Biomarkers in Alcohol Misuse: Their Role in the Prevention and Detection of Thiamine Deficiency

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Abstract — In Western countries alcohol misuse is the most frequent cause of thiamine (vitamin B1) deficiency (TD) and consequent neuro-impairment. Studies have demonstrated that between 30 and 80% of alcoholics are thiamine deficient, and this puts them at risk of developing the Wernicke–Korsakoff (WK) syndrome. The relative roles of alcohol and TD in causing brain damage remain controversial and it is important to try to determine the role played by each factor. Animal studies support an additive effect of alcohol exposure and TD, and indicate the potential for interaction between alcohol and TD in human alcohol-related brain damage. Early diagnosis of alcohol-related TD is therefore an important aspect of effective intervention and treatment. Alcohol biomarkers provide a direct and indirect way of estimating the amount of alcohol being consumed, the duration of ingestion and the harmful effects that long-term alcohol use has on body functions. Appropriate use of these markers is very helpful when considering a diagnosis of alcohol-related TD.

INTRODUCTION

Thiamine, or vitamin B1, is a water-soluble vitamin synthesized by different plants and micro-organisms. In animals, it is synthesized only in small quantities by intestinal bacterial flora. It is uncertain to what extent synthesis of thiamine occurs in man and especially in the malnourished alcoholic patient, where the uncertain production may be reduced by an inadequate supply of other nutrients, the effects of alcohol on the gastrointestinal tract and antibiotic use. However, thiamine synthesis does not protect these patients significantly against depletion. It has been established that mammals (including man) lack the necessary pathways for the synthesis of this vitamin and are therefore dependent upon obtaining sufficient quantities from the diet. In man, thiamine is absorbed from the gastro-intestinal tract by a rate-limited active transport mechanism that can be inhibited by alcohol, malnutrition or a combination of both factors. Thiamine (T) is present in the organism as free thiamine (10%), diphosphate ester (TDP, 80%) and small quantities of triphosphate (TTP) and monophosphate esters (TMP). T and TMP are detected mainly in human serum and cerebrospinal fluid; T, TMP and TDP in erythrocytes and TTP in nervous tissue. Most of the thiamine reserve is in the skeletal muscle, in heart, liver, brain and kidneys. T is converted by thiamine pyrophosphokinase to TDP, the metabolically active form. T and its phosphate esters play major roles as coenzymes in carbohydrate metabolism and in nerve cell conduction. TDP is a coenzyme for several intra-mitochondrial enzymes involved in carbohydrate and lipid metabolism and participates in the activity of three major enzyme complexes: the pyruvate dehydrogenase complex, the alpha-ketoglutarate dehydrogenase complex (Krebs cycle) and transketolase (pentose phosphate pathway) (Gubler, 1991). Free thiamine is involved in mediating the parasympathetic neural activity, and thiamine deficiency (TD) leads to impairment of nerve conduction/transmission inducing peripheral neuropathy and irreversible brain damage. Thiamine requirements are related to the calorific content of the diet and increase when the diet is rich in carbohydrates and when basal metabolism is increased (e.g. fever, trauma, pregnancy, lactation, alcoholic detoxification). Significant loss can occur in pregnancy, diuretic therapy, haemodialysis, peritoneal dialysis and diarrhoea.

Very little T is stored in the body (30–50 mg), so that the effects of depletion can be seen within 2–3 weeks of reduced thiamine intake, with reduction in liver and muscle stores and impaired function of enzymes requiring TDP as a coenzyme. The role of T in the aerobic oxidation of glucose is essential and, since the brain usually takes all its energy from aerobic oxidation of glucose, it is the first organ impaired by TD. In the case of TD, mitochondrial uncoupling and enzyme deficits result in different mechanisms involved in neuronal death (Hazell *et al.*, 1998). Neurological symptoms of TD probably arise from the direct involvement of thiamine in neuro-transmission (Devlin, 1992).

The relationship between TD and cholinergic neuronal function has been studied in an animal model (Nakagawasai, 2005). Choline acetyltransferase (ChAT) fluorescence intensity, a marker of presynaptic cholinergic neurons, was decreased in the cortex and hippocampus at an early stage (14th day) of TD and it was decreased in a wide range of brain areas at a later stage (25th day). These findings suggest that the memory deficits in the late stage of TD may be caused by a reduction in cholinergic function in the early phase of TD, and the activation of cholinergic neurons may play an important role in the improvement of TD-induced memory deficit.

TD and subsequent brain damage may occur in malnourished patients affected by different diseases. TD is reported in AIDS and asymptomatic HIV-positive patients (Müri *et al.*, 1999). It has been suggested that impaired thiamine metabolism may play a role in Alzheimer's disease (Eisinger *et al.*, 1994). Chemotherapy and radiotherapy can also cause vitamin deficiency but the indication for thiamine supplementation in malignancy is still debated (Boros *et al.*, 1998). T levels can be decreased by malabsorption, decreased intake, reduced storage, impaired utilization due to different causes such as old age, chronic use of drugs, folate deficiency and alcoholism. Pregnancy also increases the demand for T and recent case reports of TD and consequent Wernicke's encephalopathy (WE) caused by hyperemesis gravidarum have confirmed earlier reports (Indraccolo *et al.*, 2005; Phayphet *et al.*, 2007). Clinical

symptoms of TD are frequently ill-defined and most cases (80%) of WKS are diagnosed only at post-mortem (Thomson, 2000).

In Western countries, the most frequent cause of TD and consequent neuro-impairment is alcohol misuse (He *et al*., 2007). Furthermore, as reported by Thomson *et al*. (2008), 'the pattern of signs and symptoms of WE appears to be similar, regardless of whether the TD is related to nutritional problems alone or associated with alcohol misuse'.

The risk of vitamin deficiency, and in particular TD, in alcohol misusers is well known. While this is largely due to poor nutrition, chronic gastritis and diarrhoea significantly reduce the availability of vitamin B1. Intestinal absorption of thiamine can also be significantly reduced by alcohol or malnutrition acting alone or in combination (Thomson, 2000). Furthermore, the requirement of thiamine is increased by the amount of glucose in the alcohol consumed. Liver damage may deplete the liver of T reserves, therefore decreasing enzyme function. Thus, the utilization of T is further limited and ethanol neurotoxicity *per se* may interfere with the thiamine transport system in the brain. Early diagnosis is essential for effective prevention and treatment of WE.

ALCOHOL BIOMARKERS AND THIAMINE DEFICIENCY

Although health problems due to alcohol misuse are common in both hospital and clinical practice, early-phase problem drinking is frequently missed. Diagnostic screening tests for alcohol misuse should be sensitive and specific enough to identify patients drinking at an 'at risk' or hazardous fashion. Supplementing self-report with carefully selected laboratory markers of alcohol misuse, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), mean corpuscular volume (MCV) and γ-glutamyltransferase (GGT), is the best strategy to identify at-risk drinking and to monitor drinking behaviour (Miller *et al*., 2004; Aalto and Seppa, 2005; Anttila *et al*., 2005).

Until relatively recently many longitudinal studies on alcohol dependence included only male subjects. Studies of alcohol biomarkers have therefore used male populations and then generalized the results to both sexes. Recently, gender studies have been promoted to evaluate interactions between gender, alcohol effects and biomarkers and to establish sex-specific limits.

Women appear to be more vulnerable to the effects of alcohol, because of physiological, metabolic and hormonal factors (Mancinelli and Guiducci, 2004; National Institute on Alcohol and Alcohol Abuse, NIAAA, 1990) and the increase in the number of drinking females is an emerging problem not least because of the attendant risks of drinking during pregnancy (Warren and Foudin, 2001).

The use of some routine and promising alcohol biomarkers is discussed below, taking into account the gender effect, where known (Chen *et al*., 2003; Schwan *et al*., 2004).

Blood alcohol concentration (BAC)

Measurement of the BAC can be performed directly in blood samples or indirectly by sampling expired air (breath-test), saliva and urine (Taggi *et al*., 1989). The alcohol concentra-

tion in saliva is not significantly different from that in blood or breath. The alcohol peak in urine is reached ∼30 min later than in blood, saliva or breath (Taggi *et al*., 1989; Macchia *et al*., 1991). In certain situations, such as the emergency room and when the patient is unconscious, BAC determination may be critical for correct diagnosis and treatment. Gender differences in BAC may be highly significant. Experimental studies have demonstrated that, after the same alcohol intake, BAC in females was four times higher than in males (Mancinelli and Guiducci, 2004). The short half-life of ethanol is a limiting factor for the usefulness of BAC assessment.

Gamma-glutamyltransferase (GGT)

GGT is a membrane-bound glycoprotein enzyme that catalyzes the transfer of the gamma-glutamyl moiety of glutathione to the various peptide acceptors. Elevated GGT is an early indicator of liver disease. Heavy alcohol intake induces a rise in serum GGT levels that return to the reference range after 20–30 (or more) days of abstinence. Increased serum GGT is a sensitive marker (60–90%) for the diagnosis of alcohol-induced damage but only if associated with other markers of alcohol misuse and when biliary stasis is excluded. Results of the WHO/ISBRA collaborative project demonstrated that serum GGT is increased in 52% of alcohol-dependent subjects, in 28% of heavy drinkers, in 15% of light/moderate drinkers and in 10% of non-drinkers (Helander, 2003). GGT is not sensitive enough as a screen for 'at-risk' drinking in young people and in women (Conigrave *et al*., 2003). As regards the gender differences, it should be noted that in pregnancy, GGT may remain in the reference range even when a woman is drinking.

Specificity of GGT is poor since it is elevated in a number of other conditions such as pancreatitis, and prostate disease, diabetes, obesity and other liver diseases. Furthermore, GGT is induced by drugs such as barbiturates, antiepileptics and anticoagulants. Notwithstanding the above, GGT remains one of the most useful alcohol biomarker in current clinical practice.

Transaminases AST and ALT

The transaminases AST and ALT are enzymes involved in the metabolism of amino acids, the building blocks of proteins. ALT is the more specific marker of alcohol-induced liver injury because it is found predominantly in the liver, whereas AST is found in several organs, including the liver, heart, muscle, kidney and brain. Very high levels of these enzymes (e.g. >500 U/l) may indicate alcoholic liver disease, but an increase in transaminase levels is not specific to alcohol-related liver disease: it may also be related to viral hepatitis. For discriminant diagnosis, the ratio AST/ALT is used. A ratio of <1 indicates a diagnosis of viral hepatitis, while an AST/ALT ratio of >1 is suggestive of alcoholic liver disease. At present, the AST/ALT ratio is considered one of the best diagnostic tools to recognize alcohol-related liver disease, despite the fact that it performs less well in patients under the age of 30 or over the age of 70 years (Halvorson *et al*., 1993).

The total hepatic AST activity is due to mitochondrial AST (10%) and cytosolic AST (90%) isoenzymes. Alcohol-related liver damage produces a selective impairment of mitochondria, and the release of the AST mitochondrial fraction into the serum contributes to the increase of circulating AST levels (Pol *et al*., 1991). An increase in the mitochondrial fraction

was demonstrated to be a sensitive and specific marker of alcohol damage, directly related to alcohol intake within 2–3 days (Macchia *et al*., 1997).

Mean corpuscular volume (MCV)

The MCV or volume of red blood cells is elevated in chronic heavy drinkers (Neumann and Spies, 2003) and is often used as a screen for alcohol misuse. Macrocytes from alcohol misusers are typically round rather than oval and may also show excessive stomatocytosis (Lindenbaum, 1987). The prevalence of macrocytosis has been shown to be higher in female alcoholics (86%) than in their male counterparts (63%) (Morgan *et al*., 1981; Fauske and Haver, 1990). MCV values return to within reference limits after ~2–4 months of abstinence. Persistent macrocytosis following cessation of alcohol may reflect other clinical conditions such as Vitamin B12 and folate deficiency, liver disease and hypothyroidism.

Haematological parameters

Concomitant iron deficiency, increased red cell distribution width (RDW) and other dismorphisms may be markers of alcohol misuse even if MCV is normal (Seppa *et al*., 1991). Alcoholic liver disease may also be associated with spur cell haemolytic anaemia; pathological ring sideroblasts have been reported to occur in anaemic alcoholics, especially when malnutrition and folate deficiency are present. Thrombocytopaenia (low platelet count) is common in patients with recent heavy drinking. It seems that alcohol can suppress platelet production since, under experimental conditions, thrombocytopaenia occurs after alcohol intake in almost half of healthy subjects. In alcoholic cirrhosis with splenomegaly, platelets may be sequestrated in the spleen (Maher, 1998).

Blood lipid profile

Blood lipid profile is modified by alcohol intake according to dose, individual susceptibility, genetic factors and diet. Alcohol intake stimulates hepatic triglyceride (TG) synthesis and VLDL secretion. Low-to-moderate alcohol intake in non-alcoholics modifies plasma lipoprotein profile according to dose and time. Plasma TG levels are normal or slightly increased in subjects with moderate alcohol intake. Individuals who misuse alcohol frequently have raised serum TG levels: up to 80% may have hyper-triglyceridaemia following recent heavy drinking. Prolonged alcohol consumption is associated with increased levels of high-density lipoprotein (HDL) cholesterol; this returns to normal range within 2 weeks of abstinence. It is thought that ~50% of the cardio-protective effect of moderate alcohol consumption is mediated by HDL (Moorjani and Lupien, 1990; Lieber, 1997; Sacco *et al*., 1999).

Immune response

Alcoholics frequently suffer from infectious diseases, autoimmune diseases and have increased rates of some cancers, indicating that alcohol impairs the immune system. Many alcohol-specific immune effects remain poorly understood. In chronic heavy drinkers, the adducts formed by proteins with aldehydes and hydroxyl radicals from ethanol metabolism stimulate immunological responses both for IgA and IgG. IgA titres are elevated in 69% of patients with alcoholic liver disease (Vitala *et al*., 1997).

Recent studies in alcoholics have demonstrated that ethanol exposure modifies the activity of dendritic cells (DC). Ethanol affects LPS-induced maturation of DC required for efficient presentation to antigen-specific T-cells, producing phenotypic and functional alteration. The effectiveness of the immune response is decreased, the antigen presenting function of alcoholic DC is reduced and the defence from microbial effects is compromised. Experimental data *in vivo* and *in vitro* support the hypothesis that acute ethanol exposure may also block the DC maturation, leading to an increased risk of infection and inhibition of host inflammatory response (Szabo *et al*., 2004; Buttari *et al*., 2005).

Carbohydrate-deficient transferrin (CDT)

CDT is the term used to describe the group of isoforms of transferrin that are deficient in sialic acid residues (Stibler, 1991; Sillanaukee *et al*., 2001). Several methods for CDT measurement have been introduced, and different cut-off levels have been suggested. Differences in analytical procedures and units of measure, in selection criteria of cases and controls, in genetics, life-style and nutrition of the population studied, make it difficult to compare the results across different groups (Arndt, 2001; Bortolotti *et al*., 2006). CDT has been demonstrated to be an effective marker in male alcoholics. It is less effective in women in whom CDT levels are usually higher, requiring a higher diagnostic cut-off point (Lof *et al*., 1994; Sillanaukee *et al*., 1994). Iron deficiency and hormonal status are thought to play a significant role in gender differences. During pregnancy, CDT values increase independently of alcohol intake (Sarkola *et al*., 2000; Cook, 2003). CDT is a very well characterized biomarker for heavy alcohol intake and is most useful when combined with other markers (Chen *et al*., 2003). Diagnostic sensitivity and specificity of this test for the assessment of women and pregnant women require further study.

Promising biomarkers

Detection of alcohol misuse may be particularly difficult in women, especially pregnant woman. This is a serious issue, because prenatal alcohol use is a primary risk factor for foetal alcohol spectrum disorders (FASD), the most common preventable cause of mental, developmental, behavioural and social deficits, as well as some birth defects (Jones *et al*., 1973; Riley *et al*., 2003; Hoyme *et al*., 2005). The diagnostic power of traditional markers may be limited in pregnancy and in the neonate. The determination of new sensitive and specific biomarkers in meconium and hair allow for the detection of prenatal exposure to alcohol.

Fatty acid ethyl esters (FAEEs)

FAEEs are nonoxidative metabolic products resulting from the interaction between alcohol and fatty acids. They are measured as a combination of four separate molecules: ethyl myristate, ethyl palmitate, ethyl oleate and ethyl stearate (Wurst *et al*., 2004). FAEEs have been implicated in the pathophysiology of alcoholic diseases and in foetal alcohol syndrome, alcohol-related birth defects and neurodevelopmental disorders (Kaphalia *et al*., 2004).

Recent studies demonstrate that FAEEs are sensitive and specific markers for distinguishing social drinkers from heavy

or alcohol-dependent drinkers (Salem *et al.*, 2001; Wurst *et al.*, 2004) and for detecting prenatal exposure. The presence of ethyl oleate in meconium was associated with self-reported drinking during pregnancy, with high sensitivity and specificity (Bearer *et al.*, 2003). The determination of FAEE in hair is suggested as a marker for chronic heavy alcohol consumption in adults (Wurst *et al.*, 2003, 2004; Yegles *et al.*, 2004) and for prenatal alcohol exposure in the newborn.

Even if analytical determination of FAEE in hair needs specific expertise in the testing process from sample collection to results interpretation, it may be one of the most promising tools in alcohol biomedical research (Pragst and Balikova, 2006).

Ethyl glucoronide (EtG)

EtG is a non-volatile, water-soluble, direct metabolite of alcohol that forms in the liver when alcohol reacts with glucuronic acid, a substance which detoxifies drugs by turning them into water-soluble compounds that are easily removable from the body (Wurst *et al.*, 1999). EtG can be detected in body fluids and hair, and remains in the blood for up to 36 h and in the urine for up to 5 days after heavy alcohol use. EtG is not found in non-drinking subjects (Wurst *et al.*, 1999). This test appears to be effective in detecting heavy drinking, even when traditional markers and clinical evidence fail to show it (Seidl *et al.*, 1998; Wurst *et al.*, 2003).

Phosphatidylethanol (PEth)

The enzyme phospholipase D (PLD) catalyzes the formation of phosphatidic acid from phosphatidylcholin using water as a substrate. However, in the presence of primary alcohols, the reaction is diverted to transphosphatidylation and phosphatidyl alcohol is formed instead. Ethanol intake leads to formation and accumulation in the tissues of PEth, an abnormal phospholipid formed in cell membranes by a transphosphatidylation reaction (Aradottir and Olsson, 2005). PEth has been proposed as a highly specific and sensitive marker of ethanol abuse. Determination of PEth can be performed in whole blood extracts: it can be found mainly in erythrocytes, with a mean half-life in blood from alcoholics of ~4 days (Varga *et al.*, 2000). PEth remains detectable in alcoholic blood for up to 14 days after abstinence (Hansson *et al.*, 1997). Studies of alcoholics under treatment have demonstrated that PEth does not give false negatives (Wurst *et al.*, 2004) or false positives (Wurst *et al.*, 2003; Aradottir *et al.*, 2006) and showed correlation of PEth results with reported alcohol intake.

THIAMINE AND ITS ESTERS

Alcohol dependence is frequently associated with thiamine deficiency. However, analytical difficulties have meant that estimation of thiamine levels has not been part of clinical management of these patients. Recently, an improved analytical procedure for the determination of thiamine and its esters in erythrocytes was applied to clinical studies in alcoholic patients (Mancinelli *et al.*, 2003; Ceccanti *et al.*, 2005). The data obtained by direct measurement of T, TMP and TDP content in human erythrocytes showed that T and TDP content in alcoholics were significantly lower than in controls ($P < 10^{-5}$),

as expected. The highly significant decrease of T and TDP in alcoholics is further confirmation of a marked reduction in the thiamine stores in alcoholics. In chronic alcoholics, reduced concentrations of T and TDP were unrelated to the degree of liver impairment and to clinical evidence of thiamine deficiency. The diagnostic power of TDP was demonstrated to be higher than other thiamine esters: sensitivity of 84.1%, specificity of 85.4%, positive predictive value of 82.4% and negative predictive value of 88.0%. Comparison of results between males and females showed no significant differences in T and TDP mean values. However, there were striking gender differences in the T and TDP ROC curves ($P < 0.0005$) and the AUC for females was closer to 1 than that for males. On this basis, TDP itself was proposed as an alcohol biomarker to be used in combination with other well-established markers such as MCV, CDT, GGT, AST/ALT (Ceccanti *et al.*, 2005).

CONCLUSION

Alcohol biomarkers provide direct and indirect ways to estimate the amount of alcohol consumed and the duration of ingestion, as well as the harmful effects on the body resulting from long-term alcohol misuse. Furthermore, alcohol biomarkers give further information about drinking patterns in individuals and populations. They have the potential to aid the identification of alcohol-related problems such as TD, by raising the index of suspicion that there is an underlying problem as well as giving information about the nutritional status of patients.

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