Ethanol metabolites: Their role in the assessment of alcohol intake

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Abstract:

Background: Alcohol-related disorders are common, expensive in their course and often underdiagnosed. To facilitate early diagnosis and therapy of alcohol-related disorders and to prevent later complications, questionnaires and biomarkers are useful.

Methods: Indirect state markers like gamma-glutamyl-transpeptidase (GGT), mean corpuscular volume (MCV) and carbohydrate deficient transferrin (CDT) are influenced by age, gender, various substances and non-alcohol-related illnesses, and do not cover the entire timeline for alcohol consumption. Ethanol metabolites, such as ethyl glucuronide (EtG), ethyl sulphate (EtS), phosphatidylethanol (PEth) and fatty acid ethyl esters (FAEEs) have gained enormous interest in the last decades as they are detectable after ethanol intake.

Results: For each biomarker, pharmacological characteristics, detection methods in different body tissues, sensitivity/specificity values, cut-off values, time frames of detection and general limitations are presented. Another focus of the review is the use of the markers in special clinical and forensic samples.

Conclusion: Depending on the biological material used for analysis, ethanol metabolites can be applied in different settings such as assessment of alcohol intake, screening, prevention, diagnosis and therapy of alcohol use disorders.

Key words: alcohol intake, ethanol metabolites, ethyl glucuronide (EtG), phosphatidylethanol (PEth), ethyl sulphate (EtS), fatty acid ethyl esters (FAEEs)

Introduction

Alcohol use disorders (AUD) cause approximately 4 % of deaths worldwide – more than HIV, violence, or tuberculosis. In general hospitals, up to 20 % of all inpatients have an alcohol use disorder. In surgical departments rates from 16 % to 35 % were described in patients with multiple trauma (Tonnesen & Kehlet, 1999; Spies et al., 2001) leading to a number of unwanted consequences, including prolonged hospitalisation (Tonnesen & Kehlet, 1999; Spies et al., 2001; Rubinsky et al., 2012), more time in Intensive Care Units and higher rates of complications (Rubinsky et al., 2012).

Of all alcohol-dependent individuals, 80 % are treated by general practitioners, 34 % in general hospitals and a low percentage by addiction specialists (Mann, 2002).

To facilitate early diagnosis and therapy of alcohol-related disorders and thus prevent secondary complications, questionnaires like the Alcohol Use Disorders Identification Test (AUDIT) (Saunders et al., 1993) as well as reliable and valid biomarkers are useful. Biomarkers have the advantage to indicate and reflect alcohol intake, independent of recall bias of the interviewed subjects.

Both indirect and direct state markers are routinely used to detect alcohol intake. The indirect state markers such as gamma-glutamyl-transpeptidase (GGT), mean corpuscular volume (MCV), and carbohydrate deficient transferrin (CDT) are influenced by a number of factors like age, gender, and non-alcohol-related illnesses, and do not cover the entire time frame (acute, short-term, long-term) of alcohol use (Conigrave et al., 2002; Laposata, 1999; Helander, 2003; Hannuksela et al., 2007; Niemelä, 2007).

Direct ethanol metabolites have gained interest in recent decades as they are biomarkers with high sensitivity and specificity. Most frequently, EtG (ethyl

glucuronide) and PEth (phosphatidylethanol) are used in various settings and will therefore be discussed in more detail.

Ethanol metabolites

Ethanol metabolites are formed after alcohol consumption and are minor pathways of ethanol elimination. They each cover different time frames of detection and can be determined in different matrices.

Routinely measured ethanol metabolites include:

- Ethyl glucuronide (EtG) in serum, whole blood, urine and hair
- Ethyl sulphate (EtS) in serum, whole blood and urine
- Phosphatidylethanol (PEth) in whole blood
- Fatty acid ethyl esters (FAEEs) especially in hair

Generally, ethanol metabolites are detectable in serum or whole blood for hours (EtG, EtS), in urine for up to seven days (EtG, EtS), in whole blood over two weeks (PEth) and in hair over months (EtG, FAEEs) (review by Thon et al 2014).

Ethyl glucuronide (EtG):

A. Pharmacology

Ethyl glucuronide (EtG) is a phase II metabolite of ethanol, has a molecular weight of 222 g/mol and is is formed by the action of UDP-glucuronosyl transferase (Foti & Fisher, 2005). The possible effect of nutritional components such as flavonoids on EtG formation is currently under investigation and seems to be a possible partial explanation of the variability of EtG formation in humans (Schwab & Skopp, 2014).

Whereas the elimination of ethanol via glucuronidation is a minor pathway of alcohol metabolism (less than 0.1 %) EtG is a valuable biomarker of ethanol intake. EtG is non-volatile, water-soluble, and stable in storage. Depending on the amount of consumed alcohol and time spent for consumption, EtG is still detectable in the body long after completion of alcohol elimination (Schmitt et al., 1995, Wurst et al., 1999a, 2004; Dahl et al., 2002, 2011a; Borucki et al., 2005, Halter et al. 2008).

B. Specific body tissues, time frame and cut-off values

Based on a literature review, Walsham and Sherwood (2012) sum up that EtG can be detected for up to 90 hours in urine. There is no difference regarding the elimination rate between a healthy population and heavy alcohol consumers at the beginning of detoxification treatment (Hoiseth et al., 2009).

EtG is also detectable in post-mortem body fluids and tissues like gluteal and abdominal fat, liver, brain and cerebrospinal fluid (Wurst et al., 1999b), in bone marrow, muscle tissue (Schlögel et al., 2005) and finger nails (Berger et al., 2013).

Even intake of small amounts of alcohol like 0.1 L champagne can be detected via EtG for up to 27 hours. Experiments with 1 g ethanol (champagne, whisky) (Thierauf et al., 2009) as well as use of mouthwash (Costantino et al., 2006) and hand sanitizer gels (Rohrig et al., 2006) yielded ethyl glucuronide concentrations of less than 1.0 mg/L in urine. Measurable concentrations in urine were found for up to 11 hours. This aspect is of relevance regarding unintentional exposure of alcohol: pralines, non-alcoholic beer, pharmaceutical products, fruit juice, sauerkraut, mouthwash products and hand sanitizer gels may contain small amounts of alcohol. Even the intake of 21 - 42 g yeast with approximately 50 g sugar leads to measureable EtG and EtS concentrations in urine (Thierauf et al., 2010).

Inhalation of ethanol vapors may be another source for EtG in urine. Arndt et al., (2014a) emphasize that ethanol is mainly incorporated by inhalation not via the skin by using hand sanitizers by hospital employees.

Therefore, a patients' claim not having consumed alcohol may be the truth even when EtG is detectable in urine. Since patients in withdrawal treatment should avoid even the smallest amount of alcohol, they have to be informed of such hidden sources of ethanol to avoid unintentional alcohol intake. A differential cut-off of 0.1 mg/L in cases where total abstinence is the goal, and 1.0 mg/L if small amounts of alcohol intake are tolerated, have been recommended for practical reasons (Costantino et al., 2006; SAMHSA 2012) .

Based on the fact that exposure studies (by inhalation or disinfecting hands etc.) in humans never yielded results above 1.0 mg/L, a differential cut-off has been suggested (Thierauf et al., 2009; SAMHSA 2012):

a) of 0.1 mg/L in cases where total abstinence is the goal
b) of 1.0 mg/L or more for EtG to confirm drinking.
c) In addition, a recent revision of the SAMHSA advisory suggests that values between 0.5 mg/L and 1.0 mg/L could be from previous drinking as well as from recent intense extraneous exposure within 24 hours or less.

For further details, see table 1.

Due to head hair growth of 1 cm per month, hair analysis allows a cumulative and retrospective assessment of ethanol intake for a longer time period as compared to blood and urine. In hair, hydrophilic EtG is incorporated through perspiration and/or from blood (Pragst & Yegles, 2007; Schräder et al 2012).

For the assessment of chronic excessive alcohol consumption, EtG and FAEEs can be used alone or in combination to increase the validity of hair analysis (Pragst & Yegles, 2008).

According to the consensus of the Society of Hair Testing (SOHT, 2014), a concentration over 30 pg/mg EtG in the 0 - 3 cm up to 0 - 6 cm proximal scalp hair segment strongly suggests chronic excessive alcohol consumption.

For abstinence assessment HEtG should be the first choice, according to both the consensus statement of the SOHT (2014) and a systematic review (Boscolo-Berto et al., 2014). A concentration below 7 pg/mg does not contradict self-reported abstinence of a person during the corresponding time period before sampling. A HEtG concentration above 7 pg/mg in the 0 - 3 cm up to 0 - 6 cm proximal scalp hair segment strongly suggests repeated alcohol consumption.

In an alcohol drinking experiment, 32 women, who consumed 16 g alcohol per day, had EtG values of less than 7 pg in their scalp hair (Kronstrand et al., 2012). These divergent results may be explained by the fact that EtG values lower than 7 pg/mg do not exclude alcohol ingestion. Furthermore, scalp hair was cut pre-analytically in this study while previous studies pulverised the specimen: The pre-analytical preparation, such as washing, powdering (by a ball-mill) – or cutting by scissors in small pieces, extraction by solvents with or without ultrasonication, has been reported to influence the results significantly (Albermann et al., 2012a; Kummer et al., 2015; Mönch et al., 2013). These factors need some standardization, otherwise – as seen in proficiency tests or other inter-laboratory tests, the precision of analysis is decreased.

C. Methodical aspects

A DRI® ethyl glucuronide enzyme immunoassay (DRI®-EtG EIA) is commercially available. The first study showed satisfying but not convincing results (Böttcher et al.,

2008). Therefore, enquiries with medico-legal relevance need further confirmation with forensic-toxicologically acceptable methods like liquid chromatography – tandem mass spectrometry (LC-MS/MS (Weinmann et al., 2004).

D. Limitations

In recent years, the potential in vitro formation and degradation of EtG and EtS have gained attention (Helander et al., 2005; 2009; Baranowski et al., 2008; Halter et al., 2009). Initially, hydrolysis of EtG caused by microbes in urinary tract infections, especially E.coli, was reported (Helander et al., 2005). Baranowski et al (2008) confirmed complete degradation of EtG within 3 to 4 days by E.coli and C. sordellii. In contrast, the stability of EtS for up to 11 days was shown (Baranowski et al., 2008). Furthermore, Hernandez Redondo et al. (2013) reported that the bacterial degradation of EtG by E. coli can be prevented by use of dried urine on filter paper.

The WHO/ISBRA Study showed that EtG urine concentrations are influenced by age, gender, cannabis consumption and renal function. In contrast, race, nicotine consumption, body mass index, liver cirrhosis and body water content had no significant influence on EtG concentrations (Wurst et al., 2004a). The results concerning renal and liver functions have recently been confirmed by two studies, one in which EtG elimination was prolonged for 14 patients with reduced renal function (Hoiseth et al., 2013), and another study in which severity of liver disease had no influence on the validity of EtG as a marker of alcohol use (Stewart et al 2013).

The positive predictive values of EtG for patients, who claimed abstinence in the last 3 days, were 81 %. The negative predictive values were 91 %. Had the patients claimed abstinence in the last 7 days, the positive predictive values would be 97 %,

the negative predictive values would be 85 % (Stewart et al., 2013, see also table 2a and b).

Only single cases of false positive results for EtG in hair were found after use of EtG containing herbal lotions (Sporkert et al., 2012; Arndt et al., 2013). Impaired kidney function may lead to higher HEtG levels, as preliminary results indicate (Hoiseth et al. 2013).

The concentration of EtG in hair can be influenced by cosmetic treatments and thermal hair straightening tools (Ettlinger et al., 2014). Bleaching, perming and dying of hair may lead to lower concentrations of EtG or false-negative results (Yegles et al., 2004; Kerekes & Yegles, 2013; Morini et al., 2010a; Agius, 2014; Suesse et al., 2012).

The type of cosmetic hair treatment should be documented during sampling and considered during interpretation. EtG appears not to be influenced by ethanol-containing hair care products, whereas their use may lead to false positive FAEEs (Hartwig et al., 2013; Suesse et al., 2012; Gareri et al., 2011).

In contrast to hair analysis for drugs and medication, hair colour and melanin content in hair have no influence on HEtG (Kulaga et al., 2009; Appenzeller et al., 2007a). Also for gender no effect on HEtG concentration have been reported (Crunelle et al., 2014a). In segmental investigations of hair samples, a chronological correlation to drinking or abstinent phases was reported for HEtG by two studies (Wurst et al., 2008c; Appenzeller et al., 2007b), however not for FAEEs (Auwärter et al., 2004). Only for HEtG a correlation was found in 3 studies between the EtG content in hair and the amount of EtOH consumed (Appenzeller et al., 2007b; Crunelle et al., 2014b; Politi et al., 2006). Altogether, hair analysis is a useful tool to estimate overall ethanol intake over a longer time.

Ethyl sulphate (EtS)

A. Pharmacology

Ethyl sulphate (EtS) has a molecular weight of 126 g/mol, and represents, like EtG, a secondary elimination pathway for alcohol. EtS is detectable in varying inter-individual concentrations (Dresen et al., 2004; Helander and Beck, 2004; Wurst et al., 2006, Halter et al., 2008). An immunochemical detection test is currently not commercially available for EtS. For combined detection of EtS and EtG, use of rapid LC-MS/MS procedures is routinely applied.

EtS formation is catalyzed by the enzyme sulpho-transferase and the breakdown by sulphatases.

B. Specific body tissues, time frame and cut-off values

EtS is detectable in the same body tissues as EtG. A cut-off of 0.05 mg/L for repeated alcohol intake was suggested (Albermann et al., 2012b). As for ethyl glucuronide, there is evidence of prolonged elimination in reduced renal function (Hoiseth et al., 2013).

C. Methodical aspects

Schneider and Glatt (2004) developed a liquid chromatography-tandem-mass spectrometry method with 2-propylsulphates as internal standard. Helander and Beck (2004) used liquid chromatography-electro spray-ionisation-mass spectrometry (LC-ESI-MS) in a single-quadruple-modus and D_5-ethyl sulfate as internal standard for the quantification of EtS in urine samples. The disadvantage of this method is a longer period of chromatographic separation. Furthermore, the exclusive monitoring of de-protonated molecules in a single-MS does not meet forensic standards (Aderjan et al., 2000; SOFT/AAFS, 2006). At any rate, an additional fragment ion would be

required for the verification analyses according to forensic guidelines (SOFT/AAFS, 2006). Even when this requirement from forensic guidelines does not need to be met in clinical diagnostics, it is still in demand in workplace drug testing in the USA. In this context, a LC-MS/MS method with penta-deuterium EtS as internal standard and two ion transitions (Dresen et al., 2004) raises particular interest, and can be used in forensic and medico-legal cases as well as in clinical routine (Skipper et al., 2004).

D. Limitations:

In contrast to the above-described potential of in vitro formation and degradation of EtG caused by microbes, Baranowski et al. showed the stability of EtS for up to 11 days (Baranowski et al., 2008). Further studies with standardized test procedures for biodegradation showed that EtS in closed bottle test (OECD 301 D) remained stable for even longer periods whereas in the context of a higher bacterial density such as in the Manometric Respiratory Test (MRT) a reduction after 6 days was detected (Halter et al., 2009). This problem could be countered by cooling and the addition of stabilizers.

The positive predictive value of EtS for patients, who claimed abstinence in the last 3 days, was 70 %. The negative predictive values were 93 %. Had the patients claimed abstinence in the last 7 days, the positive predictive values would be 80 % respectively, the negative predictive values would be 85 % (Stewart et al., 2013, see also table 2a and b).

Fatty acid ethyl esters (FAEEs)

A. Pharmacology

In recent years, the existence of fatty acid ethyl esters (FAEEs), non-oxidative metabolic products of ethanol in blood and various organs with reduced or deficient

capacity to oxidize ethanol after consumption has been shown. Since these esters have proven to cause damage to sub cellular structures, they were postulated to be mediators of organ damage.

Two enzymes catalyse the formation of FAEEs: acyl-coenzyme a-ethanol o-acyltransferase (AEAT) and fatty acid ethyl ester-synthase. Furthermore, pancreatic lipase, lipoprotein lipase and glutathione transferase were shown to possess FAEE-synthase activity (Tsujita & Okuda, 1992; Bora et al., 1989; 1996).

Fatty acid ethyl esters are formed in the presence of ethanol from free fatty acids, triglycerides, lipoproteins or phospholipids.

B. Specific body tissues, time frame and cut-off values

Detectable FAEE levels are found in blood shortly after alcohol consumption and remain positive for more than 24 hours (Borucki et al., 2005).

Regarding hair analyses, the deposit of lipophilic FAEEs occurs in sebum (Auwärter et al., 2004). A FAEE cut-off concentration of 0.5 ng/mg for the sum of the four esters in scalp hair is considered strongly suggestive of chronic excessive alcohol consumption when measured in the 0 – 3 cm proximal segment. If the proximal 0 - 6 cm segment is used the proposed cut-off concentration is 1.0 ng/mg scalp hair.

The combined use of FAEEs and EtG (see above) can be recommended to increase the validity of hair analysis (Pragst & Yegles, 2008).

C. Methodical aspects

Of 15 different FAEEs in hair the sum of four (ethyl stearate, ethyl oleate, ethyl myristate and ethyl palmitate) are shown to function as a marker in hair analysis (Pragst & Yegles, 2007). With a cut-off of 0.5 ng/mg, a sensitivity and a specificity of 90 % were reported. A differentiation between abstinent, social and excessive

drinkers seems possible (Yegles et al., 2004; Gonzalez-Illan, et al., 2011). However, the complex GC/MS method lacks practicability for routine use.

D. Limitations:

Regarding hair samples, the analysis of FAEEs alone is not recommended to determine abstinence from ethanol, but may be used in cases of suspected false negative HEtG results, utilising a FAEEs cut-off concentration of 0.2 ng/mg for a 0 - 3 cm proximal scalp hair segment or 0.4 ng/mg for a 0 - 6 cm proximal scalp hair segment.

The concentration of FAEEs in hair can be influenced by cosmetic treatments and thermal hair straightening tools (Ettlinger et al., 2014). Regular use of alcohol-containing hair tonic can lead to false positive FAEEs results (Hartwig et al., 2003). No such false positive results are reported for HEtG (Ferreira et al., 2012).

Phosphatidylethanol (PEth)

A. Pharmacology

Phosphatidylethanol (PEth), a phospholipid, is formed in the presence of alcohol via the action of the enzyme phospholipase-D (PLD) (Alling et al., 1983). The precursors are naturally existing phosphatidylcholine (PC) homologues. PEth consists of glycerol which is substituted at positions sn1 and sn2 by fatty acids and is esterified in sn3 position with phosphoethanol (Gustavsson and Alling, 1987; Gnann et al., 2010; Isaksson et al., 2011; (Kobayashi and Kanfer, 1987). Due to the variations of the fatty acids, various homologues of PEth can be detected. In 2010, 48 PEth homologues were described in the blood of a deceased alcohol-dependent individual for the first

342 time (Gnann et al., 2010). The PEth homologues 16:0/18:1 und 16:0/18:2 are most
343 prevalent in human blood (Gnann et al., 2014) and their combined sum correlates
344 better with total PEth than PEth 16:0/18:1 or PEth 16:0/18:2 alone (Zheng et al.,
345 2011).
346 PEth is formed directly after ingestion of alcohol (Gnann et al., 2012), but has a slow
347 elimination rate with a half-life time of approximately 4 days (Hannuksela et al.,
348 2007). Therefore, PEth is a promising biomarker for the detection of alcohol abuse,
349 as it can be determined in blood of alcohol abusers even up to 3 weeks after
350 withdrawal (Viel et al., 2012; Winkler et al., 2013). Using the original high
351 performance liquid chromatography (HPLC) methods in combination with evaporative
352 light scattering detection (Varga et al., 1998; (Aradottir and Olsson, 2005)), repeated
353 consumption of more than 50 g alcohol over 2 - 3 weeks yielded positive results
354 (Varga et al., 1998), lately even with daily consumption over 40 g (Aradóttir et al.,
355 2004).
356 Whereas before 2009 the HPLC method was used, later studies employed LC-
357 MS/MS.
358
359 B. Specific body tissues, time frame, cut-off values
360 With the LC-MS/MS approach single consumption of ethanol up to a blood alcohol
361 concentration (BAC) of approximately 0.1 g/dL , yielded PEth 16:0/18:1
362 concentrations up to approx. 120 ng/mL (0.17 µmol/L) (Schröck et al., 2014) in whole
363 blood. A recent drinking experiment with healthy persons with an alcohol
364 consumption of 0.1g/dL on 5 consecutive days yielded PEth values up to 237 ng/mL
365 (0.32 µmol/L) (Gnann et al., 2012). In contrast, in alcohol-dependent patients, the
366 values were reported to be up to 4200 ng/mL (6 µmol/L) (Helander and Zheng, 2009),
367 whereas total PEth concentrations of more than 500 ng/mL (0.7 µmol/L) have been

considered as typical for prolonged alcohol misuse (Isaksson et al., 2011). In 2013, Swedish laboratories suggested a cut-off for the PEth homologue 16:0/18:1 of 210 ng/mL (0.3 µmol/L) to differentiate between moderate drinking and alcohol misuse (Helander and Hansson, 2013).

Various studies found no false positive PEth results (Wurst et al., 2003b, 2004; Hartmann et al., 2007). A linear relationship between consumed amounts of alcohol with phosphatidylethanol values has been described (Aradottir et al., 2006; Stewart et al., 2009; Stewart et al., 2010).

In 144 patients, Aradottir et al. (2004) reported sensitivity of PEth to be 99 %, of CDT, MCV and GGT to be between 40 – 77 %, as well as a correlation between the amount consumed and the PEth value. In a receiver operating characteristic (ROC) curve analysis with consumption status (active drinkers vs abstinent drinkers), as a state variable and with PEth, MCV and GGT as test variables, an area under the curve (AUC) of 0.973 for PEth could be found, the sensitivity was 94.5 % and the specificity 100 % (Hartmann et al., 2007).

These findings were confirmed in subsequent publications (Wurst et al., 2010; 2012; Stewart et al., 2010; Hahn et al., 2012). Furthermore, liver disease and hypertension (Stewart et al., 2009; 2014) showed no influence on PEth values.

PEth has been employed in various settings including judging driving ability (Marques et al, 2010; 2011), forensic psychiatry (Wurst et al., 2003b), monitoring programs (Skipper et al., 2013), identification of alcohol intake in specific risk groups (Hahn et al., 2012) and for neonatal screening of prenatal alcohol exposure (Bakhireva et al., 2013, Bakhireva et al., 2014).

C. Methodical aspects

Concerning the interpretation of results, it is important to acknowledge that publications before 2009 used the HPLC method in combination with evaporative light scattering detection. This method detects the sum of all PEth homologues. In contrast, new approaches use LC-MS, LC-MS/MS and on-line extraction LC-MS/MS methods (Schrock et al., 2014). These methods allow the detection and quantification of single homologues (Gnann et al., 2009), if a reference standard is available. Furthermore, recent publications suggested LC-HRMS (Liquid Chromatography High Resolution Mass Spectrometry) method (Nalesso et al., 2011) and a metabolic approach using LC-MS-IT-TOF (Liquid Chromatography with Quadruple Ion Trap – Time-of-Flight-Mass Spectrometry) (Loftus et al., 2011). For everyday practical application, the use of dried blood spots (DBS) may be of significant relevance (Faller et al., 2013). This method is suggested to provide results similar to whole blood analysis. Studies of PEth analysis on DBS showed good robustness and the advantage that neo-formation of PEth in the presence of a positive BAC does not occur after drying the blood on filter paper (Schrock et al., 2014). Furthermore, obtaining specimens is simplified since non-medical staff can collect capillary blood, also storage and transport are simplified, and the risks for HIV and hepatitis C infections are decreased (Faller et al., 2013).

D. Limitations

PEth concentrations were shown to be stable in blood samples stored in refrigerators (2 – 8°C) and when frozen at -80°C. However, decrease in PEth concentrations was observed during storage at -20°C. When ethanol was present in samples, neo-formation of PEth has been observed (Aradottir et al., 2004).

In-vitro formation of PEth in erythrocytes has been reported after addition of ethanol (Varga and Alling, 2002). For further details see table 2a and table 2b.

II: Practical use in specific patient's samples and settings:

EtG:

Practical use in specific patient's samples and settings:

1) Opioid maintenance therapy patients as specific high-risk group:

Many patients in opioid-maintenance therapy suffer from Hepatitis C (HCV) infection. Alcohol consumption, especially in large amounts, leads to the progression of cirrhosis (Gitto et al., 2009; Safdar & Schiff, 2004). One study in Australia (Wurst et al., 2008a) and one in Switzerland (Wurst et al., 2011) showed the usefulness and necessity of the determination of ethyl glucuronide in patients in opioid-maintenance therapy. In the former study, 42 % (n = 8 of 19) of all EtG positive patients would have not reported the alcohol consumption (Wurst et al., 2011). In the latter one, 75 % consumed alcohol according to the hair analysis for EtG, however, two thirds did not report about it (Wurst et al., 2011).

The use of direct ethanol metabolites in high-risk groups therefore allows more possibilities for therapeutic interventions, consequently leading to an improvement in the quality of life.

2) Monitoring and rehabilitation programs:

a) One example for using ethyl glucuronide successfully in monitoring programs are the Physician Health Programmes in the USA which provide a non-disciplinary therapeutic program for physicians with potentially impairing health conditions such as substance related disorders. Participating in the monitoring program, physicians with substance related disorders are allowed to keep on working whereas a regularly proof of abstinence has to be shown. Measuring EtG in urine, Skipper and colleagues (Skipper et al., 2004) showed that of 100 randomly collected samples, no sample was positive for alcohol using standard testing; however, seven were positive for EtG

(0.5 – 196 mg/L), suggesting recent alcohol use. EtG testing can provide additional information and consequently, may lead to further treatment and improvement for the patient (Skipper et al., 2004, 2013).

b) The usefulness of EtG measurement during inpatient treatment in a rehabilitation program has been shown in two studies (Junghanns et al., 2009; Wetterling et al. 2014). The results suggest that there is a considerable number of inpatients consuming alcohol during weekend leaves which is not detected by self-report or breath alcohol analyses. Of patients who lapsed during weekend leaves, a high proportion did not complete treatment. Furthermore, lapsers completing treatment did have a significantly reduced chance to reach abstinence during follow-ups.

3) Pharmacotherapy efficacy studies:

As an objective outcome parameter, EtG testing has shown to be useful in pharmaco-therapeutical studies (Dahl et al., 2011b; Mitchell et al., 2012, Jatlow et al., 2014).

4) Hangover state:

A potential role of EtG in the context of neurocognitive impairment following heavy drinking, usually referred to as hangover state, has previously been suggested (Wurst et al., 2003a; Stephens et al., 2014). Results of a recent study (Hoiseth et al., 2015) seem to consolidate this idea.

5) In addition, EtG can also be detected in specimens of dried blood, which is of relevance for forensic investigations (Kaufmann & Alt, 2008; Winkler et al., 2011; Hernandez Redondo et al., 2013)

6) Liver transplantation:

Alcohol-related liver disease accounts for up to 30 % of liver transplants (Burroughs et al., 2006). Post-operatively, 20 – 25 % of the patients' lapse or relapse to alcohol intake (Kelly et al., 2006; DiMartini et al., 2006). In 18 patients with ALD (alcohol liver

disease) Erim et al. (2007) found no self-report on alcohol consumption. One out of 127 tests for breath alcohol was positive, whereas 24 of 49 urine samples were positive for EtG. Webzell et al. (2011), who found self-reported alcohol consumption in 3 % in contrast to 20 % positive urine EtG and EtS tests, reported comparable results. Recently, Piano et al. (2014) found that the combination of AUDIT-C and EtG in urine improves the detection of alcohol consumption in liver transplant candidates and liver transplant recipients and showed higher accuracy in detecting alcohol consumption than the combination of AUDIT–C and CDT.

EtG in hair (HEtG) allows retrospective determination of alcohol consumption for up to 6 months that is the abstention period often required by transplant programs prior to listing patients. Several studies have evaluated HEtG concentrations in liver transplant patients, proposing it to be a highly specific and useful tool for the monitoring of alcohol use before, and after liver transplantation (Sterneck et al., 2013; Hilke et al., 2014) and superior to traditional markers. Further substantial advantages compared to routine methods of alcohol detection in urine or blood are, that obtaining hair samples is non-invasive and storage of the samples is easy. Despite their excellent profile, it is not advisable to use the results of hair testing for alcohol markers in isolation and conclusions always should be corroborated by a clinical assessment and interpretation.

On this background, Allen et al (2013) conclude in a recent review regarding liver transplantation that ethyl glucuronide tests in urine and hair are complementary to self-reports and questionnaires, yielding valuable information on alcohol consumption, which is relevant in diagnosis and therapy.

7) Alcohol metabolites and fetal alcohol syndrome (FAS)

Alcohol consumption during pregnancy may lead to fetal alcohol syndrome (FAS) and the fetal alcohol spectrum disorder (FASD), characterized by congenital

abnormalities, cognitive dysfunction and developmental problems. Estimations report that the prevalence of FAS and FASD is 0.2 to 1 per 100 life-births in industrialized countries (Sampson et al., 1997; Stade et al., 2009).

A recent study reported that 50% of Italian and 40% of Spanish women occasionally consumed alcohol during pregnancy (Vagnarelli et al., 2011).

Alcohol intake during pregnancy can be investigated in

a) maternal (including hair, blood, urine) and

b) fetal specimen (meconium) (Joya et al., 2012).

To date there is only one study (Wurst et al., 2008c) employing EtG in urine and hair in pregnant women assessing alcohol intake compared with self-reports: Women at the end of the second trimester were included. The AUDIT identified 25.2 % women consuming any alcohol during pregnancy. None of the participants scored above the gender-specific AUDIT score higher than the cut-off value of 4 points. However, according to the hair analysis, 12 women drank 20 - 40 g ethanol per day, and 4 had an intake over 60 g/day (Wurst et al., 2008c). The results of the study also indicate that the combination of AUDIT and biomarkers identified more alcohol use than the questionnaire alone.

These results support the application of direct alcohol metabolites in pregnant women since increases of %CDT (percent of carbohydrate deficient transferrin vs. total transferrin) and its isoforms were reported for this specific population (Bianchi et al., 2011; Kenan et al., 2011).

Studies on fetal specimen include current measures of meconium. These measures are a cumulative indicator of alcohol consumption, since it is formed between the 12^{th} and 16^{th} week of pregnancy. While the first studies investigated FAEEs concentrations, recent research focused on EtG and EtS. The largest study investigated meconium of 607 newborns. 7.9 % of specimens indicated maternal

alcohol intake during pregnancy. Low maternal education level and age were associated with biomarker values above the cut-off (Pichini et al., 2012). In contrast, Goecke et al., (2014) found in 557 births no correlation between socioeconomic or psychological characteristics and those women positively tested for alcohol use via meconium. Regarding FAEEs detection, the specimen has to be investigated promptly. One study reported that negative meconium values in 19 babies turned positive within 59 hours. Following the authors' in-vivo- and in-vitro studies, this change may be caused by contamination through nutritional components, postnatal feces and ethanol-producing germs (Zelner et al., 2012). This may also be the cause for 82.8 % EtG and 22.2 % FAEEs positive values in meconium, reported by another study (Morini et al., 2010b). Results of a recent study suggest that maternal ethanol intake was better represented by meconium EtG ≥30 ng/g than by currently used FAEE cutoffs (Himes et al., 2015).

Also the usefulness of PEth measurement in whole blood during pregnancy was described (Stewart et al., 2010; Kwak et al., 2014). In addition, capillary blood sampling for PEth analysis on DBS has been applied for neonatal screening of prenatal alcohol exposure with promising results (Bakhireva et al., 2013, Bakhireva et al., 2014) 2014)

Summary and Discussion:

In summary, ethanol metabolites reflect the spectrum between short-term intake of small amounts and long-term use of large amounts of alcohol. Cut-off values and influencing factors are summarized in tables 1 and 2. Appropriate methods of analysis and pre-analytics are crucial for a valid and reliable detection of alcohol biomarkers. For EtG, the most frequently used marker, the most recommended methods for detection are chromatographic approaches, which are considered as

549 standard methods especially in forensic cases. A commercial test-kit is available and
550 contributed to wide distribution of the test. Laboratory values always require critical
551 clinical reappraisal, especially since EtG is detectable in urine using LC-MS/MS even
552 after an ingestion of low amounts of alcohol (1 g), which also occurs in some food,
553 drugs and disinfectants. Individuals with the motivation to or obligation for abstinence
554 have to be informed about these “hidden contents” to avoid unintentional intake of
555 alcohol. For forensic purposes, the current cut-off value of 0.1 mg/L should be
556 adapted to exclude cases of unintentional alcohol use. With respect to differences in
557 formation and degradation, EtG and EtS should be analyzed together, if possible. In
558 the absence of known influencing factors, EtG in hair can be recommended as a
559 marker for alcohol intake over the last 3 months. Furthermore, guidelines for
560 interpretations of values are available from the Society of Hair Testing (SOHT, 2014).
561 While positive urine values of EtG and EtS can be in accord with
562 innocent/unintentional alcohol intake, positive values of PEth are related to previous
563 intoxications of 0.05 g/dL and more.
564 Therefore, PEth is currently used to differentiate between moderate drinking and
565 excessive alcohol use, based on thresholds derived from clinical investigations. A
566 suggested threshold of 0.3 micromole/Liter (210 ng/mL) PEth (Helander and
567 Hansson, 2013) seems promising, however needs further evaluation and verification
568 by studies with larger numbers of social drinkers. Furthermore, inter-individual
569 differences should be investigated, which might depend on differences in
570 phospholipase-D activities. New developments achieved by more sensitive analysis
571 methods and drinking experiments show the potential of PEth in abstinence
572 monitoring (Schrock et al., 2014). Due to stability issues the use of „dried blood
573 spots“(DBS) in PEth analysis is promising: a) In vitro formation of PEth in alcohol-
574 containing blood does not occur after drying blood on filter paper, b) it may facilitate

blood sampling (capillary blood instead of venous blood), storage and shipping of samples.

As biomarkers with high sensitivity and specificity covering complimentary time windows from hours to several months, depending on the biological material used for analysis and the choice of the respective biomarker, ethanol metabolites can be applied in different settings such as screening, prevention, diagnosis and therapy of alcohol use disorders and alcohol intake in general, among others.

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Table 1: Suggested cut-off values for different ethanol metabolites

Biomarkers	**Amount of alcohol consumption**	**Cut-off**	**Reference**
EtG in hair	Abstinence and low intake (< 10 g/d)	< 7 pg/mg	SOHT , 2014
	Social consumption (20 - 40 g/d)	7 – 30 pg/mg	
	Excessive drinking (>60 g/d)	> 30 pg/mg	
FAEEs in hair	Repeated alcohol intake	≥ 200 pg/mg	SOHT, 2014
	Excessive intake	≥ 500 pg/mg	
EtG in urine	Total abstinence	0.1 mg/L	Thierauf et al., 2009; SAMHSA, 2012
	- unintentional intake - recent alcohol use - previous heavy alcohol intake	0.1 mg/L– 0.5mg/L	
	unintentional intake unlikely, but possible, active alcohol intake probable	0.5-1 mg/L	
EtS in urine	Total abstinence	0.05 mg/L	Albermann et al., 2012b
PEth in blood	Abstinence and low intake	150 ng/mL (0.22 µmol/L) total PEth with HPLC 20 ng/mL (0.03 µmol/L) PEth 16:0/18:1 (LC-MS/MS) 35 ng/mL (0.05 µmol/L) PEth 16:0/18:1 (LC-MS/MS)	Varga et al., 1998; Aradottir et al., 2004 Gnann et al., 2009 ; Schröck et al., 2014 Helander et al., 2013
	Drinking experiments: a) Single consumption (once, > 40 g)	120 ng/mL (0.17 µmol/L) PEth 16:0/18:1 (LC-MS/MS)	Schröck et al., 2014

	b) Repeated drinking (5 consecutive days, >40 g each)	240 ng/mL (0.34 µmol/L) PEth 16:0/18:1 (LC-MS/MS)	Gnann et al., 2012
	Excessive drinking (>40 g/d, more than 2 weeks)	500 ng/mL (0.7 µmol/L) total PEth (HPLC) 800 ng/mL (1.14 µmol/L) PEth 16:0/18:1 (LC-MS/MS) 210 ng/mL (0.3 µmol/L) PEth 16:0/18:1 (LC-MS/MS)	Isaksson et al., 2011 Gnann H., 2011 Helander et al., 2013

EtG: Ethyl glucuronide, FAEEs: Fatty acid ethyl esters in hair, EtS: Ethyl sulfate, PEth: Phosphatidylethanol; (modified according to Thon et al., 2013) LC-MS/MS: liquid chromatography – tandem mass spectrometry

Table 2a: Factors without known influence on ethanol metabolite levels

Direct bio-markers	**Potential influencing factor**	**Reference**
EtG in urine	No influence of E. coli on EtG levels in urine, when dried urine spots are used	Hernandez Redondo et al., 2012
	Grade of liver disease, smoking, BMI, body water content	Wurst et al., 2004a;
EtS in urine	No influence of E. coli on EtS levels in urine, when dried urine spots are used	Hernandez Redondo et al., 2012
PEth	Liver disease	Stewart et al., 2009
	Hypertension	
	Storage of ethanol blood samples Refrigerator temperature, -80°C	Aradottir et al., 2004
	Gender	Wurst et al., 2010
EtG in hair	Hairsprays with ethanol, hair colour, melanin content, age, gender, BMI	Ferreira et al., 2012; Kharbouche et al., 2010; Kulaga et al., 2009; Appenzeller et al., 2007 Crunelle et al., 2014

EtG: Ethyl glucuronide, FAEE: Fatty acid ethyl esters, EtS: Ethyl sulfate, PEth: Phosphatidylethanol, BMI: body mass index, RT: room ambient temperature, E. coli: Escherichia coli, C. sordelli: Clostridium sordelli (modified according to Thon et al., 2013)

Table 2b: Factors with known influence on ethanol metabolite levels

Direct bio-markers	**Potential influencing factor**	**Type of influence**	**Reference**
EtG in urine	E. coli, C. sordelli	Decrease	Helander & Dahl, 2005; Baranowksi et al., 2008
	Reduced kidney function	Longer detection	Wurst et al., 2004a; Hoiseth et al., 2012 ; Stewart et al., 2013
	Chloral hydrate	False positives	Arndt et al., 2009
	Propyl and butyl alcohol	False positives in DRI EtG Assay	Arndt et al, 2014b
EtS in urine	Reduced kidney function	Longer detection	Hoiseth et al., 2012
	Closed Bottle test (OECD 301 D) Manometer Respiratory Test (MRT)	28 days Stable detection, depletion after 6 days	Halter et al., 2009
FAEEs in hair	Aggressive alkaline hairsprays	False negative	Hartwig et al., 2003
	Hairsprays containing ethanol	False positives	
PEth	Ethanol containing blood samples, Storage of ethanol blood samples at RT and -20°C	Increase	Aradottir et al 2004
EtG in hair	Hairspray with EtG	Increase	Sporkert et al. 2012
	Reduced kidney function	Increase	Hoiseth et al. 2013
	Bleaching, hair styling products	False negative	Yegles et al., 2004; Morini et al., 2010 Kerekes et al., 2013
	Commercial hair tonics containing EtG	Potentially false	Arndt et al, 2014b

		positve	
	Thermal hair straightening	Decrease	Ettlinger et al, 2014

EtG: Ethyl glucuronide, FAEE: Fatty acid ethyl esters, EtS: Ethyl sulfate, PEth: Phosphatidylethanol, BMI: body mass index, RT: room ambient temperature, E. coli: Escherichia coli, C. sordelli: Clostridium sordelli (modified according to Thon et al., 2013)