REVIEW ARTICLE

Alcohol Biomarkers in Clinical and Forensic Contexts

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Summary

Background: Biomarkers of alcohol consumption are important not only in forensic contexts, e.g., in child custody proceedings or as documentation of alcohol abstinence after temporary confiscation of a driver's license. They are increasingly being used in clinical medicine as well for verification of abstinence or to rule out the harmful use of alcohol.

Methods: This review is based on pertinent publications that were retrieved by a selective literature search in PubMed concerning the direct and indirect alcohol markers discussed here, as well as on the authors' experience in laboratory analysis and clinical medicine.

Results: Alongside the direct demonstration of ethanol, the available markers of alcohol consumption include the classic indirect markers carbohydrate-deficient transferrin (CDT), gamma-glutamyltransferase (GGT), and mean corpuscular volume (MCV) as well as direct alcohol markers such as ethyl glucuronide (EtG) and ethyl sulfate (EtS) in serum and urine and EtG and fatty acid ethyl esters (FAEE) in hair. Phosphatidylethanol (PEth) is a promising parameter that complements the existing spectrum of tests with high specificity (48–89%) and sensitivity (88–100%). In routine clinical practice, the demonstration of positive alcohol markers often leads patients to admit previously denied alcohol use. This makes it possible to motivate the patient to undergo treatment for alcoholism.

Conclusion: The available alcohol biomarkers vary in sensitivity and specificity with respect to the time period over which they indicate alcohol use and the minimum extent of alcohol use that they can detect. The appropriate marker or combination of markers should be chosen in each case according to the particular question that is to be answered by laboratory analysis.

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Biomarkers to detect alcohol consumption or harmful alcohol use offer the opportunity to objectively verify the information about alcohol consumption provided by a patient or subject. A distinction is made between direct and indirect biomarkers. Direct biomarkers are created when ethanol is metabolized or reacts with substances in the body. Indirect biomarkers are enzymes or cells which undergo typical changes in response to acute or chronic alcohol consumption. The parameters to be measured or even the exact summons procedure are defined for certain situations, e.g. in transplantation medicine (1) or for assessment of driving eligibility (2). By contrast, in other cases, e.g. in issues related to custody and employment law or during alcohol withdrawal programs, the choice of markers is left at the discretion of the physician or toxicologist.

These biomarkers do not only differ significantly with regard to the period during which they can be detected and the amount of alcohol consumption required for a positive test result, but also with regard to specificity. Medications or diseases may affect the analysis of the biomarkers. Thus, when choosing a biomarker, both the query (moderate alcohol consumption or abstinence) and the summons procedure (point in time at which the date of sample collection is announced) have to be taken into account.

The aim of this study is to provide an overview of the available alcohol biomarkers, their significance and their appropriate use in clinical practice.

Methods

A selective search was performed in the PubMed database for the direct and indirect alcohol biomarkers discussed in this paper, covering the period January 1958 until August 2017. In addition, the authors contributed their analytical and clinical experiences.

Detection and significance of alcohol biomarkers

Ethanol and methanol

Acute alcohol consumption can be detected by determining blood and breath alcohol levels. Ten to twelve hours after the last drink, ethanol can no longer be detected in urine (e1). If subjects are informed about the upcoming test at least 12 hours in advance, they can avoid the detection of ethanol in blood and urine by stopping the intake of alcohol in time.

TABLE 1

Specificity and sensitivity of alcohol biomarkers in relation to the reported amount drunk

Parameter	Sensitivity	Specificity	Amount drunk	References
MeOH	70%	98%	> 0.5 ‰ for several hours	(8)
CDT	46–90%	70–100%	Chronic excessive drinking	(15) (e2) (14)
GGT	37–95%	18–93%	Chronic excessive drinking	(15)
AST	25–60%	47–68%	Chronic excessive drinking	(15)
ALT	15–40%	50–57%	Chronic excessive drinking	(15)
MCV	40–50%	80–90%	Chronic excessive drinking	(15)
CDT, MCV and GGT in combination	88%	95%	Chronic excessive drinking	(9)
EtG in urine	100%	NS	1.2 g/L BAK after 24 h (Cut-off 100 ng/mL)	(20)
	50 and. 100%, resp.	NS	0.2 g/L BAK after 24 h / 12 h (cut-off 100 ng/mL)	(20)
	89%	99%	Abstinence monitoring	(9)
EtG in hair	75%	96%	Chronic excessive drinking (cut-off 30 pg/mg)	(36)
FAEE in hair	90–97%	75–90%	Chronic excessive drinking (dependent on cut-off)	(33) (34)
PEth	88–100%	48–89%	see *eTable*	see *eTable*

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BAC, blood alcohol concentration; CDT, carbohydrate-deficient transferrin; EtG: ethyl glucuronide; FAEE: fatty acid ethyl ester; GGT, gamma-glutamyl transferase; MCV, mean corpuscular volume; MeOH: methanol; NS, not stated; PEth, phosphatidylethanol.
The limits between low-risk and harmful alcohol consumption are 24 g and 12 g of pure alcohol per day for adult men and women, respectively (e3). The World Health Organization (WHO) defines chronic excessive drinking as an average daily consumption of at least 60 g ethanol per day over several months.

In this case, detection of methanol offers an additional advantage: Since the affinity of alcohol dehydrogenase to ethanol is substantially higher, methanol from drinks and endogenously produced from pectins (range: 0.35–3.2 mg/L [3]) culminates in serum above a blood ethanol concentration of 0.2–0.5 ‰ (4).

Based on scientific drinking experiments, methanol concentrations of >10 mg/L are regarded as a biomarker of preceding prolonged, continuous alcohol exposure over at least several hours (3). After analysis of drinking experimental data, the concentration of 5 mg/L was interpreted as a threshold to differentiate between patients with and without alcohol dependence (specificity of 98% [5]). Several studies used a concentration of 5 mg/L as a threshold for recent alcohol intake (end of drinking not longer than 1 day ago) (6–8).

Clinical-chemistry parameters

Carbohydrate-deficient transferrin

Excessive alcohol intake of >50–80 g ethanol per day over a period of at least 1 to 2 weeks results in the loss of carbohydrate side chains of transferrin (9, 10). Carbohydrate-deficient transferrin (CDT) is a specific, but not very sensitive biomarker (*Table 1*). Individuals with moderate consumption or an episodic drinking pattern show CDT levels in the normal range. Furthermore, false-positive results may occur due to rare genetic variations (11).

Mean corpuscular volume, gamma-glutamyl transferase, aspartate and alanine aminotransferase

An increase in mean corpuscular volume (MCV) of red blood cells and in the activity of the enzymes aspartate-aminotransferase (AST) and alanine aminotransferase (ALT), especially a ratio of AST/ALT >2, and above all of gamma-glutamyl transferase activity (GGT), may be signs of harmful alcohol consumption and alcohol-induced hepatic injury (12).

These biomarkers have the advantage that they can be measured as part of inexpensive routine testing in clinical-chemistry laboratories (*Table 2*). Furthermore, it takes several weeks after termination or reduction of alcohol consumption before they return to normal (CDT: 2–3 weeks; GGT: 2–6 weeks; AST/ALT: 2–4 weeks; MCV: 8–16 weeks [12]). A disadvantage of indirect alcohol biomarkers is their low specificity (*Table 1*). For example, non-alcohol-induced hepatic conditions, medications or liver transplant dysfunction can also increase these levels (e3–e5).

While these biomarkers can identify harmful/excessive alcohol consumption, they are not suitable for abstinence monitoring because the regular intake of ethanol in small amounts, but also binge drinking, do not lead to increases in these parameters (9, 13) *(Table 3)*.

Ethyl glucuronide and ethyl sulfate

Apart from oxidative metabolism, the phase II metabolite ethyl glucuronide (EtG; 0.02–0.06% of the ingested alcohol) and ethyl sulfate (EtS; 0.010–0.016%) are created from alcohol to a minor extent (14). They can be detected in blood not long after alcohol consumption (<45 minutes). The time during which EtG can be detected in serum is by up to 8 hours longer compared to ethanol (15). EtS can be detected in serum about twice as long as ethanol (16).

EtG and EtS are also excreted in urine not long (<60 minutes) after alcohol consumption. EtG can be detected in urine for up to about 24 hours even after consumption of small quantities; after excessive consumption, the window of detection is up to 130 hours (17). Therefore, EtG and EtS in urine are the short-term biomarkers with leading sensitivity (*Table 1*). Sensitivity is dependent on alcohol quantity, time interval between sample collection and alcohol intake as well as the cut-off level of the method applied (6,

TABLE 2

Availability of analysis

Parameter	Availability of analysis	Important notes
Breath alcohol	Breath alcohol analyzers, potentially available at the site	Results of pretest devices*1 cannot be used as evidence in court. Analyzers must be calibrated on a regular basis.
Blood alcohol	Standard parameters in clinical-chemistry laboratories	The enzymatic method alone cannot used as evidence in court. Where appropriate, samples should be sent to laboratories for additional gas chromatographic analysis, e.g. an Institute of Legal Medicine.
MeOH	Analysis offered by several specialized laboratories in Germany	Various cut-off concentrations are used.
CDT	Test method available at many clinical-chemistry laboratories	Using the HPLC method (% CDT) allows a more reliable discrimination of genetically determined changes of transferrins.
GGT, AST, ALT, MCV	Standard parameters in clinical-chemistry laboratories	Laboratory analysis of blood samples must be performed on the same day.
EtG in urine	Already available in many larger or specialized laboratories; also available as an immunoassay	The immunological method cannot be used as evidence in court. In case of a positive pretest result, the chromatographic method should be used for confirmatory analysis.*2
EtG in serum	Chromatographic method, established only in few laboratories in Germany	Detection time shorter than in urine.
EtS in urine	Chromatographic method, detectable in specialized laboratories in serum and in urine (often included in EtG method)	Parameter complementing EtG in urine; its analysis is only meaningful in combination with EtG.
EtG in hair	Analysis offered by several specialized laboratories in Germany	One strand (about the thickness of a pencil) is sampled from the occiput. At that time, sufficient material for retention samples should be obtained.
FAEE in hair	Analysis established only in very few laboratories; the EtG hair sample can be forwarded for it, if required	Parameter complementary to EtG in hair; can be included especially in controversial cases.
PEth	Analysis currently offered only by a few specialized laboratories	Correct performance of pre-analytical procedures is crucial. Analysis is performed on whole blood samples. In addition, dried blood spots should be obtained for stability reasons.

Further details on analytical methods are provided in the *eMethods*.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; CDT, carbohydrate-deficient transferrin; EtG, ethyl glucuronide; EtS: ethyl sulfate; FAEE, fatty acid ethyl ester; GGT: gamma-glutamyl transferase; HPLC, high-pressure liquid chromatography; MCV, mean corpuscular volume; MeOH, methanol; PEth, phosphatidylethanol
*1 Pre-test devices are devices used for point-of-care testing (POCT)—as opposed to the legally admissible breath alcohol analyzers of the police.
*2 For example, exposition to 1-/2-propanol–containing disinfectant formulations and the intake of chloral hydrate can lead to false-positive results of immunochemical tests *(eMethods)*.

18, 19). Since the levels in urine are dependent on diuresis, the intake of larger volume of water results in a steep decrease in EtG and EtS urine levels. This may lead to false-negative test results (e6). Therefore, it is important to interpret EtS and EtG levels based on the urine creatinine levels or to state at least a minimum requirement—usually >20 mg/dL (e7).

However, the disadvantage of the very high sensitivity of this method is that the EtG/EtS levels in urine do not allow to distinguish between a binge drinking event several days ago and a (potentially unintended) minor alcohol intake a few hours before the sample was taken. In isolated cases, positive urine findings have been observed after use of ethanol-containing mouth rinses (26.9 volume percent [vol%]) or highly concentrated ethanol-based disinfection solutions (60–96 vol%) (e8, e9). Likewise, larger quantities (e.g. 2.5 L) of alcohol-free beer can result in positive findings of EtG and EtS in urine for up to 20 hours after consumption; declaration of alcohol content is only required starting from 0.5 vol% (e10). Prior to testing, patients or subjects should be informed about this and should be asked about such behavior.

Phosphatidylethanol

Phosphatidylethanol (PEth) is an abnormal phospholipid which is produced after alcohol exposure in cell membranes of, for example, human erythrocytes (20).

TABLE 3

Use of alcohol biomarkers

Question	Suitable parameters
Abstinence	Ethanol*[1], EtG (urine, blood, hair), MeOH, PEth
Level of consumption	Ethanol*[1], EtG (hair)
Chronic excessive drinking	EtG (hair), FAEE (hair), PEth, CDT*[2], MCV*[2], GGT*[2], AST*[2], ALT*[2]
Sample collection/scheduling	**Suitable parameters**
Spontaneous check	Ethanol*[1], EtG/EtS (blood, urine)
Possible on very short notice (1–2 days)	EtG/EtS (urine), methanol, PEth
Date known some time in advance (e.g. postal summons)	PEth, EtG (hair), CDT*[2], MCV*[2], GGT*[2], AST*[2], ALT*[2]
Sample material	**Suitable parameters**
Breath	Ethanol
Blood	Ethanol, MeOH, EtG/EtS, PEth, CDT*[2], MCV*[2], GGT*[2], AST*[2], ALT*[2]
Dried blood spot (DBS) samples	PEth
Urine	Ethanol, EtG/EtS
Hair	EtG, FAEE

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CDT, carbohydrate-deficient transferrin; DBS, dried blood spots; EtG, ethyl glucuronide; EtS: ethyl sulfate; FAEE: fatty acid ethyl ester; GGT: gamma-glutamyl transferase; MCV: mean corpuscular volume; MeOH: methanol; PEth, phosphatidylethanol

*[1] Breath alcohol content (BrAC) and blood alcohol content (BAC); warning: BrAC analysis cannot be used as evidence in court; ethanol can only be detected for a very short period of time (<12 h)

*[2] By combining the clinical chemical parameters, their significance can be increased

PEth is not a single molecule but a group of glycerophospholipids with fatty acid groups of various lengths with various degrees of saturation. So far, 48 PEth species have been identified in human blood samples (e11). The PEth homolog 16:0/18:1 is currently the species most commonly determined in analyses. It accounts for the largest proportion of the PEth homologs produced after alcohol intake and is often simply referred to as PEth (21, 22). In drinking experiments, PEth was detected in blood already after 30 minutes; PEth levels peaked after 90 to 120 minutes (23). With frequent alcohol consumption, PEth accumulates in whole blood.

Most studies on PEth were carried out retrospectively from a clinical or epidemiological perspective on alcoholics in detoxification or rehabilitation facilities or on selected patient populations, such as patients with hepatic disease, pregnant women or HIV-positive (HIV, human immunodeficiency virus) patients. Only a few studies with prospective and/or experimental design assessed healthy subjects with an at most moderate drinking behavior *(eTable)*.

Since PEth can already be detected in blood after about 1 to 2 hours and for up to 12 days after one-time alcohol intake, this biomarker can be used to determine both current consumption and abstinence (24). After long-term moderate and harmful drinking behavior and also after alcohol relapse, the significance of PEth is somewhat higher than that of CDT and is only second to the detection of EtG in hair (25) (*eTable*). However, PEth analysis allows to verify faster whether a patient/subject has changed their drinking behavior.

By measuring PEth levels, daily alcohol consumption of more than 60 g ethanol can clearly be distinguished from lower alcohol consumption. Thus, PEth can be used to identify individuals with chronic excessive drinking behavior (26). Currently, no cut-offs have been officially set, but several pertinent recommendations exist.

Even though the preanalysis and analysis of PEth is complex, this biomarker can now be determined in routine analysis, thanks to the use of dried blood samples, the availability of deuterated standards and of modern analytic technologies. Since PEth can detect chronic and one-time alcohol consumption, this biomarker is well suited to monitor abstinence and drinking behavior and to identify relapse. Therefore, Germany should follow the example of Sweden and introduce this reliable biomarker into clinical laboratories (27).

Alcohol biomarkers in hair

Ethyl glucuronide in hair

There is a close correlation between level of alcohol consumption and EtG concentrations in hair (28, 29). Measuring EtG in hair offers two advantages:

- The analysis of a proximal 3–6 cm long hair segment allows to retrospectively evaluate a period of several months.
- Short-term reduction in alcohol consumption has no effect on test results.

Thus, EtG analysis can be used to detect chronic harmful alcohol consumption, potentially causing fatty liver disease or liver cirrhosis. In a forensic setting, the use of long-term biomarkers can eliminate the need for repeated short-term checks of a subject/patient.

Based on internationally adopted cut-off concentrations, abstinence from alcohol can be verified (EtG in hair <7 pg/mg) and chronic excessive drinking with a consumption of 60 g or more of ethanol per day can be detected (>30 pg/mg). Concentrations between 7 and 30 pg EtG/mg hair are regarded as a strong indicator of regular alcohol consumption (30).

Fatty acid ethyl esters

Fatty acid ethyl esters (FAEEs) are produced in the presence of ethanol from, for example, triglycerides or free fatty acids by specific FAEE synthases and other enzymes. These products of nonoxidative ethanol metabolism can be detected in blood, tissue and also in hair. The standard substrate for quantitative analysis is ethyl palmitate; in addition, other parameters can be determined (31, 32). Besides EtG, FAEE concentrations can be measured in hair as a plausibility control, but alone they are not suitable to verify abstinence. The ethyl palmitate cutoff concentration for abstinence is

0.12 ng/mg for a 3 cm proximal scalp hair segment. A concentration of 0.35 ng/mg is regarded as a strong indicator of chronic excessive alcohol consumption (30).

For both EtG and FAEEs in hair it applies that occasional alcohol consumption is not ruled out by a negative result. Consequently, alleged abstinence cannot be verified, but at best disproved. It should be noted that 3 to maximal 6 cm long proximal hair segments are recommended as samples. If this is not the case, quantitative results should be interpreted with great caution (30). An average hair growth of approximately 1 cm per month is assumed. Interpretation should take the intradermal hair segment which is not included in the analysis and the cyclical growth pattern of hair into account (33).

Cosmetic hair treatment (tinting, coloring, bleaching, perming, straightening) can significantly reduce the concentrations of the analyte. Ethanol-containing hair care products have no effect on EtG, but may lead to false-positive FAEE results (34–36).

Clinical benefit of alcohol biomarkers

In a clinical setting, alcohol biomarkers are increasingly used to objectively determine alcohol abstinence or to rule out harmful alcohol consumption. The costs of the analyses are billed according to the German Scale of Medical Fees (GOÄ) and reimbursed by health insurances.

Detection of harmful alcohol use (German Clinical Guideline: Screening, Diagnosis and Treatment of Alcohol Use Disorders [e12]), chronic excessive drinking or alcohol dependence, e.g. with craving, loss of control or development of tolerance (compare International Classification of Diseases [ICD] 10 [e13]), should trigger close medical and/or psychiatric monitoring to reduce the risk of organ disease and of further medical, social and psychological problems (e14). Referral to an addiction specialist and/or a physician with additional qualification in addiction primary care would be desirable. Survival rates in patients with alcoholic liver cirrhosis achieving abstinence are significantly better compared to those in patients who continue to consume alcohol (37). In a prospective cohort study, the 1-year and 5-year survival rates increased from 63% to 95% and from 36% to 61%, respectively (38).

During alcohol withdrawal or quit drinking programs, analysis of EtG in urine can be used to detect relapses (39, 40). In the field of liver transplantation, alcohol biomarkers are regularly used to verify alcohol abstinence prior to listing a patient for transplantation, to monitor patients on the waiting list or to detect relapses after transplantation in time. The German Medical Association's guideline on maintaining waiting lists for patients prior to liver transplantation require that EtG in urine is measured before including a patient in the waiting list and during the waiting time for an organ (1). In case a patient tests positive for EtG in urine, he or she will be confronted with the test result by the treating physician and in addition once again referred to the psychologist or psychiatrist of the transplantation program who are specialized in alcohol disease. Experience shows that in this situation, many patients admit to the earlier denied alcohol consumption and are more open to addiction treatment. If this is not the case, the further procedure is decided by the local interdisciplinary transplantation conference: Either the patient is not listed/removed from the transplant waiting list or their status is only temporarily inactivated during further follow-up. It should be noted that this decision is not solely based on the detection of EtG in urine, but always takes all findings, including the medical history, psychological/psychiatric assessments, other laboratory/alcohol parameters, and, if indicated, liver histology and the patient's prognosis into account. To reduce the risk of a false-positive EtG test, e.g. due to the intake of alcohol-containing food, a cut-off concentration of 0.5 mg/L instead of 0.1 mg/L is used in transplantation medicine (1, 6, 18). Furthermore, patients are first informed in detail that they must stay away even from small amounts of alcohol in food, such as desserts or sauces.

BOX

Case study 1

The 55-year-old male patient with Child B cirrhosis and hepatocellular carcinoma within UNOS T2 criteria first presented for liver transplantation because of alcoholic liver cirrhosis in January 2016. The patient reported an alcohol consumption of half a bottle of hard liquor plus several bottles of beer daily until he was diagnosed with cirrhosis in December 2014. Thus, the requirement of alcohol abstinence for a period of half a year prior to liver transplantation was fulfilled, based on the information provided by the patient. However, the following laboratory parameters were abnormal: bilirubin 10.7 mg/L, AST 111 U/L, ALT 126 U/L, and GGT 1347 U/L. The patient consented to alcohol marker testing in serum and urine, but the results were not indicative of current alcohol consumption: urine EtG negative, ethanol 0 ‰, methanol 1.3 mg/L (cut-off 5 mg/L), and CDT 1.4%. However, in a sample consisting of a 3-cm-long strand of hair an extremely high concentration of 149 pg EtG/mg was measured, indicating alcohol consumption during the last 3 to 6 months. In the psychological reassessment, the patient admitted after confrontation with this result that he continued to drink alcohol until December 2015. The patient joined an addiction program and was initially not included in the liver transplant waiting list because of an unfavorable psychological evaluation result.

ALT, alanine aminotransferase (normal range <50 U/L);
AST: aspartate aminotransferase (normal range <50 U/L);
CDT, carbohydrate-deficient transferrin (cut-off 2.6%);
EtG, ethyl glucuronide (cut-off 0.5 mg/L in urine; cut-off 30 pg/mg of hair);
GGT, gamma-glutamyl transferase (normal range <60 U/L);
UNOS: United Network of Organ Sharing

KEY MESSAGES

- Besides ethyl glucuronide (EtG) in urine, tests to confirm abstinence include primarily phosphatidylethanol (PEth) in blood and EtG in hair. Using EtG in hair, alcohol consumption can retrospectively be estimated over a period of several months.
- EtG in urine is a suitable parameter both for alcohol withdrawal or quit drinking programs and for abstinence checks prior to proposed liver transplantation or inclusion in the waiting list.
- The clinical-chemistry parameters alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) can be used to identify existing alcoholic liver damage. However, their specificity is comparably low.
- A combination of various biomarkers and matrices is often advisable, because they differ in their underlying pathomechanisms.
- Alcohol biomarker results should never be interpreted in isolation, but always in the context of all relevant factors, including the clinical picture, medical history and mental and physical state of health of the patient.

Alcohol biomarkers have proven to be a very useful approach to monitor patients after liver transplantation who had first been diagnosed with alcoholic cirrhosis. Using these biomarkers, alcohol relapse can be better detected compared to medical consultation, patient-reported information and elevated liver enzyme levels (6, 7). The aim is to motivate the patient to remain abstinent to prevent irreversible damage to the implant. A recently completed study has provided evidence of the diagnostic advantage of the alcohol biomarker PEth in the transplantation situation (25). The case studies in the *Box* and *eBox* illustrate the benefit of alcohol biomarkers in transplantation medicine.

Likewise, alcohol biomarkers are generally used to determine the etiology of fatty liver disease or liver cirrhosis. However, it should be kept in mind that alcohol is often only a co-factor in the development of liver disease; thus, it is crucial to perform a diagnostic evaluation of other potential causative factors too.

Conclusion

The advantage of the clinical use of alcohol biomarkers is that the actual alcohol consumption of a patient can be assessed. This insight should be used to support a patient in such a way that damages resulting from harmful alcohol use are prevented.

The simple and cost-effective EtG urine test is better suited than ethanol-based tests to confirm alcohol abstinence during withdrawal programs, in patients on the transplant waiting list, in a forensic setting, and during primary care follow-up. By combining it with CDT and in the future also with PEth testing, where indicated, harmful alcohol consumption during the preceding 1 to 2 weeks can be detected too. Even though EtG in hair is the most telling test to confirm chronic harmful alcohol consumption, this biomarker is typically only determined in special situations, e.g. to help answer forensic questions.

Finally, it is important to emphasize that alcohol biomarker test results should never be interpreted in isolation, but always in the context of medical history, clinical findings and the patient's mental and physical state of health.

Conflict of interest
The authors declare that no conflict of interest exists.

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► **Supplementary material**
For eReferences please refer to:
www.aerzteblatt-international.de/ref1818

eMethods, eBox, eTable::
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Supplementary material to:

Alcohol Biomarkers in Clinical and Forensic Contexts

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eTABLE

Overview of the studies on phosphatidylethanol

Study population/number	Aim/methods	Diagnostic information	Notes	References
Studies on PEth in selected patient populations				
Patients with chronic liver disease (n = 222)	Self-evaluation, %CDT vs. PEth LC-MS/MS PEth 16:0/18:1 LOD: 8 ng/mL	Cut-off 80 ng/mL: sensitivity 91%, specificity 77% with ≥ 4 drinks/day in the last month	AUROC PEth: 0.90 AUROC %CDT: 0.79 supplementary to clinical assessment, relapse marker	(e15)
Outpatient alcohol withdrawal patients (n = 40), follow-up approx. 2 years	Monitoring, PEth, 9 homologs LC-MS/MS LOD, cut-off: 0.1µM Cut-off %CDT: 1.7%	Start: 70% Peth+, 55% CDT+ significant reduction in positive findings over time	PEth 16:0/18:1 is as sensitive as total PEth; relapse rate: PEth+ (43%), CDT+ (38%)	(e16)
HIV patients (n = 77) Blood collections at start and after 3 weeks	Checking of the amount drunk information, BrAC (daily) LC-MS/MS PEth 16:0/18:1 PEth 16:0/16:0 PEth 18:1/18:1 LOD: 10 ng/mL	Total patient population: Sensitivity: 88% Specificity: 88.5% ≥ 3 drinks/week: Sensitivity: 100% Specificity: 47.5% (LOD)	AUROC PEth 16:0/18:1: 0.92 > AUROC PEth 16:0/16:0 and 18:1/18:1; independent of age and BMI	(e17)
DBS of newborns (analysis up to 12 months after sample collection) (n = 250)	Prevalence, feasibility LC-MS/MS, PEth 16:0/18:1 LOD: 8 ng/mL	4% positive (PEth ≥ 8 ng/mL) Signs of alcohol consumption during last month of pregnancy	Reduction of PEth in DBS: ≤ 34.1% within a period of 12 months	(e18)
Patients before (n = 51) and after (n = 61) liver transplantation	Evaluation, comparison with other biomarkers LC-MS/MS PEth 16:0/18:1	Sensitivity PEth: 100 % Sensitivity ethanol, methanol and EtG (urine): 45–92 % last alcohol consumption ≤ 7 days	EtG in hair detects longer ago alcohol consumption	(25)
Women of reproductive age (n = 80)	Evaluation of PEth and alcohol consumption, LC-MS/MS PEth 16:0/18:1 or 18:1/16:0 LOD: 20 ng/mL (cut-off)	Significant association between alcohol amount and PEth 93% positive with ≥ 2 drinks/day	AUROC 0.77 (>1 drink/day) AUROC 0.80 (>2 drinks/day)	(e19)
Drivers in an interlock program (n = 147)	Compliance, comparison with other biomarkers LC/ELSD, LC-MS/MS LOD: 0.25 or. 0.005 µM	Ignition lock-out: LC-MS/MS: 88.5% positive, LC/ELSD: 71.2% positive LC-MS/MS positive, even with no or very few lock-outs	Method comparison (MS/MS vs. ELSD): r2 = 0.86 PEth 16:0/18:1 ≈ 45% total PEth	(e20)
Selected prospective or experimental studies on PEth				
moderate alcohol consumption (16 g women, 32 g men) (n = 44), Study duration: 3 months, no control group	Biomarker comparison LC-MS/MS PEth 16:0/18:1 LOD 3.5 ng/mL Cut-off %CDT 0.86%	PEth is always detectable after alcohol intake; Sensitivity: 100 % Specificity: 78 %	AUROC PEth: 0.92 AUROC %CDT: 0.82 The combination of PEth with %CDT does not result in an increase in AUROC.	(22)
Alcohol dose: 0.25 and 0,5 g/kg (n = 27), respectively; blood collections up to 14 days	Pharmacokinetics PEth 16:0/18:1 PEth 16:0/18:2 LC-MS/MS LOD: 5 ng/mL BrAC	PEth detected in all subjects, Cmax: 90–120 min, HL: 1.0–13.1 days, Samples with PEth 16:0/18:1 positive, but 16:0/18:2 negative, and vice versa	AUC (combined): overlapping between the doses; different formation and degradation rates of homologs; BrAC explains only 31% of PEth variability	(e21)
Alcohol dose: 1 g/kg (n = 16), blood collections up to 14 days	Window of detection after one-time consumption PEth 16:0/18:1 LC-/MS/MS EtG (urine)	Cmax: 37–208 ng/mL (8 h) mean HL: 3 days Detection period: 3–12 days		(e22)
Alcohol dose: 5 x 1 g/kg on 5 consecutive days (n = 11), Study duration: 16 days	Window of detection after repeated consumption PEth 16:0/18:1 LC-MS/MS BAC	PEth Cmax: 74–237 ng/mL (after 3–6 days) BACmax: 0.99–1.83 ‰	Low PEth concentrations compared to levels after alcohol abuse	(e23)

BrAC, breath alcohol concentration; AUROC, area under the receiver operating characteristic curve; BAC, blood alcohol concentration; BMI: body mass index; CDT, carbohydrate-deficient transferrin; CDT+, CDT-positive cases; C_{max}, maximum concentration; DBS, dried blood spot; EtG, ethyl glucuronide; HIV, human immunodeficiency virus; HL, half-life; LC/ELSD, high-pressure liquid chromatography with an evaporative light-scattering detector; LC-MS/MS, high-pressure liquid chromatography-tandem mass spectrometry; LOD, limit of detection; n, number of subjects; PEth, phosphatidylethanol; PEth+, positive PEth cases. The number combination behind the analyte identifies the PEth homolog (e.g. 16:0/18:1) used for the analysis *(see eMethods)*.
Interlock program: Vehicle-integrated breath alcohol analyzer combined with an immobilizer, including traffic-psychological support for the driver

eBOX

Case study 2

In January 2016, a 58-year-old male patient presented with decompensated hepatic cirrhosis Child C and dialysis-dependent renal failure associated with hepatorenal syndrome in the transplantation outpatient department. According to the patient's history, he had consumed 4 to 10 bottles of beer per day since age 15 until last year. He stated that he was currently drinking only 2 bottles of non-alcoholic beer per day. Liver function tests showed normal enzyme levels (GGT 60 U/L, AST 47 U/L, ALT 18 U/L). The patient was three times asked to visit the outpatient department. With his consent, alcohol biomarkers were tested and the results were always normal: EtG in urine <0,5 mg/L, ethanol 0 ‰, methanol <3mg/L (cut-off 5 mg/L), CDT <1%. EtG in hair could not be measured, because his hair was too short.

After positive psychological evaluation with regard to transplantation, the patient was included in the waiting list. During an outpatient follow-up in August 2016, a high urine EtG concentration of 111 mg/L was detected using LC/MS-MS, while the concentration of ethanol with 0 ‰, methanol was normal with 1.6 mg/L and CDT non-detectable. At the same time, GGT increased slightly to 112 U/L, AST to 51 U/L and ALT to 34 U/L. The patient denied any alcohol consumption. A liver biopsy was performed which showed only 5% focal macrovesicular steatosis and only isolated single-cell necrosis within the cirrhosis; thus, no evidence of current alcohol consumption. Likewise, the psychological re-evaluation of the patient did not show a lack of adherence. Thus, the transplantation conference decided to interpret the positive urine EtG results as a false-positive finding related to the consumption of non-alcoholic beer in the presence of dialysis-dependent renal failure, while also taking into account that this biomarker is not validated for this patient group. The patient remained on the waiting list, but was asked to stop the consumption of non-alcoholic beer and to come in for regular follow-ups. Over time, the patient's renal function improved so that dialysis treatment could be discontinued in October 2016. Yet, with a GFR of 55 mL/min, positive urine EtG levels were measured on two occasions, up to a concentration as high as 39 mg/L. At the same time, the levels of GGT increased to 303 U/L, of AST to 110 U/L and of ALT to 170 U/L. While ethanol was not detected at any time, increased concentrations of methanol of 6.3 and 5.1 mg/L, respectively, were found. CDT remained with 0.6% within the normal range. The patient continued to deny any alcohol consumption. Finally, the patient was asked without notice to come to the outpatient department on the next day when a significantly increased urine EtG concentration of 169 mg/L was found. At this point, the patient admitted for the first time that he had consumed alcohol. Due to his lack of insight into the illness and an unfavorable psychological evaluation result with regard to future abstinence, the patient was removed from the waiting list and enrolled in an addiction program.

ALT, alanine aminotransferase (normal range <50 U/L); AST: aspartate aminotransferase (normal range <50 U/L);
CDT, carbohydrate-deficient transferrin (cut-off 2.6%); ETG, ethyl glucuronide (cut-off 0,5 mg/L in urine);
GGT, gamma-glutamyl transferase (normal range <60 U/L); GFR, glomerular filtration rate (normal range >90 mL/min);
LC/MS-MS, high-pressure liquid chromatography-tandem mass spectrometry

eMETHODS ALCOHOL BIOMARKER ANALYSIS

Ethanol in the blood

Clinical chemistry laboratories normally use enzymatic tests as the method of analysis to determine ethanol levels in the blood. In order for results to be valid for forensic purposes, it is required that double measurements are performed using two different methods (e24). At least one of the methods must include an analysis by gas chromatography (e24).

Breath alcohol testing is less invasive and produces an indicative result. However, it should be noted that the results of the analyzers in clinical use lack reliability as the measuring principle is prone to errors and could even be manipulated; consequently, it cannot be used as evidence in court.

Should a positive breath alcohol test have legal consequences, such as loss of job or denial of a treatment place, among others, it has to be kept in mind that blood samples should be analyzed according to forensic criteria to obtain objective results that can be used as evidence in court. For the enzymatic measurement of blood alcohol levels, relative deviations of 15% with blood alcohol concentrations up to 0.6 g/L and of 9% with concentrations above 0.6 g/L are deemed acceptable in a clinical context, according to the German Medical Association's Guideline on Quality Assurance in Medical Laboratory Examinations ("Rili-BÄK") (e25). According to the forensic guideline, deviations from a target value are permissible if they do not exceed ± 0.05 g/kg with concentrations of up to 1.0 g/kg or ± 5% with higher concentrations (e24).

Methanol in the blood

As with the chromatographic measurement of ethanol, methanol in the blood is analyzed using headspace gas chromatography (HS-GC). This method offers the advantage that after a defined heating and calibration period only the highly volatile components present in the headspace above the sample at that time are injected into the gas chromatograph. In this way, clean chromatograms are obtained—endogenous substances in the blood cause little disturbance. Typically, a flame ionization detector (FID) is used (6, 8). The method has to be more sensitive than the one used to measure ethanol in the blood, because in the case of methanol concentrations of mg/L have to be measured instead of g/L. Therefore, for methanol analysis capillary columns are best suited. The packed columns used for chromatographic ethanol analysis do not provide sufficient levels of separation and sensitivity.

Clinical-chemistry parameters

Gamma-glutamyl transferase, alanine and aspartate aminotransferases, mean corpuscular volume

Enzyme-based assays of various manufacturers are commercially available for clinical-chemistry parameters such as gamma-glutamyl transferase (GGT), alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The sample should be collected in a heparin plasma tube.

The mean corpuscular volume (MCV) of erythrocytes is measured using flow cytometry as part of the blood count. EDTA (ethylenediaminetetraacetic acid) tubes should be used for blood sample collection.

To prevent artefact formation, clinical-chemistry analyses should be performed on the same day.

Carbohydrate-deficient transferrin

Various methods are available to measure desialyzed isoforms of transferrin. The most commonly used and commercially available methods are high-pressure liquid chromatography (HPLC) and (multi-) capillary electrophoresis as well as nephelometric immunoassays and methods based on isoelectric focusing (e26–e28). While the latter are high-throughput techniques suitable for fast screening applications, HPLC methods generate reliable results which can be used as evidence in court. As a confirmatory analysis, HPLC can verify positive screening results (e29). Reports of increased levels of carbohydrate-deficient transferrin (CDT), e.g. due to type 2 diabetes mellitus or anticonvulsant treatment, may rather be related to interferences with less specific analytical methods than with actual clinical or pharmacological effects. Genetic variants can also be more reliably distinguished using HPLC methods (e30). The sample is obtained in a serum tube and stable for 24 hours when stored in a fridge. If longer storage times are required, the sample should be centrifuged early and the serum should be stored frozen.

Ethyl glucuronide and ethyl sulfate in urine and serum

Besides immunochemical methods (EIA) which are currently only commercially available for ethyl glucuronide (EtG) and especially used for screening applications in a clinical setting, combinations of liquid chromatography-mass spectrometry (LC/MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) are the methods most widely adopted in clinical practice. The latter fulfill all requirements for clear identification and reliable quantification and can thus be used to answer forensic questions. In addition, they offer the opportunity to measure EtG and ethyl sulfate (EtS) in one run, allowing to verify a positive result and call attention to irregularities: For example, contamination of urine samples with ß-glucuronidase from *Escherichia coli* in the presence of urinary tract infection or as the result of intentional sample counterfeiting, affects the EtG concentration, but not the EtS concentration (e31). Urine and serum samples should be taken to the laboratory as quickly as possible and be stored in a fridge until then.

In the case of immunological tests, attention should be paid to the significant cross-reactivity with glucuronides of other short-chain alcohols (especially 2-propyl β-D-glucuronide, cross-reactivity 69–84% [e32]). Consequently, false-positive test results of enzyme immunoassays may be found after use of propyl- or isopropyl-alcohol-containing disinfectants and their passive inhalation (e33). Furthermore, false-positive immunochemical results were also observed after intake of chloral hydrate (e34). This reaction is explained by the interference of trichloroethyl glucuronide (e34). Therefore, the initially only indicative immunochemical results should be verified using the corresponding chromatographic method in clinical practice, too.

Phosphatidylethanol analysis

For the analysis of phosphatidylethanol (PEth), whole blood samples are generally used; tissue homogenates are almost exclusively used in research. Recently, PEth has been detected in breath samples which can be obtain in a non-invasive manner (e35).

Whole-blood samples should preferentially be stored in tubes with added EDTA, heparin or (less common) fluoride/oxalate and should not be centrifuged. Blood samples can be stored at room temperature for up to 24 hours and at 4 °C for up to 3 weeks; for longer periods, a storage temperature of –80 °C is required. Alternatively, dried blood spots (DBS) can be prepared (e36). Over a very broad range of concentrations, DBS analysis yielded results matching those obtained with native blood samples of corresponding concentrations (e36). In DBS samples stored at 4 °C and –80 °C, PEth was stable over a period of 9 months; at room temperature, an average decline by 13.5% was found after this period (e37). DBS offer the additional advantage that any ethanol, which may be present in the blood sample, will evaporate, preventing the formation of PEth after sample collection.

PEth is typically isolated from blood samples by a multi-step extraction process involving the use of 2-propanol, followed by n-hexane or n-heptane after the addition of an internal standard. For DBS, methanol was used for extraction too (e18). As the internal standard, phosphatidylpropanol (1,2-di-[octadecenoyl]-sn-glycero-3-phosphopropanol) or phosphatidylbutanol (1,2-dihexadecanoyl-sn-glycero-3-phosphobutanol) was initially used. Recently, deuterated compounds (PEth-d5, PEth-d31) have become commercially available.

Thin-layer chromatography (TLC) was used in initial studies, but then abandoned in favor of more sensitive and specific chromatographic methods due its lack of sensitivity. Analyses based on gas chromatographic separation and mass spectrometric detection failed because of non-reproducible derivatization reactions (e38). A lately developed enzyme-linked immunosorbent assay (ELISA) test kit, using serum, not whole-blood samples, did not achieve satisfactory levels of diagnostic accuracy (e39). Capillary electrophoretic separation with ultraviolet (UV) detection, a rarely used technique, and high-pressure liquid chromatographic separation with evaporative light scattering detector (ELSD), a much more common technique introduced in 1998, are today no longer used. Both methods are less sensitive and less specific compared to the LC-MS/MS methods currently in use (28). UV detectors are only of limited use in lipid analysis because they only detect lipids with conjugated double bonds or unsaturated fatty acid groups, while in case of ELSD the lipid droplets formed in the measuring cell scatter the incoming laser light. Using these detector types, contaminants or matrix components cannot reliably be distinguished from each other. However, until recently total PEth has routinely been measured using LC/ELSD in many studies.

LC-MS(MS) technologies can ionize and detect all PEth homologs ("PEth species"), using electrospray ionization in negative ion mode. Isobar compounds can be separated from each other with the help of tandem mass spectrometry. Gradient systems, composed of water, acetonitrile, methanol, and 2-propanol, as well as nonpolar columns (e.g. C4 to C8) achieve almost complete separation of the complex lipid mix. Due to the limited number of commercially available standards for quantitative analysis of individual components, PEth 16:0/18:1, the most common homolog, is preferentially used, while PEth 16:0/18:2, 16:0/16:0 and 18:1/18:1 are measured in rarer cases (e36).

Evidence from currently available studies indicates that measuring the PEth 16:0/18:1 homolog alone is not inferior to measuring additional PEth species or total PEth. However, one publication found a higher identification rate when PEth 16:0/18:2 was measured in addition to PEth 16:0/18:1 (e21). This can be caused by different production and elimination rates (e21). The two homologs have been detected in both alcoholic and moderately drinking patients, but PEth 18:0/18:1 could not be measured in the latter patient group (26).

Analysis of ethyl glucuronide in hair

Ethyl glucuronide (EtG) in hair is analyzed using a multi-step process. First, the hair sample obtained from the patient is trimmed to the segment from 0 to 3 cm. Prerequisite for this processing step is that the scalp end of the hair sample is clearly recognizable, i.e. it was marked at the time of sample collection. Next, a typically multi-step decontamination of the hair sample is performed to remove externally adhering material and dissolve the lipid layer from the hair. Homogenization and disintegration of the hair material is achieved either by cutting it with scissors to fine pieces (about 1 mm in length) or by pulverization in a ball mill. For this examination, typically an aliquot of 50 mg hair material is used.

For the actual extraction of EtG, a polar extracting agent, usually water, is used, since EtG is a highly polar substance. To facilitate this process, ultrasound and/or higher temperatures are applied. Since the limit of determination to be reached lies in the low pg-per-mg range, mass spectrometry-coupled methods (usually LC/MS/MS or GC/MS/MS) are used (e40, e41). Here, d5-ethylglucuronide serves as the internal standard (e40, e41). For hair analysis validation and quality control, commercial control materials and various inter-laboratory tests (Society of Toxicological and Forensic Chemistry [GTFCh] or Society of Hair Testing [SoHT]) are available.

Fatty acid ethyl ester in hair

For the determination of fatty acid ethyl esters (FAEE) in hair, the samples are first prepared and decontaminated in the same way as for EtG testing. However, care must be taken that suitable nonpolar solvents are used for the washing process.

It is recommended to use a 3 cm or 6 cm long proximal strand of hair for analysis (31). If longer or shorted segments of hair are used, result interpretation cannot be based on the corresponding cut-off concentrations.

First, the fatty acid esters ethyl myristate, ethyl palmitate, ethyl oleate, and ethyl stearate are extracted from the finely cut or pulverized hair. Then, after thickening using headspace solid-phase microextraction (HS-SPME), the extract is measured in combination with gas chromatography/mass spectrometry. As internal standards, the corresponding deuterated ethyl esters are used (e42, e43).