

Biomarkers of alcohol misuse: recent advances and future prospects

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Abstract

Alcohol abuse and dependence are highly prevalent in many cultures and contribute considerably to the global burden of health and social issues. The current inability to accurately characterise long-term drinking behaviours is a major obstacle to alcoholism diagnosis and treatment. Therefore, it is of great importance to develop objective diagnostic tools to discern subjects with excessive alcohol use and alcoholism or to confirm abstinence. Research over past years has revealed several biochemical compounds with considerable potential for accurate reflection of alcohol intake. This review will address the issue of alcohol biomarker definition, the types of molecules used as so-called traditional biomarkers, and the compounds that can serve as novel biomarker candidates or components of biomarker panels.

Introduction

The use of alcoholic beverages is probably the most ancient social habit worldwide, it is highly prevalent in many cultures and contributes considerably to the global burden of health and social issues. Chronic and acute alcohol intoxication has been linked to a multitude of diseases, including cancers, cardiovascular diseases, liver cirrhosis, neuropsychiatric disorders, injuries, and foetal alcohol syndrome, as well as social problems, such as suicides, homicides, road and industrial accidents, and many criminal offences [1, 2].

The inability to properly assess alcohol drinking behaviours (see Table I for the main patterns of alcohol misuse) presents a significant barrier to the diagnosis and treatment of hazardous alcohol use. It is important to emphasise that there are no pathognomonic clinical signs or symptoms of alcoholism. Medical complaints and clinical presentation of individuals consuming excessive amounts of alcohol depend on when in the course of the condition they seek medical attention. One approach for detecting hazardous drinking is to ask a simple screening question, followed by a more focused self-report questionnaire if required. There are

a number of relatively easy questionnaires available for quantifying alcohol intake, including CAGE (named after the key questions asked in the questionnaires), the Michigan Alcoholism Screening Test (MAST), the Alcohol Use Disorders Identification Test (AUDIT), and its shortened version (AUDIT-C) [3–5]. Although meticulous efforts have been made to construct these interview formats there are serious limitations associated with these approaches. A number of patients fail to admit to their true alcohol consumption, particularly when they are forced to deny or minimise the magnitude of drinking behaviour in order to mitigate personal, professional, or legal ramifications of alcohol abuse, or in the context of altered mental states or mental illnesses [5]. The issue coupled with physician discomfort, inadequate training, or judgmental attitudes makes the diagnostic process even more complex.

Therefore, it is of great importance to have objective diagnostic tools to discern subjects with excessive alcohol use and alcoholism or to confirm abstinence. This review will address the issue of alcohol biomarker definition, the types of molecules used as so-called traditional biomarkers, and the compounds that can

Table I. The main patterns of alcohol misuse [8, 9]

Pattern of alcohol misuse	Level of alcohol consumption/ number of units*	Risk/problems
Social drinking	Usually drinking not more than 2–3 units of alcohol/day	The term refers to substance use that poses low risk of future harmful consequences for the user
Hazardous drinking	Women who drink more than 1–2 units of alcohol/day and men who drink more than 3–4 units/day	The term refers to substance use that increases the risk of harmful consequences for the user. The pattern is of public health significance despite the absence of any current disorder in the individual user
Binge drinking (also called single occasion drinking)	Occasionally (i.e. in one drinking session) drinking more than 5 units of alcohol/day	The term refers to an episode of heavy drinking over a short period of time (e.g. over the course of an evening or over an hour or two), or drinking to intoxication or to drunkenness
Heavy drinking (also called harmful drinking or alcohol abuse)	Drinking regularly more than 6 units of alcohol/day	The term refers to drinkers already experiencing or showing evidence of health harms (physical or mental), but not showing evidence of alcohol dependence. If immediate harm appears, they are called problem drinkers
Dependent drinking	Drinking alcohol chronically	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

*1 unit of alcohol = 10 ml of absolute ethanol.

serve as novel biomarker candidates or components of biomarker panels.

Biomarkers of alcohol consumption

In accordance with the definition established by the US National Institute of Health, a biomarker is “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” [6]. Thus, in the context of alcohol abuse, a biomarker should be an accurate indicator of an individual’s alcohol drinking pattern, or any genetic predisposition toward alcohol abuse and alcoholism. These two kinds of alcohol biomarkers are described as state markers (i.e. biochemical measures that allow evaluation of a patient’s history of alcohol consumption) and trait markers (i.e. biochemical tools that reveal a person’s inherited risk of developing alcoholism due to chronic exposure), respectively [7]. Importantly, the potential utility of a biomarker is strictly defined by its diagnostic power. To qualify an alcohol-related biomarker as useful; it should meet several criteria, of which sensitivity (the diagnostic method has to be accurate for most if not all drinking subjects) and specificity (the diagnostic method has to be linked to alcohol consumption but not to other conditions or problems) are of utmost importance. From practical point of view, the test used to measure the biomarker should also be precise and accurate [6, 7].

State markers of excessive alcohol intake can be grouped into two types due to pathophysiological matters: indicators of alcohol consumption (acute as well as chronic) and indicators of alcohol induced organ damage. Biochemical measures traditionally linked with hazardous drinking are γ -glutamyl transferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), mean cell volume (MCV), and carbohydrate-deficient transferrin (CDT) [7–9]. However, in a broad sense, their utility as diagnostic tools for alcohol abuse is greatly hampered due to variable results in different populations and low sensitivity and specificity (Tables II and III). Nevertheless, it is worth emphasising that CDT is the first test approved by the U.S. Food and Drug Administration for the monitoring of alcohol consumption over an extended period of time [8, 9].

Novel state markers

Research in recent years has revealed several biochemical markers with considerable potential for more accurate reflection of excessive alcohol intake than traditional markers. These markers may be related to compounds produced when the body metabolises alcohol (so-called direct markers) or may reflect changes in other molecules, cells, or tissues that result from chronic or acute alcohol exposure (so-called indirect markers). Most of them have been developed in a research context and are still awaiting validation and possible introduction into commercial settings [7–10].

Table II. Summary characteristics of traditional alcohol biomarkers [7, 8, 66, 67]

Parameter	Biomarker			
	γ -Glutamyl transferase (GGT)	Aspartate amino-transferase (AST)	Alanine amino-transferase (ALT)	Mean corpuscular volume (MCV)
Type of drinking characterised	Probably at least 5 drinks/day for several weeks	Unknown, but heavy and lasting for several weeks	Unknown, but heavy and lasting for several weeks	Probably at least 5 drinks/day for c.a. 2 weeks
Dose-response of alcohol	80–200 g/day	≥ 40 g/day	≥ 40 g/day	> 50 g/day
Time to elevation	24 h – 2 weeks	3–7 days	3–7 days	1–2 weeks
Time to descent to normal levels	2–6 weeks of abstinence ($T_{1/2} = 14$ –26 days)	2–4 weeks of abstinence ($T_{1/2} = 12$ –24 h)	2–4 weeks of abstinence ($T_{1/2} = 37$ –57 h)	2–3 weeks of abstinence ($T_{1/2} = 15$ days)
Sensitivity for detecting excessive alcohol consumption	37–95%	25–60%	15–40%	55–90%
Specificity	18–93%	47–68%	50–57%	80–90%
Relapse sensitivity	50%	Not reported	Not reported	55–76%
Current clinical use	Identifying chronic alcohol abuse. Screening for heavy drinking. Useful for monitoring abstinence in treatment programs	Identifying chronic alcohol abuse. Screening for heavy drinking	Identifying chronic alcohol abuse. Screening for heavy drinking	Screening for heavy drinking
Strengths in clinical use	High specificity in patients with suspected alcohol abuse. Elevation precedes alcohol-induced liver damage. Effective marker for patients suspected of binge drinking. Inexpensive	Highly sensitive and specific for alcohol-induced liver damage	Highly sensitive and specific for alcohol-induced liver damage	Accuracy similar in male and female subjects. Indicates chronicity of drinking. Routine laboratory test
Limitations in clinical use	Many factors cause false positives. Poor screening tool in general population (due to low sensitivity). Poor marker of relapse	Enzyme elevation can be detected only after periods of heavy drinking. Elevation secondary to liver damage at hepatocellular level	ALT seems to be less sensitive than AST. Enzyme elevation can be detected only after periods of heavy drinking. Elevation secondary to liver damage at hepatocellular level	Many factors cause false positives. Poor screening tool for alcohol abuse (due to low sensitivity). Poor marker of relapse
				Low sensitivity; more valuable to confirm than to exclude heavy drinking. Poor screening tool for alcohol use in general population. Cost and low availability of testing

Table III. Factors affecting the serum level of traditional alcohol biomarkers [8, 68]

Parameter	Biomarker			
	γ -Glutamyl transiferase (GGT)	Aspartate amino-transferase (AST)	Alanine amino-transferase (ALT)	Mean corpuscular volume (MCV)
Age	Less likely \uparrow in subjects < 30 years of age and possibly in subjects > 60 years of age	Less likely \uparrow in subjects < 30 years of age and possibly in subjects > 70 years of age	Less likely \uparrow in subjects < 30 years of age and possibly in the elderly	Less likely \uparrow in subjects < 30 years of age
Gender	More likely \uparrow in men	Not reported	Not reported	More likely \uparrow in women
Medical conditions	(\uparrow): Liver and biliary disorders, obesity, hypertriglyceridaemia, diabetes, pancreatitis, hyperthyroidism, hypertension, heart diseases, kidney diseases, venous embolism and thrombosis, serious injuries	(\uparrow): Obesity, liver and biliary disorders, muscle diseases, extreme exertion	(\uparrow): Obesity, liver and biliary disorders	(\uparrow): Folate or vitamin B ₁₂ deficiency, bleeding, haematological conditions (including haemolysis, haemoglobinopathies, marked increases in white cell count, bone marrow diseases), non-alcoholic liver disease, hypothyroidism, hyperglycaemia
Medications	(\uparrow): Liver microsomal enzymes inducers, e.g. anticolvulsants, anticoagulants, non-steroidal anti-inflammatory drugs, barbiturates, benzodiazepines, tricyclic antidepressants	(\uparrow): Almost any medication, e.g. anticolvulsants, non-steroidal anti-inflammatory drugs, antibiotics, statins, herbal preparations	(\uparrow): Almost any medication, e.g. anticolvulsants, non-steroidal anti-inflammatory drugs, antibiotics, statins, herbal preparations	(\uparrow): Chemotherapy, antivirals, anticolvulsants, oral contraceptives, trimethoprim, colchicine, neomycin, nitrous oxide
Other	Smoking (\uparrow), fruit intake (\downarrow), carbohydrate (\downarrow), coffee (\downarrow)	Coffee (\downarrow)	Coffee (\downarrow)	Smoking (\uparrow), coffee (\downarrow)
				Not reported
				(\uparrow): Iron deficiency, hormonal status in women (including pregnancy), cystic fibrosis, carbohydrate-deficient glycoprotein syndrome, galactosaemia, non-alcoholic liver disease (e.g. hepatitis C, hepatocellular carcinoma, primary biliary cirrhosis), liver and pancreas transplantation, dementia, depression, solvent intoxication
				(\uparrow): Probably anticolvulsants (\downarrow): Probably angiotensin II receptor blockers (sartans)

Ethyl glucuronide, ethyl sulphate

Ethyl glucuronide (EtG) and ethyl sulphate (EtS) are direct conjugated metabolites of ethanol formed in low amounts primarily in the endoplasmic reticulum of liver, and the reactions are catalysed by uridine diphosphate-glucuronosyltransferase and sulphotransferase, respectively [7, 9, 11]. Compared with ethanol testing, EtG and EtS are superior markers of recent alcohol intake due to a longer detection window. Namely, EtG is present in blood for up to 36 h (c.a. 8 h following complete elimination of ethanol) and in urine for 3–5 days after heavy alcohol consumption [7, 9], whereas EtS is detectable in urine c.a. 16–27 h longer than ethanol [12]. When people test positive for EtG, it is likely that they have consumed alcohol recently, even if there is no ethanol left in their bodies. This makes EtG especially useful for detecting drinking relapses, particularly in alcoholism treatment programs. In addition to blood and urine, EtG can be also detected in other body fluids, hair, and body tissues [7, 9, 13]. A few studies have demonstrated that EtG measurements in hair have a relatively high specificity and sensitivity in the detection of alcohol abuse: 80–95% and 70–90%, respectively [14, 15].

Importantly, EtG/EtS results should be interpreted in the context of all available clinical and behavioural information. It has been reported that incidental exposure to alcohol in daily use products (i.e. hand sanitisers, mouthwash) may result in detectable levels of EtG and/or EtS. In addition, upper respiratory infections as well as β -glucuronidase hydrolysis may lower levels of EtG, but do not seem to affect EtS [16, 17]. Furthermore, EtG in hair is vulnerable to cosmetic treatments [18]. Another weakness of EtG as a biomarker of alcohol misuse is a rather sophisticated method required for an accurate reading of EtG from urine, and so far, attempts to produce a measure for urine-based EtG using simpler techniques or to measure EtG in other body fluids or hair have yielded less than satisfactory results [7, 9, 11].

Acetaldehyde, acetaldehyde adducts, and anti-adduct antibodies

Acetaldehyde is a first product of oxidative metabolism of ethanol. The circulating compound exists on its own, but has also been demonstrated to react with various proteins (including haemoglobin, albumin, and other serum proteins, CYP450 2E1, or red blood cell membrane proteins), resulting in acetaldehyde-protein adduct formation [7, 10, 19]. Following alcohol intake, concentration of free acetaldehyde is highly variable with a life-time of c.a. 3 h, but some acetaldehyde-protein adducts may be detected up to 3 weeks after alcohol consumption, and haemoglobin-bound acetaldehyde (HAA) accumulates in red blood cells over their

120-day average life span [7, 10, 20]. A single high-dose of alcohol (2 g/kg) increases blood HAA when the conventional markers, such as MCV or GGT, show no change [9, 10]. Approaches aimed at detecting both free and bound acetaldehyde in blood have been developed. So-called whole blood-associated acetaldehyde assay (WBAA) has great potential as a highly specific, precise, and extremely sensitive tool to test for heavy alcohol consumption and to monitor people in alcoholism treatment programs. The ability of the WBAA or HAA assays to measure alcohol consumption patterns over time make them unique among the biomarkers of alcohol misuse [7].

Alternatively, circulating antibodies against acetaldehyde adducts may be measured as biomarkers of alcohol intake. Binding with human proteins, acetaldehyde gives rise to a molecular adduct having acetaldehyde as hapten, and therefore forming neo-antigen that can induce antibodies toward themselves [9, 21]. The increased reactivity of IgA with acetaldehyde-modified serum proteins has been reported in heavy drinkers and alcohol-dependent persons, but not in social drinkers. Furthermore, an increased ratio of IgA/IgG is highly indicative of alcoholic liver disease [9]. However, the sensitivity and specificity of the circulating anti-adduct antibodies are 65–73% and 88–94%, respectively [22]. Interestingly, a single high-dose of alcohol (2 g/kg) has been shown to increase the level of salivary IgA. As saliva is an easily and non-invasively obtained material, salivary anti-acetaldehyde adducts IgAs seem to show promise in binge drinking detection [23].

Fatty acid ethyl esters

Fatty acid ethyl esters (FAEEs) represent products of non-oxidative ethanol metabolism. The compounds are formed by conjugation between fatty acyl chains (such as oleic acid, steric acid, and palmitic acid) and ethanol. The reaction is most often catalysed by enzymes (such as FAEE synthase, microsomal acyl-CoA:ethanol o-acyltransferase, carboxylesterase, lipoprotein lipase, cholesterol esterase, or triglyceride lipase), but FAEEs can also be formed spontaneously [7]. These alcohol metabolites are present throughout the body, including pancreas, liver, heart, brain, white blood cells, adipose tissue, hair, blood, and meconium, and they accumulate preferentially in adipose tissue and hair [24, 25].

Along with acetaldehyde, FAEE formation is an important pathway of ethanol disposition. Fatty acid ethyl esters measured in liver and adipose tissue have been used as a postmortem marker of alcohol consumption. Human and animal studies have demonstrated that when measured in adipose tissue, FAEEs may be useful as an alcohol consumption marker for up to 12 h af-

ter death; whereas when measured in liver tissue, they may be useful for up to 24 h after alcohol consumption [26]. Such a tool is needed because traditional measures, e.g. blood alcohol levels, can be artificially high as a result of postmortem alcohol formation. Furthermore, FAEs measured in hair look very promising as a marker providing long-term insight into drinking behaviour, as there is no possibility to flush them out of hair and they build up over a long period of chronic drinking. Interestingly, FAEs have been found to accumulate within the proximal 5–10 cm of hair, and then to decrease to a plateau afterwards, irrespective of alcohol dose. Therefore, through segmental analysis of the hair, it seems to be possible to confirm a current period of prolonged abstinence if FAE levels consistent with heavy alcohol exposure are not found in newly grown segments [25]. The FAE hair test that measures cumulative levels of four separate molecules (ethyl myristate, palmitate, oleate, and stearate) seems to be a sensitive and specific marker for chronic excessive alcohol use in adults, distinguishing social drinkers from heavy or alcohol-dependent drinkers [24, 27]. With the appropriate cut-off level (0.5 ng of cumulative FAEs per mg hair) it has been found to be 90% sensitive and specific in the detection of heavy alcohol use. Hair FAE levels between 0.2 and 0.5 ng/mg have been found to be indicative of social use, typically excluding strict abstainers, whereas levels in excess of 1.0 ng/mg are nearly 100% specific to heavy alcohol use, but offer lower sensitivity (~75%) [27, 28]. One should remember that FAEs in hair can be affected by cosmetic treatments and hair care, although preliminary studies seem to indicate that these effects are not likely to be of clinical significance [29]. Additionally, FAEs through their measurement in meconium may prove to have unique value as a reliable test providing evidence of prenatal exposure to alcohol in newborn infants and therefore for drinking during pregnancy [30, 31].

Phosphatidylethanol

Phosphatidylethanol (PEth) represents an abnormal cellular membrane phospholipid formed only in the presence of ethanol. Though the reaction catalysed by phospholipase D has been detected in the whole body, for the purpose of serving as an alcohol consumption biomarker PEth is sampled in the blood cells, where it can be readily accessed and measured [32]. *In vitro* studies have demonstrated that the quantity of PEth produced in human red blood cells is directly proportional to the ethanol concentration and the exposure time, and there is no correlation between the rate of PEth formation and haematological indexes (i.e. red blood cell count, mean corpuscular volume, haemato-

crit). Furthermore, the absence of PEth enzymatic degradation activity in human erythrocytes results in its accumulation in cellular membranes, which suggests a potential use of the compound for the measurement of prolonged as well as binge alcohol consumption. Importantly, PEth is considered to be less sensitive than EtG or EtS to small amounts of ethanol and does not detect single drink episodes [32]. The threshold of total ethanol intake resulting in a positive PEth assay was estimated at c.a. 1000 g in 3 weeks, with a daily consumption of at least 50 g [33].

Since the formation of PEth is specifically dependent on ethanol, the diagnostic specificity of PEth as an alcohol biomarker is theoretically 100%. Remarkably, its sensitivity has been found to reach high values, between 94.5 and 100% [34, 35]. Differing from the traditional indirect biomarkers used for diagnosing a chronic excessive drinking behaviour (i.e., MCV, AST, ALT, GGT, and CDT), blood PEth concentration seems not to be influenced by age, gender, other ingested substances, or non alcohol-associated diseases, such as hypertension, and kidney and/or liver diseases. In contrast to EtG or EtS, PEth is considered to be insensitive to incidental ethanol exposures, such as mouthwash and antibacterial hand cleansers [33, 34]. The utility of PEth for the diagnosis of alcohol abuse is determined by the short-term nature of this marker, its mean half-life in blood of alcoholics is approximately 4 days, with a range of 3–5.3 days. In clinical studies, the compound was detectable in blood of chronic heavy drinkers up to 28 days after sobriety [33]. Despite such high performance, the existing methods for detecting PEth are still too challenging for routine clinical usage, although they are capable of effectively measuring single molecular species of PEth in blood in the nanomolar range.

β -Hexosaminidase

β -Hexosaminidase (β -HEX) is a lysosomal exoglycosidase present in most cell types, and it is normally involved in the metabolism of carbohydrates and gangliosides in the hepatocytes, particularly in release of *N*-acetylhexosamines from the non-reducing end of oligosaccharide chains of glycoconjugates [9, 10, 36]. Heavy alcohol consumption, i.e. more than 60 g per day for at least 10 consecutive days, results in marked changes of the enzyme activity in the body fluids. One proposed mechanism is lysosomes damage and subsequent leakage of the enzyme from lysosomes and cells into body fluids [36]. The diagnostic sensitivity of increased activity of the serum β -HEX B isoenzyme and urine total β -HEX has been reported to achieve 69–94% and 81–85%, respectively. Furthermore, in alcohol-dependent persons, the enzyme levels fall rapidly to nor-

mal following abstinence (7–10 days, $T_{1/2} = 6.5$ days). [10, 37]. However, β -HEX serum, urine, or saliva levels may also increase following isolated consumption of ca. 2 g/kg bw of alcohol (so-called “binge drinking”) [38]. Despite relatively high specificity (84–98%), subjects with liver disorders (such as cholestasis and cirrhosis), hypertension, diabetes mellitus, cerebral or myocardial infarction, thyrotoxicosis, pregnancy, or after oral contraceptive pills may present with a false-positive elevation of the enzyme activity [10, 37]. The distinct advantage of β -HEX as a potential marker of prolonged alcohol abuse is that it is a standard and inexpensive technique of detection.

Plasma Sialic Acid Index of Apolipoprotein J

The term “Plasma Sialic Acid Index of Apolipoprotein J” (SIJ) expresses the ratio of moles of sialic acid per mole of apolipoprotein J (Apo J). Apolipoprotein J (or clusterin) is a multifunctional *N*-glycoprotein found in high-density lipoprotein (HDL) complexes, which has been implicated in a plethora of physiological and pathological processes. Among others, it is considered to be implicated in lipid transfer between lipoproteins, especially cholesterol. The glycoprotein is highly sialylated, i.e. human Apo J was demonstrated to contain 28 moles of sialic acid residues per mole of Apo J, compared with 4–6 moles of sialic acid per mole of transferrin [9]. This is highly significant and may make it easier to measure changes in sialic acid content caused by alcohol consumption. As with the molecule transferrin, long-term ethanol intoxication decreases sialylation of plasma Apo J, mainly by increasing the activity of sialidase and decreasing cellular glycosyltransferases (i.e. mannosyltransferase, galactosyltransferase, *N*-acetyl-glucosaminyltransferase, sialyltransferase) [9, 39]. Consequently, SIJ is decreased in alcoholics (on average by 50–57%, with specificity ~100%) and its levels progressively return to normal range over a period of several weeks of abstinence ($T_{1/2} = 4$ –5 weeks) [35, 40]. Furthermore, plasma SIJ correlates with relapse in alcohol-dependent subjects (~90% sensitivity) [9].

More studies are needed, but preliminary findings show promise for SIJ as a highly specific marker for alcohol misuse. However, widespread use in any clinical laboratory setting will require simplification of the method of measuring sialic acid in plasma Apo J. Although the methods used in most studies have been relatively straightforward and cost-effective, they are highly sophisticated and time-consuming, and they can be carried out only in specialised laboratories at the present time.

Total serum sialic acid

In humans, serum sialic acids are mostly attached to carbohydrate chains of glycoproteins, e.g. transferrin and Apo J, and glycolipids. Apart from these two fractions of sialic acid, serum total sialic acid (TSA) also comprises a minor fraction of serum free sialic acid (FSA) generated by desialylation of glycoproteins. The serum levels of TSA and FSA during excessive alcohol consumption seem to depend on the changes in the most sialylated glycoproteins [41].

Literature data have demonstrated that TSA concentration has clear potential as a marker for excessive alcohol consumption. Compared with social drinkers, both male and female alcoholics have elevated amounts of TSA in the serum, saliva, and urine, although the exact mechanisms that generate this increase still remain a matter of speculation [9, 41, 42]. The diagnostic value of TSA as an alcohol abuse biomarker showed 48–58% sensitivity and 64–96% specificity [9, 41]. Unfortunately, various diseases and states, such as cancer, diabetes, renal diseases, cardiovascular disease, or pregnancy, may increase serum TSA concentration, thus decreasing its specificity [43]. However, in a research context the TSA levels did not differ between subjects with elevated and normal liver enzymes activity, in contrast to a fraction of lipid-bound sialic acid [42]. Despite the lack of specificity, TSA can be recognised as a good test of alcohol abuse, independent of the presence of hepatocellular injury. Because TSA levels take longer than either CDT or GGT to decrease during periods of abstinence, the TSA test might not be useful for treatment programs assessing patients for relapse.

Interestingly, preliminary studies have demonstrated elevated concentrations of FSA in the sera of alcoholics. The diagnostic accuracy reached the value of 85–94%, although the sensitivity was low (~40%). Compared with traditional markers of alcohol abuse, the clinical usefulness of FSA is markedly lower than that of CDT and GGT, even though the specificity and positive predictive value of those tests were similar. At present, the clinical significance of FSA is limited to inherited diseases, including sialidosis, Salla disease, infantile sialic acid storage disease, and neuraminidase deficiency [41, 44].

Cholesteryl ester transfer protein

Cholesteryl ester transfer protein (CETP) is a hydrophobic glycoprotein synthesised by hepatocytes and circulating in plasma bound mainly to HDL particles. It promotes the redistribution of cholesteryl esters, triacylglycerols, and phospholipids between lipoproteins [9]. Research data have demonstrated that alcohol consumption markedly reduces both the plasma concentration and activity of CETP. As a consequence

the direction of cholesteryl esters transfer is reversed, which leads to an increase in the plasma HDL cholesterol concentration (a common laboratory abnormality in alcohol-dependent persons) [9, 37]. The possible clinical usefulness of plasma CETP is considered to be comparable with conventional alcohol markers, such as MCV, GGT, AST, and ALT. Its specificity is, however, limited due to some factors affecting plasma levels (e.g. various diseases, differences in diet, drugs) [9]. Another disadvantage of CETP as a marker of alcohol misuse is its complicated and laborious method of determination.

5-Hydroxytryptophol, 5-hydroxyindole-3-acetic acid

5-hydroxytryptophol (5-HTOL) comprises one of the minor metabolites of the neurotransmitter serotonin, and it is a normal constituent of urine. Alcohol and its primary oxidative metabolite, acetaldehyde, affect the metabolism of serotonin so that 5-HTOL concentration increases dramatically following alcohol consumption. Elevated 5-HTOL can be detected in urine for 5–15 h (depending on dose) after alcohol intake, compared to standard measurements, which can detect ethanol in the urine for a little over an hour for each drink consumed [45]. Preliminary work indicates that testing for 5-HTOL in urine is both sensitive and specific for detecting recent heavy alcohol consumption and may prove to be especially useful in forensic toxicology. Furthermore, there is a possibility to use the test to monitor subjects involved in treatment maintenance programs (excluding persons treated with the anti-drinking medication disulfiram, which can also lead to increases in 5-HTOL levels) [45].

The ratio of 5-HTOL to another serotonin metabolite, 5-hydroxyindole-3-acetic acid (5-HIAA), is considered to be alternating marker that can be used to verify the presence of ethanol in the body [37]. The 5-HTOL:5-HIAA ratio was found to have 100% sensitivity up to 4 h after a moderate dose of ethanol, but its reliability decreases fairly rapidly after 7 h [46]. The short time frame and the sophisticated method of determination currently used limit the diagnostic utility of these markers for assessing the history of ethanol abuse, and hamper their translation into routine clinical practice [37].

Salsolinol

Salsolinol, formed in brain and other tissue following alcohol intake, is a potential neurotoxin suspected to contribute to alcohol abuse. Chemically it is a biologically active alkaloid with morphine-like effects [47]. The compound primarily constitutes a non-enzymatic condensation product of the neurotransmitter dopamine and the ethanol oxidation product acetaldehyde, although it may

also be the effect of the enzymatic reaction between alcohol and pyruvate (a glucose metabolite that is used by cells for energy) [7]. The usefulness of salsolinol as a potential marker for chronic alcohol consumption depends considerably on the method of determination – and especially on the tissue in which it is detected [48]. Following acute alcohol consumption, the total urinary salsolinol output and the plasma concentration of salsolinol have been demonstrated to change in different ways. Some alcohol-ingesting subjects showed a significant increase of both urine and plasma concentrations, whereas others presented with decreased or unchanged salsolinol levels. Furthermore, compared with non-alcoholics, alcoholics who have been abstinent for as little as 1 week have decreased salsolinol levels in one type of white blood cell (namely, lymphocytes) [49]. Studies of salsolinol levels in the brain, in contrast, found no difference in salsolinol levels between alcoholics and non-alcoholics [48]. A few studies have reported that salsolinol from dietary sources (e.g. bananas) is a major contributor to its plasma levels [50].

Poor assay specificity and possible artefact formation of the alkaloids during work-up and storage have been suggested to be responsible for controversial reports on the detection of the compound in mammalian tissues and fluids after alcohol intake. Moreover, the analytical technique for determining the compound in human urine, plasma, brain, or cerebrospinal fluid requires sophisticated and expensive special equipment and is therefore not suitable for routine analysis and is unlikely to become clinically valuable [48]. More experimental work is necessary to determine whether alcohol really has an influence on the biosynthesis of salsolinol and if it may be a sufficient clinical marker to distinguish between alcoholics and non-alcoholics.

Dolichol

Dolichol, a homologous group of α -saturated long-chain polyisoprenoid alcohols, is synthesised from acetate and accumulates in tissues during ageing. Being a glycosyl carrier, the compound is involved in the translational modification of proteins to *N*-linked glycoproteins. Its function, however, is easily influenced by free radicals, e.g. those generated due to alcohol consumption [9, 10].

Both dolichol and ethanol are substrates for a single enzyme, alcohol dehydrogenase, and due to this substrate competition, elevated dolichol levels in the blood and urine have been suggested as markers of alcohol abuse [48]. Importantly, human studies have demonstrated that moderate alcohol consumption (60 g/day) did not affect urinary dolichol levels. Increased urinary dolichol levels have been reported in

chronic alcoholics and in newborns of alcoholic mothers, and as related to urinary creatinine they were 2.5–4 times higher than that of non-alcoholic social drinkers. The half-life of urinary dolichol is c.a. 3 days, whereas in serum it exceeds 7 days [37, 51, 52]. In alcoholic patients elevated urinary dolichol levels returned to normal by the fifth day of abstinence [51]. Despite the fact that the urinary dolichol test showed high specificity (96%), its sensitivity is moderate (68%) or even low (9–19%) [37].

Circulating cytokines

Cytokines are a class of multifunctional proteins implicated in cellular communication and activation. Being involved in processes such as inflammation, cell death, cell proliferation, cell migration, and healing mechanisms, they are critical to the development and functioning of both innate and adaptive immune response [53].

Both acute and long-term alcohol consumption have been demonstrated to influence considerably inflammatory cell and adaptive immune responses and directly suppress a wide range of immune responses. Among other mechanisms, alcohol is known to alter cytokine levels in a variety of tissues including plasma, liver, lung, and brain [53–56]. As the measurement of serum levels of many cytokines has recently become more available in clinical practice, it is possible for circulating cytokines to contribute to diagnostic tools for alcohol abuse. The most promising candidates comprise tumour necrosis factor- α (TNF- α), interleukin (IL)-1 α , IL-1 β , IL-6, IL-8, IL-12, and monocyte chemoattractant protein-1 (MCP-1) [53]. Serum levels of TNF- α have been shown to be higher in alcoholics than in the general population, regardless of alcohol consumption level [57]. Circulating TNF- α , IL-1, and IL-6 are found to be elevated in both acute and chronic alcohol-induced liver disease. Furthermore, chronic alcohol consumption without associated liver disease has also been linked with significantly increased production of TNF- α , IL-1 β , IL-6, and IL-12 [53]. On the other hand, subjects with alcohol liver cirrhosis, who were actively drinking, showed abnormally low levels of inflammatory cytokines. Interestingly, no significant alterations in cytokine levels were observed in patients with alcohol liver cirrhosis, who were in alcohol abstinence [58].

Although a growing body of evidence suggests the potential use of circulating cytokines as an indicator of alcohol intake, given their broad biological role, it is unlikely that they will be used as standalone alcohol biomarkers. Similarly, the role of other factors in cytokine release associated with alcoholism, including nutrition, age, gender, method of analysis, and co-morbid drug use, still remains to be elucidated.

Proteomic techniques in the alcohol misuse field

Proteomics is defined as the analysis of many or all of the proteins in a given sample. The central premise of such analysis is the comprehensive characterisation of the proteins in a cell, tissue, or organ that will provide insights into the status of the system. Based on the methodological considerations, proteomics addresses the physical arrangement of amino acids into a protein (structural proteomics), the actual physiologic activity of proteins (functional proteomics), and the patterns of protein expression and modification in health and disease (expression proteomics). One of the aims of proteomics is to identify biomarkers of disease [59].

Literature data directly indicate that proteomic techniques may constitute powerful tools in the discovery, characterisation, and validation of the complex new biomarker panels for alcohol misuse [60]. The instrumentation and the computational power for this type of analysis is becoming more and more sophisticated, resulting in characterisation and validation of new protein changes in response to chronic alcohol consumption. Furthermore, proteomic experiments are now able to detect thousands of proteins in a single run. In nonhuman primates an experimental 17-plasma protein panel (combining plasma cytokines, growth factors, and other proteins) correctly classified abusive drinking with 100% sensitivity and differentiated any level of drinking from alcohol abstinence with 88% accuracy [61].

Exemplary human serum proteins that can serve as novel biomarker candidates or components of biomarker panels to discern subjects with excessive alcohol use or confirm abstinence include AT-rich interactive domain-containing protein 4B, phosphatidylcholine-sterol acyltransferase, hepatocyte growth factor-like protein, ADP-ribosylation factor [62], serum amyloid A4, clusterin, fibronectin [61], α 2-HS glycoprotein, apolipoprotein A-I, glutathione peroxidase 3, heparin cofactor II, pigment epithelial-derived factor [63], a fragment of α fibrinogen, isoform 1 [64], gelsolin, selenoprotein P, serotransferrin, tetranectin, and haemopexin [65].

Concluding remarks

Because of the many limitations and weaknesses of currently used biomarkers of alcohol consumption, none of them has become widely accepted, and the search for ideal (i.e. more sensitive and specific) biomarkers continues. While it may be tempting to think of biomarkers as single molecules, a growing body of evidence indicates that panels of biomolecules in combination may function best in terms of sensitivity and specificity.

Further investigations should elucidate important pathophysiological bases of alcohol drinking behaviour and ethanol-induced organ damage and ultimately lead to better forms of prevention and therapy. Importantly, future alcohol biomarkers need to be able to differentiate between a variety of drinking behaviours occurring in real clinical practice (abstinence vs. light drinking vs. heavy drinking), as opposed to just differentiating between nondrinking and drinking. Furthermore, they should also enable assessment of both average intake and drinking patterns (e.g. binge drinking).

Another challenge will be to translate often sophisticated and expensive analytical techniques for determining the chosen compounds in easily available fluids and tissue into cost-effective and straightforward diagnostic tools that can be used in routine clinical practice.

Conflict of interest

The authors declare no conflict of interest.

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