly the β-HEX B isoenzyme in serum and total β-HEX in urine, is a very sensitive marker of prolonged alcohol abuse [40]. The increased activity of serum and urinary β-HEX has been reported in alcohol-dependent patients and in healthy volunteers after drinking more than 60 g of alcohol daily, for at least 10 successive days, with sensitivities of 70–90% [10]. Increased β-HEX activities have also been reported after a single binge drinking session [41]. Various mechanisms have been proposed to increase the activity of HEX in body fluids after alcohol drinking, e.g. change of lysosomal membrane permeability and leakage of the enzyme from lysosomes and subsequently from cells into body fluids, delayed removal of enzyme by impaired liver, enhanced synthesis by activated reticuloendothelial cells and leakage from the degenerating cells of various body organs [38]. Since deglycosylation of glycoconjugates may be a critical initial step leading to their subsequent proteolysis [6], we can speculate that high activity of β-HEX in heavy drinkers/alcohol-dependent persons may affect levels/activity of other glycoconjugate markers of alcohol abuse. Although high specificities have been reported for β-HEX (~90%), increased serum levels of β-HEX have been noted in hypertension, diabetes mellitus, cirrhosis, pregnancy, after oral contraceptive pills, or in cerebral and myocardial infarction [10,40]. In alcohol-dependent persons, β-HEX levels fall rapidly to normal following abstinence (7–10 days). β-HEX can be measured using standard and inexpensive laboratory techniques (spectrophotometry and fluorimetry).

SAs are mostly bound to the carbohydrate chains of glycoproteins and glycolipids in serum [17]. The concentrations of TSAs (total SAs) are elevated in the serum, saliva and urine of heavy drinkers. The rise in serum SA may be due to de-sialylation of glycoproteins, e.g. transferrin, apoA (apolipoprotein A) or CETP, by the increased activity of sialidase. The diagnostic value of SA as an alcohol abuse biomarker, by using a colorimetric assay, showed 48–58% sensitivity and 64–96% specificity of TSA [42,43]. Various diseases and states, such as cancer, diabetes, renal diseases, cardiovascular disease or pregnancy, may increase SA residues in serum, thus decreasing its specificity [28]. Despite the lack of specificity, SA determination helps differentiate between alcoholic and non-alcoholic liver disease [9].

EtG (ethyl glucuronide) is a direct metabolite of ethanol, formed in the liver by the conjugation of ethanol with activated glucuronic acid [17]. It is detectable in blood for up to 8 h and in urine for 3–5 days, after heavy alcohol consumption. After moderate drinking, urinary EtG is a
superior marker of recent ethanol consumption in healthy subjects [15]. It is also detectable in hair and tissues. EtG testing was performed clinically during monitoring programmes in the U.S.A.; however, the chromatographic methods for measuring EtG limit its use in routine clinical laboratories [17,40].

**Other markers**

An elevated MCV has been reported in 4% of adults, and 65% of these are likely to be alcohol-related [9]. Usually at least 1 month of drinking more than 60 g of alcohol daily increases the MCV above the reference range. However, chronic moderate levels of drinking (less than 40 g/day) show values that at population level increase MCV by 1–2 fl. The sensitivity and the specificity are 40–50% and 80–90% respectively [9,10]. MCV is a more sensitive marker in women, and specificity decreases in vitamin B12 or folic acid deficiency, liver diseases, haematological disorders, hypothyroidism, reticulocytosis and smoking [9].

Approx. 2–4 months of abstinence are required for MCV normalization. Although various mechanisms underlying alcohol-induced MCV elevation were proposed (e.g. direct haematotoxic role of ethanol and its metabolites, folate acid deficiency or immunological effect), the damage to the structure of glycoconjugates in red blood cell membranes is very likely to decrease stability and increase membrane permeability and erythrocyte volume [9]. Acetaldehyde and ROS, generated during alcohol intoxication, may modify glycosylated proteins and lipids in the cellular membranes, increasing membrane fragility. In addition, the ATP required for membrane stabilization is markedly limited due to an elevated NADH/NAD ratio [44]. When incorporated into membranes, FAEEs were shown to cause an increase in membrane fluidity. Thus disorder in the membrane bilayer and fragility developed. Ethanol and water compete with each other on target membrane molecules. Glycoproteins attract a large volume of water (up to 95%). Thus displacement of water by ethanol from hydrogen-bonded sites creates the opportunity for allosteric changes that lead to conformational changes of membrane glycoconjugates [45]. All of the aforementioned mechanisms may be involved not only in destabilization of erythrocyte membranes, but also in destabilization of liver cell membranes, releasing other biomarkers of alcohol abuse, e.g. AST, ALT, GGT or β-HEX.

Other glycosylated proteins have also been suggested as promising markers for alcohol detection. Cytokines are signalling glycoproteins whose levels may be altered by alcohol [46,47]. Of these, IL (interleukin)-1α, IL-1β, IL-6, IL-8 and IL-12 may further contribute to the diagnostic biomarker panel [47]. Using proteomic technologies, elevated levels of IgA, α1-antichymotrypsin, haptoglobins, apoprotein A-I, or decreased apoprotein A-II and fibrinogen αE chain, were identified [48]. The increase in glutamate dehydrogenase and urinary alanine aminopeptidase, the decrease in erythrocyte aldehyde dehydrogenase, or increase in serum hyaluronate, were also suggested as a glycoconjugate-related markers of alcohol-dependence and alcohol-induced tissue damage [15,40,49]. However, all the aforementioned markers need further confirmatory research.

There are also genetic markers of glycoprotein production that help identify individuals protected against alcohol-dependence (e.g. alcohol dehydrogenase (ADH3*1, ADH2*2) and aldehyde dehydrogenase (ALDH2*2) genotypes) or individuals at risk of dependence (e.g. with lower platelet adenylyl cyclase and monoamine oxidase activity and the A1 allele of the D2 dopamine receptor) [40].

**Concluding remarks**

The biological roles of the oligosaccharide units of glycoconjugate biomarkers include: maintaining their conformation and stability, modulation of functions, and the control of their half-life [50]. As the bulk of ingested ethanol and serum glycoconjugates, particularly glycoproteins, are metabolized in the liver, both metabolic processes interfere with each other, resulting in glycoconjugate metabolic errors. Ethanol and its metabolites such as acetaldehyde, ROS, FAEEs and the ethanol–water competition mechanism, are all involved in deregulation of glycoconjugate metabolism. These metabolic errors result in the formation of novel glycoconjugate markers of alcohol abuse.

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