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STUDIES ON THE RELIABILITY OF BIOMARKERS FOR ALCOHOL USE AND ABUSE

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"I'd rather have a bottle in front of me than a frontal lobotomy"

Tom Waits

ABSTRACT

Alcohol is consumed by the vast majority of the population, but prolonged excessive drinking is associated with various negative health and social consequences. It is therefore important to identify individuals with at-risk alcohol consumption, before it turns into abuse or dependence. Early detection of alcohol use and abuse can be done by the use of biomarkers such as ethyl glucuronide (EtG), carbohydrate-deficient transferrin (CDT), and phosphatidylethanol (PEth) that provide objective information about current consumption. However, since misleading test results can have devastating consequences, the use of reliable biomarkers is substantial. The aim of this thesis was to evaluate several factors, both clinical and analytical, that could generate erroneous test results when testing for alcohol use by these biomarkers.

Measurement of urinary EtG levels was done in 482 samples using different liquid chromatography-mass spectrometry procedures. Accurate determination of EtG concentrations was done according to specific criteria suggested by international guidelines. The sensitivity and specificity were calculated for each of four methods by comparing EtG results obtained with a fifth reference method that demonstrated the highest selectivity. These results showed that meeting the guideline criteria does not always guarantee correct identification, and the likelihood of different analytical methods to provide reliable analytical results depends on the reporting limit applied.

Evaluation of the analytical performance of CDT testing was done by comparing two different methods in routine use, capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC). Most of the problems encountered by CE could be solved by using the HPLC method, and it was therefore advised to have access to a confirmatory HPLC analysis, when a high throughput method like CE is employed.

Evaluation of the clinical performance of CDT in pregnancy was done by measuring serum transferrin glycoforms in 171 samples collected from 24 healthy women during and after pregnancy. A gradual increase in the CDT (%disialotransferrin) level was observed during pregnancy, and in many subjects the level approached the upper limit of the reference interval. For use in pregnant women, the cutoff value for CDT used to detect risky drinking needs to be raised slightly to minimize the risk for false-positive results.

The possible interference by transferrin glycation on CDT testing was also evaluated. Samples subjected to *in vitro* glycation and samples collected from diabetic patients were tested for CDT by HPLC. No interferences were observed in samples from diabetics, which contrasted to the effect seen *in vitro* by transferrin glycation. The results indicated that CDT, and also PEth, are reliable markers to identify risky drinking in diabetic patients.

Taken together, the results of the present studies have identified and suggested ways to overcome a number of analytical and clinical interferences with these alcohol biomarkers, and thus helped to improve their routine use.

LIST OF PUBLICATIONS

- I. Helander A, Kenan N, Beck O.
Comparison of analytical approaches for liquid chromatography/mass spectrometry determination of the alcohol biomarker ethyl glucuronide in urine. *Rapid Commun Mass Spectrom* 2010, 24(12):1737-43.
- II. Kenan N, Husand S, Helander A.
Importance of HPLC confirmation of problematic carbohydrate-deficient transferrin (CDT) results from a multicapillary electrophoresis routine method. *Clin Chim Acta* 2010, 411(23-24):1945-50.
- III. Kenan N, Larsson A, Axelsson O, Helander A.
Changes in transferrin glycosylation during pregnancy may lead to false-positive carbohydrate-deficient transferrin (CDT) results in testing for riskful alcohol consumption. *Clin Chim Acta* 2011, 412(1-2):129-33.
- IV. Helander A, Kenan Modén N.
Effect of transferrin glycation on the use of carbohydrate-deficient transferrin (CDT) as an alcohol biomarker. (Manuscript)

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LIST OF ABBREVIATIONS

5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-Hydroxytryptophan (serotonin)
5-HTOL	5-Hydroxytryptophol
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
AUDIT	Alcohol use disorder identification test
BAC	Blood alcohol concentration
CDG	Congenital disorder of glycosylation
CDT	Carbohydrate-deficient transferrin
CE	Capillary electrophoresis
CYP2E1	Cytochrome P450 2E1
EDTA	Ethylenediaminetetraacetic acid
EtG	Ethyl glucuronide
EtS	Ethyl sulfate
FAEE	Fatty acid ethyl ester
FeNTA	Ferric nitrilotriacetic acid
GC	Gas chromatography
GGT	γ -Glutamyl transferase
HPLC	High-performance liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
MAST	Michigan alcohol screening test
MCV	Mean corpuscular volume
MEOS	Microsomal ethanol oxidizing system
NAD ⁺	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
PA	Phosphatidic acid
PC	Phosphatidylcholine
PEth	Phosphatidylethanol
pI	Isoelectric point
PLD	Phospholipase D
SPE	Solid phase extraction
UGT	Uridine 5'-diphospho-glucuronosyltransferase

1 INTRODUCTION

Alcohol has been a part of the human diet since pre-historical times, but excessive consumption is associated with intoxication and an increased risk for illness, disability, and mortality [1]. Many medical conditions are attributable to alcohol including liver and pancreas diseases, cancer, and neuropsychiatric diseases [2], and according to the World Health Organization's (WHO) latest global status report on alcohol and health, 6.2% of all male deaths (1.1% for female deaths) are attributable to alcohol [3]. Beside the physical and psychological harm to the drinker, alcohol consumption has an impact on society by means of medical costs, reduced productivity, disrupted relationship with family and friends, and harms caused to others (e.g. maternal drinking, drunk driving, and violence) [2, 3].

Hazardous drinking, harmful use, and alcohol dependence are all part of the alcohol-use disorders (AUD), and are defined as:

- *Hazardous drinking*: "A pattern of substance use that increases the risk of harmful consequences for the user. In contrast to harmful use, hazardous use refers to patterns of use that are of public health significance despite the absence of any current disorder in the individual user" [4].
- *Harmful use*: "A pattern of psychoactive substance use that is causing damage to health. The damage may be physical or mental. Harmful use commonly, but not invariably, has adverse social consequences" [4].
- *Alcohol dependence*: "A cluster of behavioral, cognitive, and physiological phenomena that develop after repeated substance use and that typically include a strong desire to take the drug, difficulties in controlling its use, persisting in its use despite harmful consequences, a higher priority given to drug use than to other activities and obligations, increased tolerance, and sometimes a physical withdrawal state" [5].

Guidelines from the Swedish National Institute of Public Health (FHI) [6] state that drinking is considered hazardous if the weekly alcohol consumption is exceeding 9 standard drinks for women or 14 for men, where a standard drink contains 12 g ethanol. Heavy episodic drinking (binge drinking) is also regarded as hazardous drinking, referring to consumption of 4 standard drinks or more on the same occasion

for women, and 5 drinks for men. Moreover, even lower alcohol consumption can be considered hazardous under certain circumstances, for example during pregnancy, in traffic, workplace, or some medical conditions.

According to the Swedish Council for Information on Alcohol and Other Drugs (CAN) [7], approximately one million Swedes are classified as hazardous drinkers, and around 300,000 have developed alcohol dependence.

Nevertheless, heavy alcohol consumption does not necessarily lead to alcohol dependence, but a strong and positive relationship exists between high alcohol consumption and the risk to develop dependency [8, 9]. Consequently, identification of individuals with at-risk alcohol consumption before the onset of dependency or other complications can reduce the harms associated with alcohol [2, 10].

1.1 ETHANOL IN THE BODY

Following alcohol ingestion, ethanol is absorbed mainly from the small intestine and to a lesser extent from the stomach [11]. There are several factors affecting the rate of ethanol absorption such as the feeding state, dose taken, gastric emptying rate, meal composition, and beverage type [12]. Soon after ingested, ethanol is distributed throughout the water in the body owing to its chemical properties being a small, polar, and water soluble molecule that can move easily across cell membranes by passive diffusion. The extent of ethanol distribution varies between individuals since it is determined largely by the total body water [12] that in its turn depends on age, weight, and gender [13].

A small fraction of the digested ethanol (2–5%) is excreted unchanged in urine, sweat, or breath, while most ethanol (~95%) is metabolized enzymatically in the liver through oxidation [12] (Fig. 1). The first step in ethanol degradation is its conversion to acetaldehyde, a reaction catalyzed by cytosolic alcohol dehydrogenase (ADH) with NAD^+ as the coenzyme. Acetaldehyde is a reactive and toxic substance, and is rapidly further oxidized to acetate in the second step, mediated by mitochondrial aldehyde dehydrogenase (ALDH) and NAD^+ . The genes encoding for ADH and ALDH display polymorphism that can result in an altered, but functioning, enzyme activity and thereby causing variations in alcohol metabolism [14].

An additional pathway to metabolize ethanol involves the microsomal ethanol-oxidizing system (MEOS) whose activity is increased after chronic alcohol consumption. In this reaction (Fig. 1), ethanol is converted to acetaldehyde by the CYP2E1 enzyme, a member of the cytochrome P450 family [15].

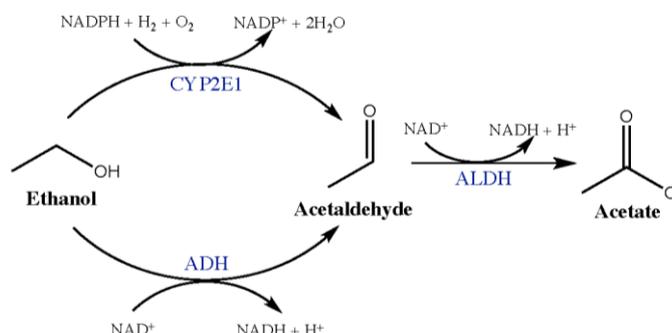


Figure 1. Oxidative pathways of ethanol metabolism.

Apart from oxidative pathways, a minor part of ethanol can undergo non-oxidative metabolism which result in the formation of fatty acid ethyl esters (FAEE), phosphatidylethanol (PEth), ethyl glucuronide (EtG), and ethyl sulfate (EtS).

1.2 ALCOHOL BIOMARKERS

Early identification of hazardous drinking is important for the initiation of suitable intervention strategies and thereby lowering the risks and costs associated with unhealthy drinking habits. One way to assess information about a patient's harmful alcohol consumption is through self-report questionnaires, such as the MAST [16], CAGE [17], and AUDIT [10]. The information obtained, however, might not always reflect the patient's actual intake of alcohol, since it relies on the patient's memory and honesty [18].

The use of measurable clinical biomarkers is another, more objective, way to evaluate drinking behavior [19]. An alcohol biomarker can be defined as an analyte in a diagnostic test that can indicate alcohol consumption. Biomarkers can be products of normal biological processes that are formed after alcohol ingestion (e.g. oxidative and non-oxidative ethanol metabolism), or pathogenic processes (e.g. alcohol-induced tissue damage or dysfunction) [19, 20].

An alcohol biomarker can be used as a classifier that categorizes subjects into being positive or negative for a certain condition, for example heavy vs. non-heavy drinking. This sort of test yields four potential results (Fig. 2):

- True positive (TP) - A patient with the condition who test positive.
- False negative (FN) - A patient with the condition who test negative.
- False positive (FP) - A patient without the condition who test positive.
- True negative (TN) - A patient without the condition who test negative.

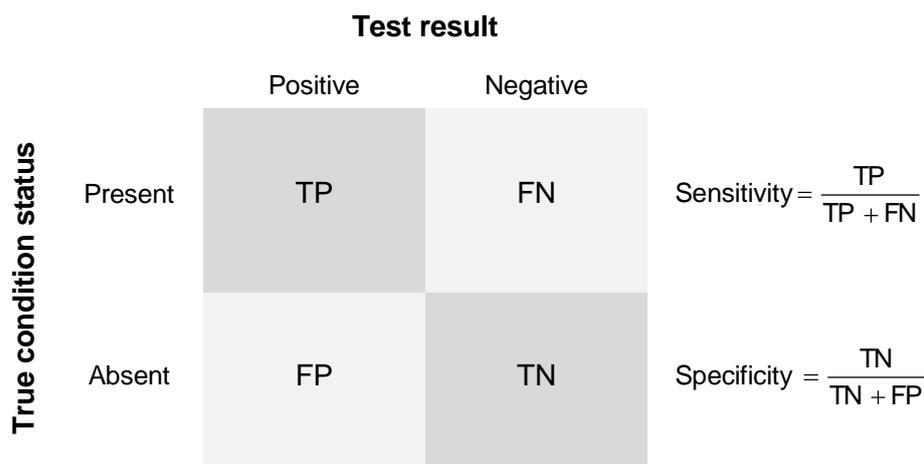


Figure 2. Diagnostic test characteristics.
 TP=true positive, FN=false negative, FP=false positive, TN=true negative.

Based on these results, it is possible to calculate the biomarker’s sensitivity and specificity - two measures that reflect its diagnostic accuracy (Fig. 2). The term sensitivity refers to the probability that the test result is positive given that the condition is present, while specificity is the probability that the test result is negative given that the condition is absent [21]. The sensitivity and specificity of a biomarker can vary depending on the cutoff value applied. This value is chosen according to the purpose of the test (e.g. screening, monitoring abstinence) and serves as a decision threshold, or a limit, to define positive and negative test results. In case the test value is increased by a condition, use of a higher cutoff will make it more difficult to detect the presence of the condition (lower sensitivity), but the risk for false positive test results is lower (higher specificity). On the other hand, lowering the cutoff values will result in higher sensitivity but lower specificity. Depending on the clinical application, it is thus possible to adjust the cutoff value in order to maximize either sensitivity or specificity. For example, high specificity is important whenever a test has legal consequences, and

therefore a higher cutoff level is preferable. In contrast, a lower cutoff can be used when screening for problematic drinking since high sensitivity is desirable.

Alcohol biomarkers are useful in clinical setting where they can be used for screening, detection of relapse, and evaluation of treatment outcome [22]. Furthermore, they can be used in other settings such as occupational, traffic, and forensic medicine [23-25]. Depending on their window of assessment, alcohol biomarkers can be roughly divided into short- and long-term markers.

1.3 SHORT-TERM LABORATORY BIOMARKERS

1.3.1 Blood alcohol concentration (BAC)

The most objective way to detect recent alcohol consumption is the direct measurement of ethanol in the body. Shortly after ingestion, ethanol can be detected in all body fluids but measurements are usually done in whole blood or breath, using several chemical and enzymatic methods [26]. One disadvantage of the direct measurement of ethanol is the short assessment window due to the relatively fast elimination from the body [27].

One way to extend the time of detection is by measuring metabolites of ethanol that remain detectable in the body for hours to days after ethanol has been eliminated [26].

1.3.2 Fatty acid ethyl ester (FAEE)

Fatty acid ethyl esters are non-oxidative metabolites of ethanol, produced by esterification of ethanol with fatty acids (Fig. 3) or fatty acetyl-CoA [28]. FAEE was found suitable as an alcohol biomarker since it is present in blood shortly after alcohol intake, and remained detectable for 13-99 hours after the last intake [29-31]. In addition to blood and various organs, FAEEs can be also detected in skin surface lipids, hair, and meconium [32, 33].

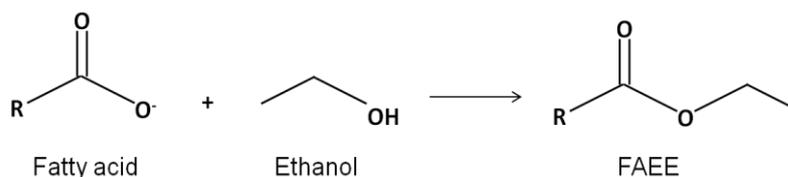


Figure 3. FAEE formation through esterification reaction between fatty acid and ethanol.

1.3.3 5-Hydroxytryptophol (5-HTOL)

One pathway of serotonin (5-hydroxytryptamine, 5-HT) catabolism involves oxidative deamination that result in 5-hydroxyindoleacetaldehyde (5-HIAL), a reaction catalyzed by monoamine oxidase (MAO) (Fig. 4). In subsequent reactions, 5-HIAL is either oxidized to 5-hydroxyindoleacetic acid (5-HIAA), or reduced to 5-hydroxytryptophol (5-HTOL). The former reaction is catalyzed by an NAD^+ -dependent ALDH, while the reduction reaction is mediated by an NADH-dependent ADH [34].

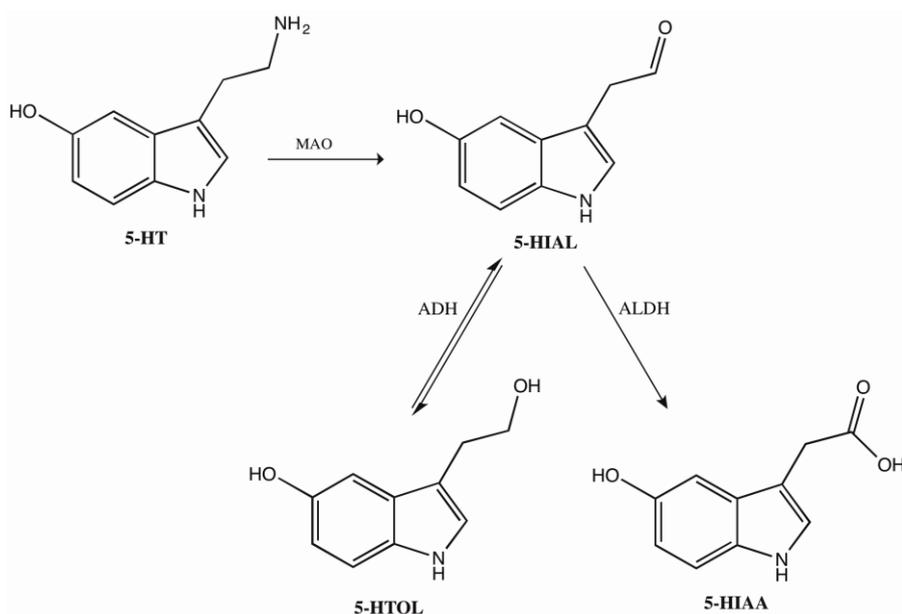


Figure 4. Pathways of serotonin (5-HT) metabolism. 5-HIAL = 5-hydroxyindoleacetaldehyde, 5-HIAA = 5-hydroxyindoleacetic acid, 5-HTOL = 5-hydroxytryptophol.

Under normal conditions, 5-HTOL is only a minor metabolite of serotonin but after alcohol ingestion, a dose-dependent increase in 5-HTOL formation on the expense of 5-HIAA is observed [35]. This shift in 5-HT catabolism is explained by competitive inhibition of ALDH by acetaldehyde, and/or by the increased levels of NADH which favors the formation of 5-HTOL [36].

Elevated urinary levels of 5-HTOL can be measured several hours after ethanol is no longer detectable, and can be therefore used to monitor recent alcohol consumption [37]. It should be noted, however, that it is preferable to use the ratio of 5-HTOL/5-HIAA to compensate for urine dilution [38] and the elevated levels of both metabolites following ingestion of serotonin-containing food [39]. Although very sensitive as an alcohol biomarker, 5-HTOL is limited by its short detection window (~24 h) [40]. An

alternative biomarker, showing similar sensitivities but longer assessment window, is ethyl glucuronide (EtG) [41, 42].

1.3.4 Ethyl glucuronide (EtG)

Approximately 0.02% of the ingested ethanol is eliminated in a minor pathway involving enzymatic formation of EtG [43] (Fig. 5). In this conjugation reaction, glucuronic acid is added to ethanol through the action of UDP-glucuronosyltransferase (UGT), an enzyme capable of glucuronizing a wide range of xenobiotics and endogenous compounds and thereby assisting in their elimination in the urine [44].

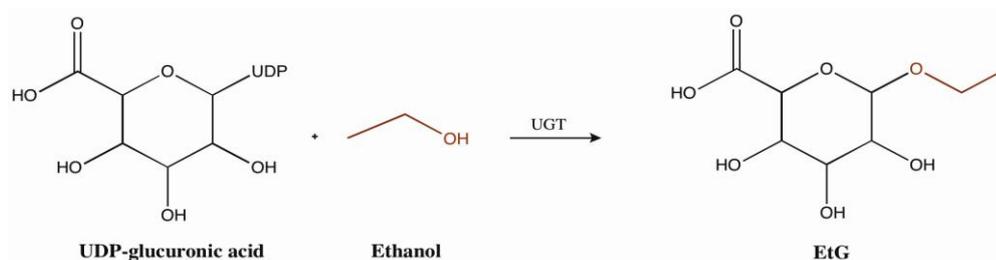


Figure 5. Ethyl glucuronide (EtG) formation by the addition of glucuronic acid to ethanol through the action of UDP-glucuronosyltransferase (UGT).

EtG is eliminated slower than ethanol [43], and depending on the amount alcohol ingested, it can be detected for up to several days [45]. Measurements of EtG can be done in urine, whole blood, serum, meconium [46, 47], oral fluid [48], and hair [49] using different analytical procedures, including LC-MS(/MS), GC-MS, capillary electrophoresis, and antibody-based enzyme immunoassay.

Even though elevated EtG levels provide a good indication of recent alcohol intake, several factors generating unreliable test results have been identified. One cause is diuresis, caused by consuming large amounts of fluid prior to sampling, which results in decreased urinary levels of EtG [43]. Compensation for urine dilution can be done by expressing EtG as a ratio to creatinine and thereby avoiding falsely low concentrations [43, 50]. An additional risk factor for false results is bacterial contamination which can lead to the degradation or even formation of EtG [51-53]. Such contaminations are commonly present in samples originating from patients suffering from urinary tract infection [51], or in post-mortem samples [52]. Reducing or preventing bacterial contamination can be achieved by storing samples in cooler temperature, adding preservatives to collection tubes [51], or collecting urine sample on a filter paper [54].

Unintentional exposure to ethanol through hand sanitizers [55-57], mouthwash [58, 59], or certain food [60] were also suggested to cause positive EtG test results, but this risk seems to rely largely on the cutoff applied.

1.3.5 Ethyl sulfate (EtS)

Another minor (<0.1%) elimination pathway in ethanol metabolism involves the formation of EtS through sulfate conjugation with 3'-phosphoadenosine 5'-phosphosulfate, catalyzed by cytosolic sulfotransferase [61, 62] (Fig. 6). EtS is detectable in urine for several days after drinking alcohol, depending on the dose ingested [47, 63]. Measurements of EtS and EtG in urine can be done simultaneously using LC-MS/MS methods [64, 65].

Just as for EtG, EtS levels are sensitive to urine dilution, and it is therefore recommended to express EtS as a ratio to creatinine to avoid falsely low results [65]. An advantage of EtS, comparing to EtG, is its stability against bacterial contamination which can result in post-collection formation or degradation of EtG but not EtS [51, 53]. However, false-positive results for EtS, but not EtG, were observed after consumption of non-alcoholic wine containing EtS and EtG, indicating higher bioavailability for EtS [58].

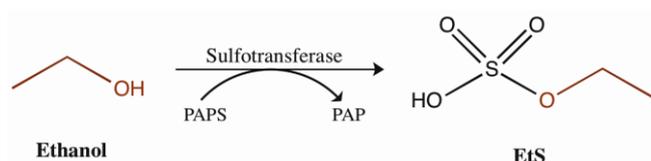


Figure 6. Ethyl sulfate (EtS) formation through sulfate conjugation with 3'-phosphoadenosine 5'-phosphosulfate (PAPS), catalyzed by sulfotransferase.

1.4 LONG-TERM LABORATORY BIOMARKERS

1.4.1 Mean corpuscular volume (MCV)

The enlargement of red blood cells, known as macrocytosis, is associated with chronic alcohol intake [66] in a dose-dependent manner [67]. Measurements of MCV can be therefore used for the screening of alcohol abuse [68]. However, macrocytosis is also associated with vitamin B₁₂ and/or folic acid deficiency, smoking, liver diseases,

reticulocytosis and medications [69, 70], resulting in decreased specificity of MCV as alcohol biomarker [68].

1.4.2 Gamma-glutamyltransferase (GGT)

GGT is an enzyme found on the cell membrane in several tissues and is involved in glutathione metabolism [71]. In addition to its use as a marker for liver dysfunction, elevated GGT levels in serum may indicate excessive alcohol consumption [72]. GGT has, however, low sensitivity and varying specificity due to inter- and intra-individual variability in the response of GGT to alcohol consumption, and by several other factors, including age, gender, medical condition, coffee consumption, and smoking that can affect test results [73].

1.4.3 Aspartate- and Alanine aminotransferases (AST and ALT)

The enzymes AST and ALT are involved in amino acid metabolism and are located mainly in the liver [73]. Elevated serum level of AST and/or ALT are indicators of liver injury, either acute or chronic [74], but can also serve as biomarkers for excessive drinking [20]. In order to distinguish between alcoholic and non-alcoholic liver damage, it has been suggested to calculate the enzyme ratio of AST to ALT, where a ratio above 2 is indicative for an alcohol related elevation [75]. The sensitivity and specificity of these enzymes as alcohol biomarkers are low [76], and factors such as age, obesity, medication, and coffee intake can affect the levels of AST and ALT [73].

1.4.4 Carbohydrate-deficient transferrin (CDT)

1.4.4.1 Transferrin structure

Human serum transferrin is a glycoprotein with a molecular mass of about 80,000 Da that is synthesized mainly in the liver. Transferrin consists of a single polypeptide chain containing 679 amino acid residues and two N-linked complex type glycan chains at positions 413 and 611 [77]. Transferrin exhibits a high degree of microheterogeneity (i.e. structural variations) as a result of genetic variants of the polypeptide chain, differences in the glycan structure, and varying iron load [77]. Genetic transferrin variants are quite common with transferrin C being the most prevalent phenotype in all populations, whereas allelic B (lower pI) and D (higher pI) variants, with a different primary structure but a normal set of carbohydrate chains, occur at low frequencies [78]. Transferrin displays also a high degree of glycan

microheterogeneity, which can be biantennary, triantennary, or even tetraantennary. Furthermore, glycans may differ in their terminal sialic acid residues content, which gives rise to nine different glycoforms, namely asialo- to octasialo transferrin (total of zero to eight sialic acid residues, respectively) [77, 79] (Fig. 7). The major glycoform found in serum, accounting for ~80% of total transferrin, has two disialylated biantennary glycans and is named tetrasialotransferrin [80]. Other detectable glycoforms are pentasialo- (~14%), trisialo- (~4–5%), disialo- (<2%), and hexasialotransferrin (<1%) [80].

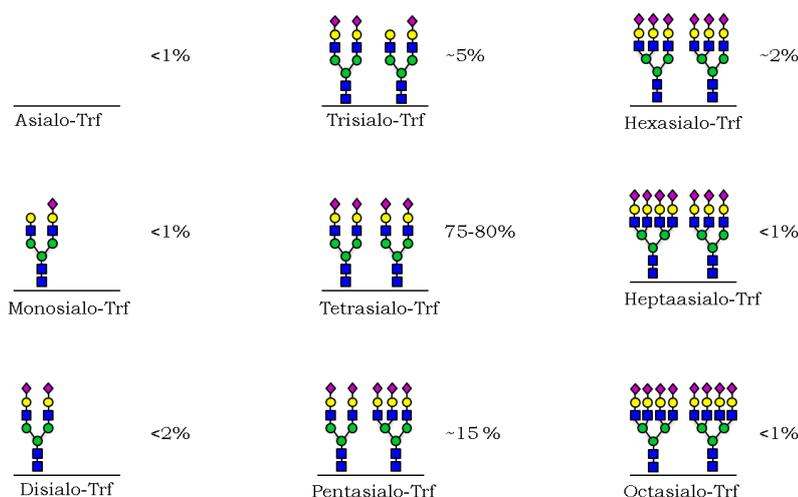


Figure 7. Different transferrin (Trf) glycoforms found in human serum and their relative amount (%). ♦ sialic acid ● galactose ● mannose ■ N-acetylglucosamine.

1.4.4.2 CDT

CDT refers to the altered pattern of serum transferrin that occurs as a result of prolonged heavy alcohol consumption, and it has emerged as a useful biochemical marker for identifying chronic alcohol abuse and for monitoring abstinence from alcohol during treatment [81]. Regular high alcohol intake, averaging at least 40 g/day for two weeks or more, generally results in a different transferrin glycoform profile in which the relative amount of disialotransferrin and asialotransferrin increases [82]. Transferrin half-life is ~1.5–2 weeks and after drinking is stopped, the glycoform pattern will slowly normalize [81], but it may require abstinence for one month or longer to reach a stable baseline level [83].

1.4.4.3 CDT analysis methods

Determination of CDT can be done by several analytical techniques that are based on different properties of the transferrin molecule. In high-performance liquid

chromatography (HPLC) [84] and capillary electrophoresis (CE) [85] methods, the glycoforms are separated based on their charge, and detected by a photometer. Another method, immunonephelometric CDT assay, is based on a monoclonal antibody that specifically recognizes the structure of transferrin glycoforms that lack one or both of the complete glycan, (i.e. asialo-, monosialo-, and disialotransferrin) [86].

A drawback in CDT analysis is the lack of standardization [87] which is an important process to ensure the reliability of measurements, both within and between testing sites [19]. The use of several analytical methods for CDT measurements, each with its own characteristics and reference interval, hampers the comparison between studies. Consequently, a working group on standardization of CDT (WG-CDT) has been established, aiming to define and validate the analyte, select a reference method, work out procedures for the production of reference materials, and make suggestions for the clinical usage of CDT [87]. According to the WG-CDT recommendations, disialotransferrin should be the primary target molecule for CDT measurements, the analytical principle should be HPLC, and CDT should be expressed in a relative amount (%CDT) [87].

1.4.4.4 CDT analysis interferences

Even though an increased level of serum %CDT is considered conclusive evidence of heavy drinking, several other factors have been previously suggested to affect CDT levels [88]. Later on, most of these factors were shown to be connected with the unspecific methods used as that time, and that age, gender, ethnicity, and other causes do not interfere markedly with the currently recommended analytical procedures [80, 87, 89]. However, there are few known factors that might interfere with CDT analysis, including genetic transferring variants [90], congenital disorder of glycosylation (CDG) [91], and liver abnormalities [92, 93].

1.4.5 Phosphatidylethanol (PEth)

In the early 1980's, an abnormal phospholipid was detected in organs from alcohol-fed rats [94], and was later on identified as PEth [95]. The term PEth does not refer to a single molecule but a group of phospholipids having a similar structure. The PEth molecules contain a glycerol backbone, a non-polar phosphoethanol head group, and

two fatty acids (Fig. 8) with a chain length of 14–22 carbon atoms and varying number of double bonds [96].

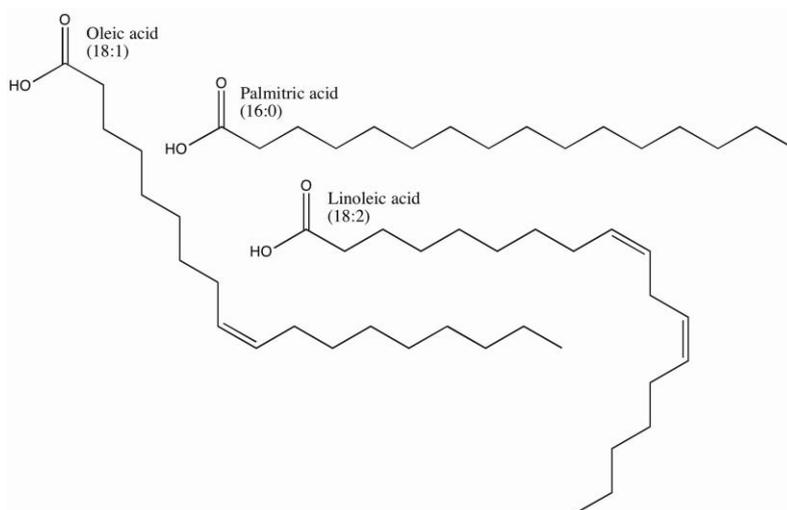


Figure 8. Three out of many fatty acids that can be observed in PEth molecules (number of carbon atoms: number of double bonds).

Formation of PEth takes place in cell membranes [94] through the enzymatic action of phospholipase D (PLD) that catalyzes the hydrolysis of the phosphodiester bond of phosphatidylcholine (PC) [97]. In addition to the hydrolysis reaction, PLD is also capable of catalyzing transesterification in the presence of ethanol, a reaction in which the phosphatidyl group of PC is transferred to alcohol and thereby forming PEth [98] (Fig. 9).

Measurement of PEth can be done in whole blood, using LC-MS(/MS) [96, 99, 100], CE [101], and immunological methods [102].

Several inter- and intra-individual variations exist regarding PEth formation, degradation and composition of the molecular species, which might complicate the interpretation of results [103]. Formation of PEth has been found to correlate with the amount of alcohol ingested [104, 105] but the relationship varies between individuals [93, 105]. Degradation of PEth is also subject to variation between individuals, and the half-time of PEth was found to range between 4–10 days [106, 107]. An additional inter- and intra-individual variation can be observed in the composition of PEth species as a result of the numerous combinations of fatty acids on the molecule [100, 108]. These combinations give rise to many molecular species of which 48 could be identified in blood from a heavy drinker [96]. However, the predominant species in

blood collected from social and heavy drinkers are PEth 16:0/18:1 and 16:0/18:2, which together account for 60–70% of total PEth [100, 108] and correlate well with the total PEth amount [99].

One of the advantages of PEth as an alcohol biomarker is its high specificity for alcohol, since it is formed from ethanol [99]. Moreover, PEth is not influenced by gender [109, 110] or liver diseases [110], which offers an additional advantage in comparison to some other long-term biomarkers [93, 111]. However, a potential cause of false-positive PEth result is post-collection formation of PEth in improperly stored samples containing ethanol [112].

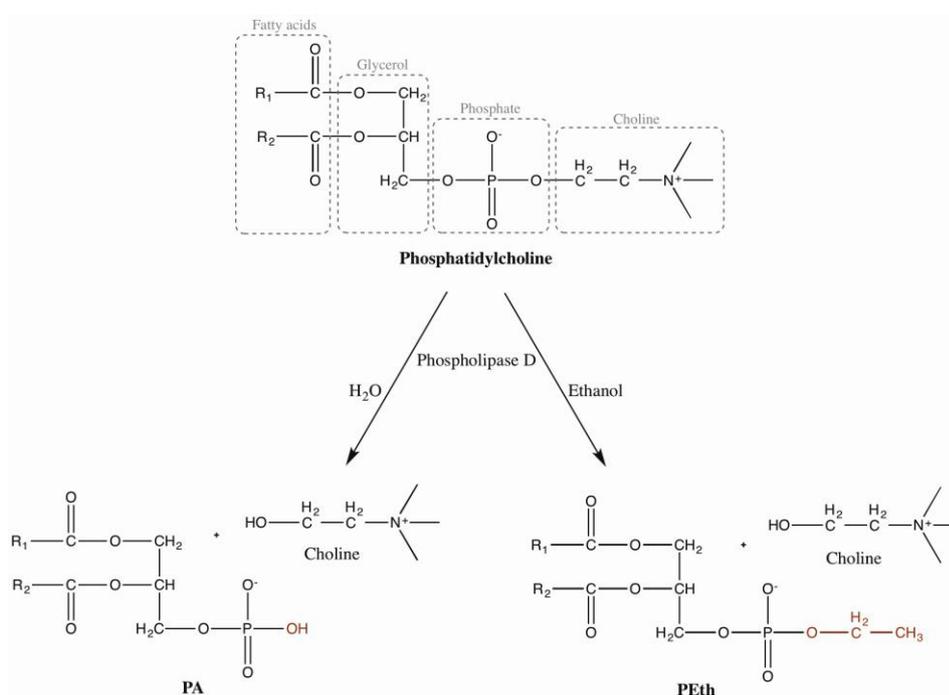


Figure 9. Formation of PEth. Phospholipase D catalyzes the hydrolysis of phosphatidylcholine producing phosphatidoc acid (PA) and choline. In the presence of ethanol, the transphosphatidyl transfer reaction takes place, and PEth is formed. R₁ and R₂ are fatty acid chains with varying length and degree of saturation.

2 AIMS

The general aim of this thesis was to study the reliability of alcohol biomarkers, taking into considerations both analytical and clinical aspects. Specific studies were undertaken:

- To evaluate whether the international guidelines concerning the performance and the design of analytical methods can ensure reliable identification when applied to EtG.
- To compare the analytical performance of CE and HPLC in samples giving rise to various interferences in the CE system during CDT analysis.
- To study the change in transferrin glycoform pattern during pregnancy, and evaluate the possible risk for false positive CDT results.
- To study the effect of glycated transferrin on CDT measurement, following both in vitro and in vivo glycation. An additional aim was to examine whether glycation interferes with CDT analysis in diabetic patients, by comparing with PEth results.

3 MATERIAL AND METHODS

3.1 CLINICAL SAMPLES

3.1.1 Urine samples for EtG and EtS analysis

3.1.1.1 Paper I

De-identified urine specimens were collected from leftover volumes from drinking experiments, clinical studies, and the routine samples pool at the Alcohol Laboratory (Karolinska University Hospital, Stockholm). A total of 482 urine samples were selected according to their EtG concentrations that were previously determined by routine LC-MS measurements [113]. About one third of the samples were EtG negative (levels below 0.5 mg/L), another half were EtG positive at levels up to 1 mg/L, and the remaining samples contained EtG levels above 1 mg/L. The urine samples were stored frozen at -20°C, and later on were thawed and centrifuged prior to analysis.

3.1.2 Samples for CDT/PEth analysis

3.1.2.1 Paper II

The serum samples used in this project were de-identified, and sent to us from a routine laboratory in Oslo, Norway. A set of 183 samples were initially analyzed by CE, and in cases of abnormal peak profile or other interferences in the electropherogram, samples were reanalyzed following Sebia's sample cleanup procedure. If the problem remained, or otherwise when confirmatory testing was required, samples were submitted for an additional analysis by an HPLC candidate reference method. Upon arrival, samples were stored at -20°C until analysis.

3.1.2.2 Paper III

In this study, 171 serum samples were collected from 24 healthy pregnant women having normal spontaneous pregnancies. The first sample was taken in gestation week 9–21, and the last sample was collected 8–15 weeks after delivery. In between, samples were collected every ~4th week throughout pregnancy. Samples were stored at -20°C until analysis.

3.1.2.3 Paper IV

The samples used in this study were de-identified leftover volumes from the routine sample pool at the Karolinska University Laboratory (Stockholm, Sweden). The samples were originally submitted for routine CDT or HbA1c testing. For the *in vitro* glycation study, five serum specimens were collected on the basis of their CDT (%disialotransferrin) concentration, covering the range of 1.0–2.5%. For CDT and PEth measurements in diabetic patients, 50 EDTA whole blood samples with low HbA1c concentration (<44 mmol/mol) and 50 samples showing high levels (>68 mmol/L) were selected. For PEth analysis, 200 μ L of whole blood were transferred into smaller tubes and placed at -70°C until analysis. The remaining whole blood volumes were then centrifuged and the plasma fractions used for CDT measurements were collected and stored at -20°C until analysis.

3.2 ANALYTICAL METHODS

3.2.1 EtG and EtS

3.2.1.1 Solid phase extraction

Sample cleanup was done by using a HyperSep SAX SPE cartridge (Thermo Scientific, Waltham, MA) which was previously found suitable for EtG [114]. The SPE cartridge was initially conditioned with methanol, deionised water, and acetonitrile (1 mL of each component). A sample mixture consisting of 50 μ L urine or standard specimen, 100 μ L internal standard (IS), 200 μ L water, and 700 μ L of acetonitrile, was then allowed to flow through the cartridge. The column was subsequently washed with 1 mL deionised water and 1 mL acetonitrile, and EtG was eluted with 1 mL of a solution containing acetonitrile, water, and formic acid (95:4:1, v/v). Nitrogen gas was then used to evaporate the solvent and EtG was finally dissolved in 110 μ L deionised water.

3.2.1.2 LC-MS

Urinary EtG measurements were done using an ESI-LC-MS system (Agilent 1100) operating in negative ion mode, as previously described [113]. Chromatographic separation was carried out on a porous graphite column (HyperCarb, 5 μ m, 2.1 \times 100 mm, Thermo Scientific), equipped with a 2.0 \times 10 mm HyperCarb guard cartridge. The

mobile phase consisted of 25 mmol/L of formic acid and 5% acetonitrile dissolved in water, and the flow rate was 0.2 mL/min.

Using selected ion monitoring (SIM), the following species could be detected: m/z 221 for EtG, m/z 125 for EtS, m/z 226 for EtG-d₅ (IS) and m/z 130 for EtS-d₅ (IS). EtG and EtS concentrations were determined from the peak-area ratio of EtG/EtG-d₅ and EtS/EtS-d₅ by reference to a calibration curve. The criteria for correct identification of EtG was based on the presence of a peak with correct relative retention time (within 0.5% of the retention time for standards), and a detector response with a signal-to-noise (S/N) ratio >3.

3.2.1.3 LC-MS/MS

The LC-MS/MS experiment was done using two instruments depending on EtG concentration. For samples containing more than 1 mg/L according to the routine measurements, analysis was performed on a Perkin-Elmer series 200 LC systems connected to a Sciex API 2000 mass spectrometer (Applied Biosystems, Canada). The ESI interface was used operating in the negative ion mode with the same chromatographic conditions as described above (LC-MS).

For samples having lower EtG concentration (<1 mg/L), the MS used was an Acquity UPLC connected to a Quattro Premier XE tandem mass spectrometry (Waters, Milford, MA, USA). Chromatographic separation was achieved on a high-strength silica (HSS) trifunctional C₁₈ column (1.8 μm, 2.1 × 100 mm, Waters) preceded by a 0.2 μm column filter (Waters). The mobile phases consisted of (A) 0.1% formic acid in water, pH 2.82, and (B) 100% acetonitrile, flowing at 0.4 mL/min.

In the LC-MS/MS studies, EtG identification was performed by monitoring the deprotonated molecule (m/z 221) and the two most intense product ions m/z 75 (qualifier) and m/z 85 (quantifier). EtG concentrations were determined from the peak-area ratio of EtG/EtG-d₅ by reference to a calibration curve, covering the range of 0.1–100 mg/L. The criteria for correct identification were identical to those of LC-MS, but in addition, the product ions (i.e. m/z 85/75) ratio needed to be within ±20% of the expected value determined for a standard solution prepared in EtG-negative urine.

3.2.2 CDT

3.2.2.1 HPLC

CDT measurement in serum was done by an HPLC method [84] that enables the separation and relative quantification of the different transferrin glycoforms. Prior to analysis, 100 μL of each serum sample was saturated with iron by adding 20 μL ferric nitrilotriacetic acid (FeNTA) solution (10 mmol/L, pH 7.0). Thereafter, serum lipoproteins were precipitated by adding 20 μL of a solution containing dextran sulfate and CaCl_2 (20 g/L and 1 mol/L, respectively). The samples were mixed gently and kept cold (4°C) for 30–60 min followed by 10 min centrifugation at $3000 \times g$. Finally, 100 μL of the clear supernatant was diluted with 400 μL water and transferred into glass vials.

The HPLC system was an Agilent 1100 Series LC, equipped with a quaternary pump, degasser, thermostated autosampler, and a multiple wavelength detector. The transferrin glycoforms were separated on a strong anion-exchange column (SOURCE 15Q 4.6/100, GE Healthcare) followed by salt gradient elution. Detection of the different glycoforms was done by measuring the absorbance of the transferrin-iron complex at 460–470 nm.

3.2.2.2 Capillary electrophoresis

CDT measurement by CE was performed using the Capillarys CDT assay run on the Sebia Capillarys 2 multicapillary analyzer for serum protein analysis. This method is fully automated and all reagents used were supplied with the test kit. In this technique, samples were iron-saturated at the anodic end of the capillary, and submitted to high voltage (8200 V) which initiated the separation of the glycoforms in an 18 cm long (25 μm i.d.) fused-silica capillaries. Detection of the different glycoforms was done thorough absorbance measurements at 200 nm in the cathodic end of the capillary. Following analysis, the relative amount of each glycoform is calculated automatically but it is possible to modify peak integration manually. According to the manufacturer's instructions, samples showing $\text{CDT} \leq 1.3\%$ are considered normal and $\text{CDT} > 1.6\%$ are positive. CDT results between 1.3 and 1.6% are considered inconclusive. Additionally, using the complementary sample treatment solution is recommended by the manufacturer in cases of poor electrophoretic patterns.

3.2.3 PEth

3.2.3.1 Extraction

Isolation of PEth species was done according to an established procedure [100] in which lipids are extracted from whole blood by stepwise addition of 100 μL blood to 600 μL isopropanol and 25 μL IS (phosphatidylpropanol, 1.35 $\mu\text{mol/L}$) under constant vortex-mixing. After 10 min of gentle mix, heptane was added ($2 \times 450 \mu\text{L}$) with mixing after every addition. After another gentle mix for 10 min, samples were centrifuged for 10 min at 2000g at 4°C and the clear supernatants were transferred to new glass tubes and evaporated to dryness under a stream of nitrogen gas. The dried extract was then dissolved in 50 μL hexane, and mixed with 50 μL acetonitrile and 75 μL isopropanol.

3.2.3.2 LC-MS

The instrument used for PEth measurements was ESI-LC-MS (Agilent, 1100 series). The lipid extracts were separated on a HyPurity C₄ column (Thermo Scientific) of dimension 50 mm \times 3 mm id, and 5 μm particle size. A binary pump was used, and the mobile phases consisted of 20% 2 mmol/L ammonium acetate and 80% acetonitrile, and 100% isopropanol, flowing at 0.2 mL/min. The MS instrument was operated in the negative mode and the data was acquired using SIM of the deprotonated molecule of the different PEth species and PProp (IS). PEth concentrations were determined from the peak-area ratio of PEth/PProp by reference to a calibration curve.

4 RESULTS

4.1 PAPER I

The aim of this study was to try to find a compromise between theoretical considerations for analytical selectivity and practical experience in the verification step. In this work, EtG and EtS were used as model compounds to compare the bioanalytical identification requirements, as listed in various international guidelines.

Among the five methods tested, the procedure involving initial sample cleanup by SPE followed by LC-MS/MS analysis (Method 1) achieved the lowest limit of detection (LOD; <0.001 mg/L) and was therefore chosen as the reference method in this study. Among 482 sample analyzed by this method, EtG was identified according to the compound identification criteria in 429 (89%) samples, showing EtG concentrations in the range of 0.006–444 mg/L. The relative retention time for EtG compared with EtG-*d*₅ was 0.985 ± 0.003 (mean \pm standard deviation (SD); range 0.956–0.996) and the mean product ion ratio (*m/z* 85/75) ranged between 0.879–0.934 in different analytical runs.

The measured levels of EtG obtained by the different methods were compared to results from the reference method, using regression analysis (Fig 10). Analysis of 429 samples resulted in correlation coefficients between 0.962 and 0.978, over the entire concentration range, with slopes ranging from 0.919 to 1.000. Similar result were obtained in the lower concentration range (<2.0 mg/L EtG by Method 1, N = 348), with correlation coefficients of 0.956–0.966 and slopes between 0.900 and 1.000. Analysis by different methods caused large differences in EtG concentrations for a number of samples, which remained also after re-analysis of these samples.

In addition to the quantitative comparison, the analytical sensitivity and specificity of the other methods were compared to the results obtained from the reference method at five selected cutoff levels, ranging from 0.10 mg/L up to 1.00 mg/L. For each reporting limit, the frequency of true positives plus true negatives for each method was compared with the SPE-LC-MS/MS results. For direct-injection LC-MS/MS, the overall agreement was in the range 81.6–97.2% (cutoff 0.1–1.0 mg/L, respectively), for SPE-LC-MS 90.2–97.0%, for direct- injection LC-MS 85.5–97.7%, and for combined direct-injection LC-MS analysis of EtG and EtS 85.8–97.7%.

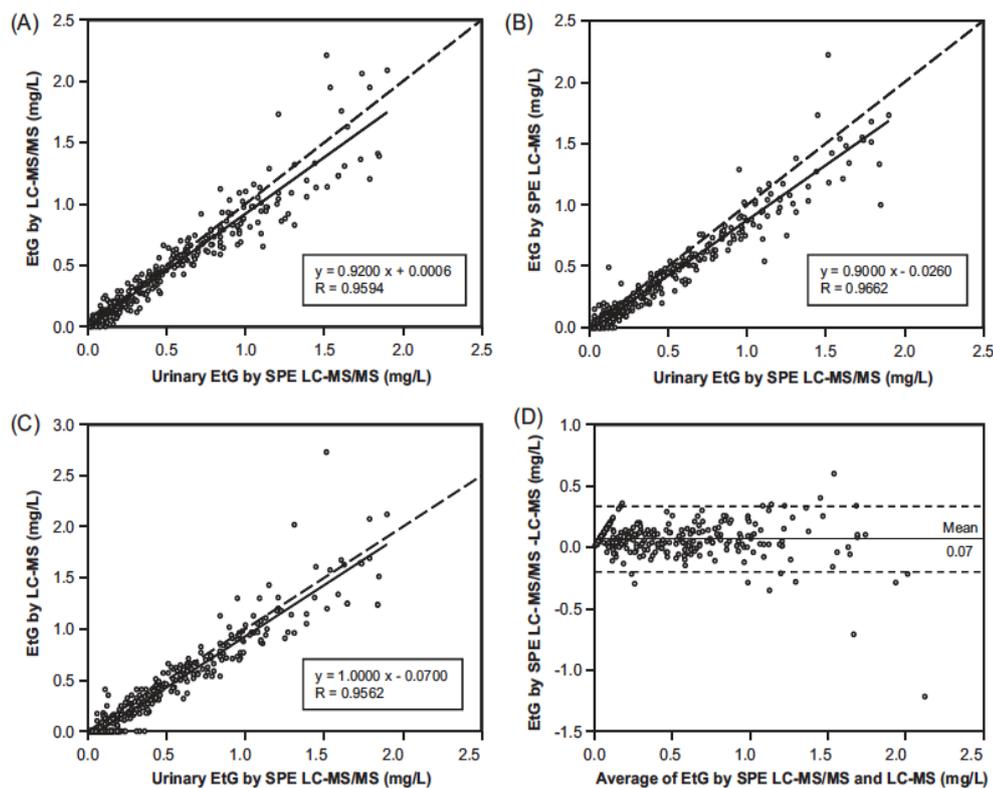


Figure 10. Method comparison of urinary EtG results obtained by different LC-MS and LC-MS/MS analytical procedures. Data shown are (A–C) Passing & Bablok regression for 348 urine samples containing <2 mg/L EtG according to the SPE-LC/MS/MS reference method. Broken lines are $x = y$. (D) Bland and Altman plot for urinary EtG concentrations <2 mg/L obtained by the SPE-LC/MS/MS reference method and a direct injection LC-MS method. The solid line represents the mean value and the broken lines are ± 2 SD.

For the LC-MS methods, most cases of disagreement with the reference method were due to uncertainty in quantification for samples showing EtG concentration close to the cutoff levels. For the direct-injection LC-MS/MS, the additional criteria of product ion ratio was an equal contributor to the disagreement with the reference method, accounting for 51% of all negative cases. Product ion ratios outside the range of $\pm 20\%$ were more frequent at low concentrations, but even for six samples having EtG concentrations in the range 0.50-0.93 mg/L.

The overall agreement for the different procedures comparing to the reference method, at different cutoff values was plotted (Fig. 11). By all analytical procedures, the highest accuracy for urinary EtG was achieved at the 0.5 mg/L decision limit.

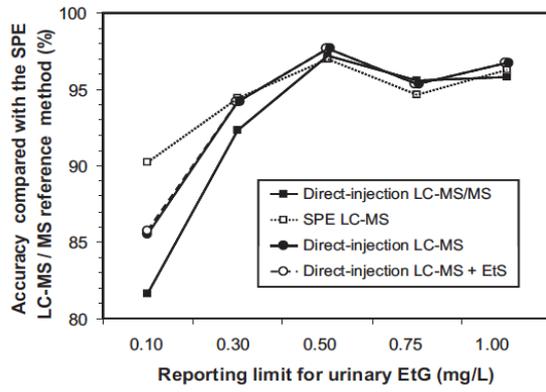


Figure 11. Overall accuracy of the LC/MS and LC/MS/MS procedures for urinary EtG compared with the SPE-LC/MS/MS reference method. The frequency of true-positive plus true-negative results out of all results is shown for each of the analytical procedures at the five selected thresholds.

4.2 PAPER II

The aim of this study was to demonstrate the importance of HPLC confirmatory analysis of CDT results by CE. This study was done by comparing two analytical methods, CE and HPLC, for CDT analysis. Measurements of CDT levels in 58 unproblematic samples (i.e. not showing analytical interferences) revealed a good correlation between the methods over the entire concentration range (Fig. 12A). However, the HPLC method generated higher values in the lower concentration range (CDT < 2%) (Fig. 12B)

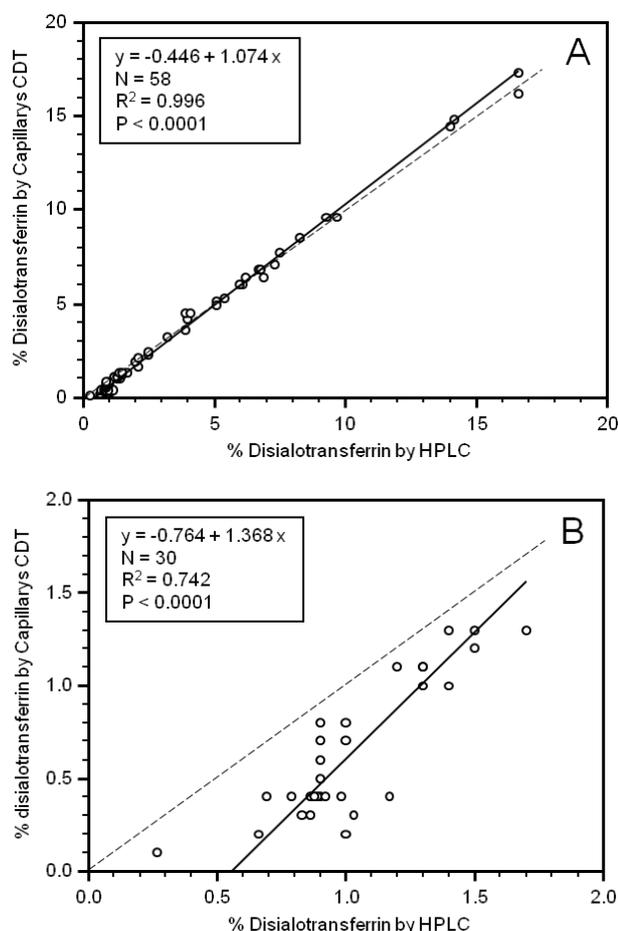


Figure 12. Passing and Bablok regression analysis for serum disialotransferrin values obtained by CE (y-axis) and an HPLC (x-axis) over the entire concentration range (A), and (B) CDT levels below 2%.

Due to several analytical interferences, a total of 183 samples could not be properly quantified by the CE method and were therefore submitted for re-analysis by HPLC. The frequency of samples encountering this problem was calculated to 0.6%, of which half could be successfully analyzed after performing the Sebia Sample treatment procedure. The remaining samples (0.3%) continued to display abnormal

peak profiles or other disturbances in the electropherogram, and were consequently subjected to a confirmatory HPLC analysis.

The analytical interferences resulted in poor electropherograms, exhibiting altered glycoform profiles, disturbing, or even missing peaks, and thereby disabling correct quantification of CDT by the CE method.

Genetic transferrin variants can disrupt CDT analysis by showing a different peak profile, which is automatically recognized by the Capillarys software. However, HPLC analysis revealed that among the 51 samples detected as “genetic variants” by CE, only 63% were correctly identified, while the remaining 37% showed normal glycoform profile.

In another 41 samples (22%), no CDT value was given since the Capillarys software failed to detect or quantitate the disialotransferrin peak. In 71% of these samples, incomplete separation was observed between disialo- and trisialotransferrin by the HPLC method. The remaining samples were successfully quantified by HPLC but the total transferrin concentration (based on total peak area) in many samples was low.

Another 37 (20%) samples were re-analyzed by HPLC because their CDT values were at, or slightly above the CE cut-off level (1.3%). A confirmatory analysis showed that 9 (24%) samples had normal CDT levels and only 3 (8%) cases displayed elevated disialotransferrin level. However, the majority of the samples (68%) were showing poor separation between disialo- and trisialotransferrin by HPLC, of which 54% had disialotransferrin values >1.7%.

Disturbing peaks in the electropherogram, either under the disialotransferrin peak or under the entire area, were observed in 41 samples. A normal glycoform profile was seen in 35 (85%) samples of which 6 (15%) had elevated CDT levels.

Another reason for interferences in the CE method was caused by samples collected in tubes containing EDTA. Quantification of disialotransferrin in these samples was possible by the HPLC method despite the characteristic peak that co-elutes with asialotransferrin.

4.3 PAPER III

The aim of this project was to study whether pregnancy related changes in transferrin glycosylation pattern could result in an increased risk for false positive CDT results. Measurement of serum transferrin glycoforms was done in 171 serum samples collected from 24 pregnant women (range 5–7 samples/woman, mean 7.1, median 7.0). During the course of pregnancy, all subjects displayed a gradual elevation in total transferrin concentration which was associated with an altered glycosylation pattern. The average transferrin concentration increased from 2.52 ± 0.28 g/L (mean \pm SD) in the initial sample to 3.89 ± 0.69 g/L in the last sample taken before delivery, which corresponds to a 50% increase.

Measurements of the relative amount of each of the five glycoforms showed a continuous increase in disialo-, pentasialo-, and hexasialotransferrin, while levels of trisialo- and tetrasialotransferrin decreased. The change in glycoform profile was observable from 14 weeks into pregnancy but was even more apparent from week 28 until delivery.

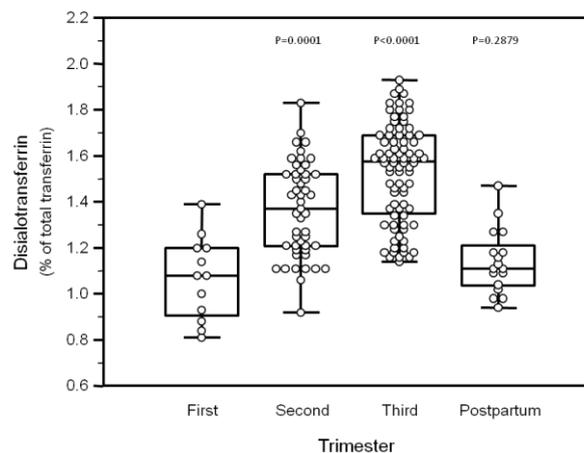


Figure 13. Box-and-whisker plot for the relative amount of serum disialotransferrin concentrations. The first trimester is roughly until the end of week 13, the second from week 14–27, and the third is from week 28 to the end of the pregnancy. The box-and-whisker plot shows the median, 25th and 75th percentiles, minimum and maximum values.

The relative amount of disialotransferrin increased with ~50% during pregnancy, from $1.07 \pm 0.17\%$ (mean \pm SD) in the initial samples to $1.61 \pm 0.23\%$ before delivery (Fig. 13). A similar increase averaging 50%, was observed for pentasialotransferrin, with corresponding levels of $13.2 \pm 1.40\%$ and $19.7 \pm 1.70\%$, respectively, and hexasialotransferrin levels were $0.78 \pm 0.38\%$ and $2.63 \pm 0.72\%$ (>300% increase). A decrease in the relative amount was seen for the trisialo- and tetrasialotransferrin

glycoforms, with initial values of $5.28 \pm 0.88\%$ to $3.81 \pm 0.70\%$ before delivery (~30% decrease), and from $79.6 \pm 1.35\%$ to $72.2 \pm 2.18\%$ (~10% reduction), respectively.

Analysis of the samples collected 8-15 weeks delivery (Fig. 13) showed that the relative amount of all glycoforms was returning towards their initial values, but still, the levels of tetrasialo-, pentasialo-, and hexasialotransferrin were significantly different.

The highest %disialotransferrin (i.e. %CDT) value observed in 37.5% of the pregnant women was $\geq 1.70\%$, with a maximum value of 1.93% (Fig. 14). For non-pregnant controls, %disialotransferrin levels of 1.70% and 1.90% are equivalent to the mean + 2SD and mean + 3SD [80, 84] (Fig.14).

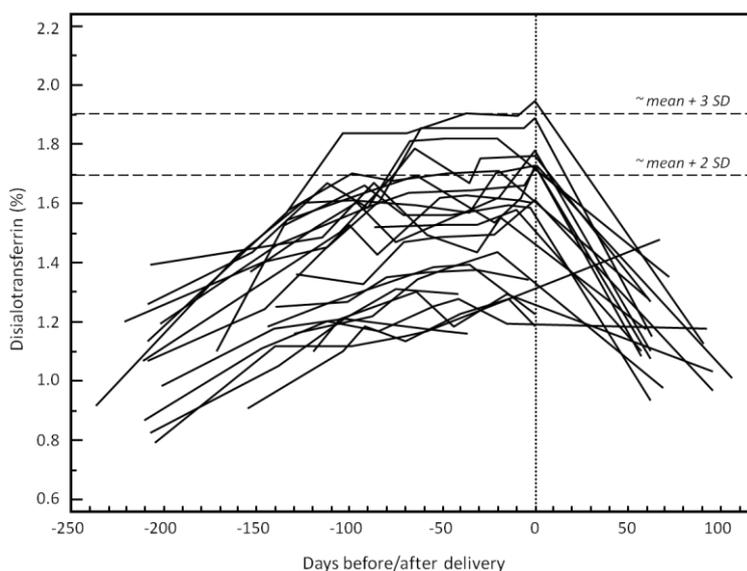


Figure 14. Line graphs showing individual changes in %disialotransferrin values in serum samples during the progress of gestation and once after delivery. The mean + 2 SD and mean + 3 SD for control populations are given.

4.4 PAPER IV

The aim of this project was to study whether transferrin glycation (i.e. non-enzymatic reaction with glucose) could affect the use of CDT as an alcohol marker, since glycation was previously shown to impair iron binding to transferrin [115].

HPLC analysis of serum transferrin undergoing *in vitro* glycation revealed an altered glycoform pattern. Following incubation with glucose, time- and dose-dependent changes were observed, resulting in wider peaks and poorer separation, mainly for the highly sialylated glycoforms. The relative amount of disialotransferrin was decreased to 87% and 74% following 24 h incubation with 20 or 200 mmol/L glucose, respectively (Fig. 15). The corresponding level of disialotransferrin in samples incubated without glucose was 99% on average. Following 48 h of incubation, the disialotransferrin levels continued to decline, and separation between peaks was even poorer, especially for tetrasialo-, pentasialo-, and hexasialotransferrin (Fig. 15).

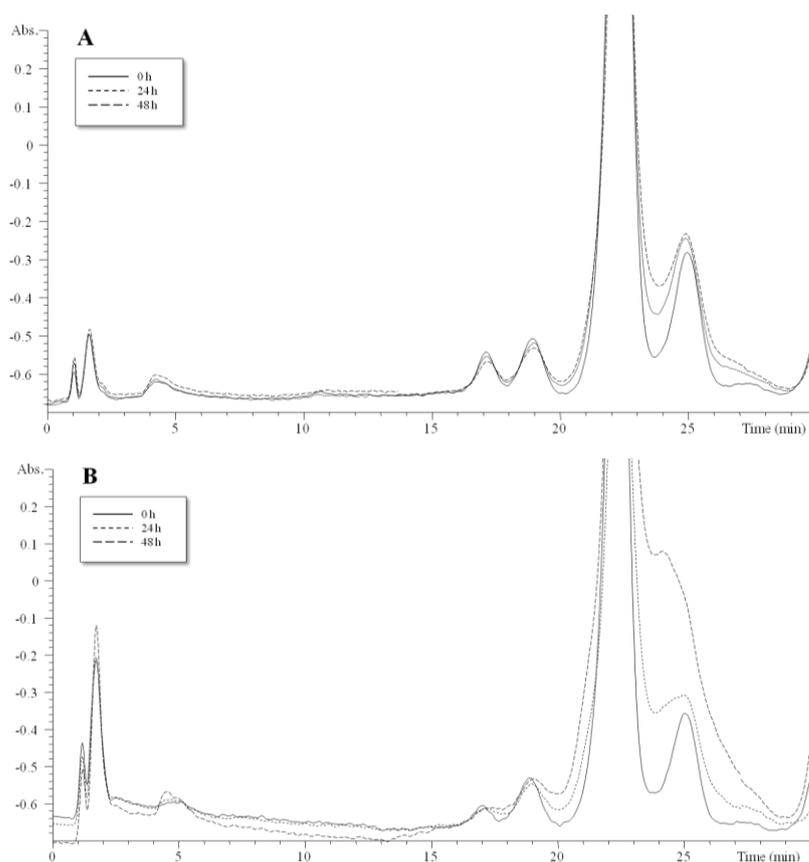


Figure 15. HPLC chromatographic changes in transferrin glycoform pattern following incubation with (A) 20 mmol/L and (B) 200 mmol/L glucose at baseline (0 h) and after 24 h and 48 h.

Analysis of transferrin glycoform and CDT measurements were done in 50 clinical samples showing normal/low HbA1c level (<44 mmol/L; range 29– 43 mmol/mol, mean 37), and 50 samples with elevated concentrations (>68 mmol/mol; range 69– 128 mmol/mol, mean 88). Comparison of peak width for the different glycoforms showed no significant differences between the two groups. However, on average the %CDT (%disialotransferrin) level was significantly higher in samples with elevated HbA1c levels (mean 1.21% disialotransferrin) compared to samples with normal/low HbA1c levels (mean 1.06% disialotransferrin) (Fig. 16).

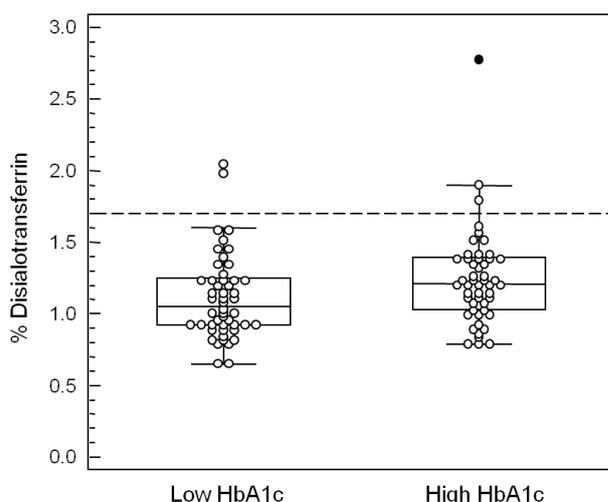


Figure 16. Box-and-whisker plot showing %CDT values in samples with low (< 44 mmol/mol) or elevated (> 68 mmol/mol) HbA1c values.

An additional significant difference was found in the relative amount of trisialotransferrin, showing lower levels (mean 4.6%) in samples with higher HbA1c, and higher levels (mean 5.0%) in samples with a low/normal HbA1c. Overall, five samples showed CDT levels >1.7% (mean $2 \pm \text{SD}$ for controls) [84], of which three had elevated HbA1c, and two low HbA1c.

The mean concentration of PEth showed no significant difference between samples with elevated HbA1c (0.37 $\mu\text{mol/L}$) and samples low HbA1c (0.30 $\mu\text{mol/L}$) (Fig. 17). Similarly, measurements of the major PEth species (16:0/18:1) revealed no significant difference for sample with elevated and low HbA1c levels (0.15 and 0.10 $\mu\text{mol/L}$, respectively). However, the PEth levels in 17% of samples exceeded the recommended cutoff value [99], suggestive for recent high alcohol intake. Positive PEth levels were found in both samples with elevated HbA1c (frequency 18%) and low HbA1c (16%).

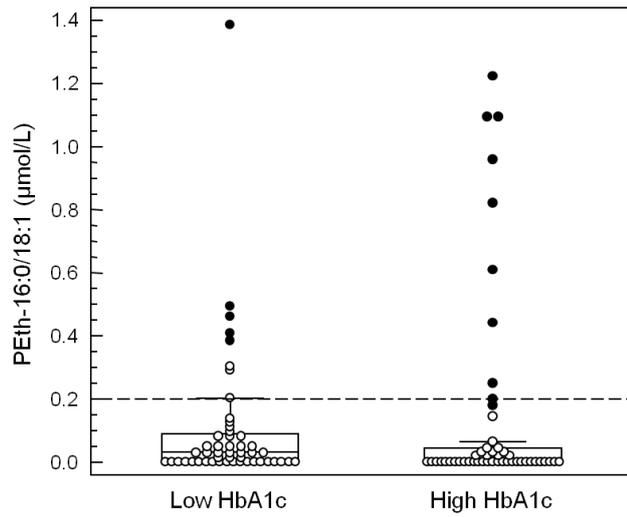


Figure17. Box-and-whisker plot showing PEth-16:0/18:1 values in blood samples with low (< 44 mmol/mol) or elevated (> 68 mmol/mol) HbA1c values.

5 DISCUSSION

Alcohol biomarkers can be used for different purposes, including identification of individuals who are at risk for alcohol-related problems, measuring AUD treatment outcome, and monitoring individuals in occupation and traffic medicine settings [23]. Given the potentially serious medical–legal problems that may be associated with a positive test value, the use of a valid biomarker is essential.

Traditional alcohol biomarkers such as MCV, GGT, AST and ALT are indirect measures that reflect alcohol toxicity or damage, and suffer from low specificity for alcohol [70, 73, 76]. In contrast, the direct markers (metabolites) PEth and EtG are formed only in the presence of ethanol and thus specific for alcohol [43, 98]. Moreover, the sensitive methods used for PEth and EtG analysis enables the detection of even a single intake of alcohol (EtG) or low/moderate drinking levels (PEth) [99, 100]. Improvements have also been done in CDT methodology [84] and previous reports suggesting several causes of false-positive results were dismissed [89].

There are several concerns that need to be addressed when evaluating a new biomarker. A first concern is the analytical validity, which refers to the ability of a test to measure the biomarker accurately [116]. Another aspect is the clinical validity, referring to the biomarker's ability to predict the presence or absence of a certain condition, expressed in terms of diagnostic accuracy (sensitivity and specificity) [117]. Lastly, the clinical usefulness should be considered [116], which hopefully leads to an improved outcome for the patient.

The first study in this work dealt with analytical aspects, in which different analytical strategies for accurate determination of EtG were compared. The identification point (IP) system, according to the EU criteria for compound identification, was then applied for the different LC-MS analytical procedures and the diagnostic performance of EtG was studied. The results showed that meeting the identification criteria and scoring higher IP does not necessarily eliminate the risk for false positive/negative results. Moreover, depending on the cutoff value applied, accurate determination of EtG could be accomplished by less sophisticated, less expensive, and faster procedures.

In the second paper, both analytical and clinical considerations were taken into account, when CDT results from two different routine methods were compared. Analyzing CDT by either CE or HPLC offers the advantage of visual presentation of transferrin glycoforms, by which abnormal patterns can be easily observed. However, the unselective wavelength (200 nm) used for detection in CE may lead to interferences in the electropherogram when analyzing samples containing high levels of other biomolecules, such as C-reactive protein, immunoglobulin and complement factors [118]. The different detection procedures might explain the disturbing peaks observed in 22% of the samples analyzed by CE while no interferences were apparent by HPLC analysis. Another problem concerning both methods is the poor chromatographic separation between disialo- and trisialotransferrin, causing difficulties in result interpretation. Mass spectrometric investigation of such samples revealed alterations in the glycan structure, and this phenomenon was reported to occur in higher prevalence among patients with liver abnormalities [92, 93, 119].

Overall, the CE method could not provide reliable CDT results in ~0.6% of the routine samples, which is in agreement with other observations [120, 121]. However, most of the problems encountered by CE could be solved by the HPLC method. Consequently, the combination of high throughput CE with an option for a confirmatory procedure by the HPLC candidate reference method [87] will provide an efficient and high qualitative workflow for the routine analysis of CDT.

Apart from instrumental limitations that can lead to decreased reliability of the biomarker, pathological factors should be considered as well. As observed in study II, genetic transferrin variants and possible liver abnormalities interfered with interpretation of CDT result when analyzed by CE. Another physiological state that interferes with CDT analysis is pregnancy, as demonstrated in study III. During pregnancy, a gradual increase in CDT level was observed, and for many subjects, these levels reached the upper limit of the reference interval. These results were later on confirmed by two independent studies [122, 123]. Overcoming the problem of decreased specificity of CDT in pregnant women can be done by either applying a slightly higher cutoff value, or using an alternative biomarker. PEth, which is highly specific for ethanol [100], could be an especially useful biomarker during pregnancy since it can be detected even after low/moderate alcohol intake [99], which are often

the levels of interest in this group. Moreover, PEth was found to be a sensitive indicator of drinking in women during reproductive age [105].

An additional pathological condition that might interfere with CDT testing is diabetes. The results demonstrated that in vitro glycated transferrin caused changes in the glycoform pattern, showing decreased levels of %disialotransferrin (%CDT). However, the transferrin glycoform pattern in samples originating from diabetic patients with elevated HbA1c was apparently unaffected. Additionally, no indication of reduced CDT levels was found in those specimens when compared to samples with low HbA1c. Instead, significantly higher %CDT values were detected in samples from diabetics showing a high HbA1c. Among all patients, about 5% had elevated %CDT values indicating risky drinking, while nearly 17% showed elevated PEth levels, implying regular high alcohol intake. Results from this study showed that the use of CDT and PEth is useful in identifying diabetes patients with risky alcohol consumption. The higher frequency of PEth results comparing to CDT can be explained by its higher sensitivity [103], thus enabling detection of lower drinking levels. Additionally, it seems like transferrin glycation in vitro is different from that in vivo, as demonstrated for hemoglobin [124], since no chromatographic interferences were detected in the latter.

6 CONCLUSIONS

The present criteria for reliable compound identification by MS analysis, as suggested by the EU and other directives, do not always guarantee the exclusion of false test results. Consequently, the current criteria might need to be further developed, to include more requirements on sample pre-treatment and LC separation.

Confirmatory analysis of %CDT (%disialotransferrin) by HPLC provides accurate quantification, or at least an estimation of the level in serum samples showing analytical interferences by CE analysis. Therefore, a routine setting employing an initial high-throughput CE analysis of CDT should have access to a more sensitive and specific confirmatory HPLC analysis.

A gradual increase in the %disialotransferrin (%CDT) level was observed during pregnancy. To minimize the risk for false-positive CDT results, the cutoff value used to indicate heavy drinking in pregnant women needs to be raised slightly.

Transferrin glycation in vivo was suggested to differ from that in vitro, and did not interfere with CDT analysis by HPLC. CDT and also PEth were indicated to be suitable markers for detection of excessive alcohol intake in diabetic patients.

Taken together, the results of the present studies have identified and suggested ways to overcome a number of analytical and clinical interferences with these alcohol biomarkers, and thus helped to improve their routine use.

7 ACKNOWLEDGMENTS

It's not what you know but who you know.

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