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# PHOSPHATIDYLETHANOL (PEth) CONCENTRATIONS IN BLOOD ARE CORRELATED TO REPORTED ALCOHOL INTAKE IN ALCOHOL-DEPENDENT PATIENTS

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**Abstract — Aims:** Phosphatidylethanol (PEth) is an abnormal phospholipid formed only in the presence of ethanol by the enzyme phospholipase D. PEth in blood is a promising new marker for ethanol abuse. None of the biological markers used at the present time is sensitive and specific enough for the diagnosis of alcoholism. **Methods:** The most frequently used alcohol markers [carbohydrate deficient transferrin (CDT), gamma-glutamyltransferase (GGT), and mean corpuscular volume (MCV)] were studied together with PEth in actively drinking alcohol-dependent patients (inpatients and outpatients), with regard to correlation to ethanol intake and diagnostic sensitivity of the markers. The relation between the markers was also studied. **Results:** PEth, CDT, and GGT correlated to ethanol intake, with the strongest correlation found for PEth. The diagnostic sensitivity for PEth was 99%, and for other markers it varied between 40 and 77%. Only when CDT was combined with GGT was a sensitivity of 94% reached. PEth correlated to CDT and GGT but not to MCV. CDT did not correlate to GGT or MCV. **Conclusions:** Blood concentrations of PEth are highly correlated to ethanol intake, and the present results indicate that its diagnostic sensitivity is higher than that for previously established alcohol markers.

## INTRODUCTION

Due to the difficulty in assessing alcohol-drinking behaviour from an objective point of view, a number of biological markers have been introduced and evaluated in different clinical settings. Biological markers comprise markers of toxic effects such as mean corpuscular volume (MCV) and gamma-glutamyltransferase (GGT), indirect markers such as carbohydrate-deficient transferrin (CDT) and 5-hydroxytryptophol (5-HTOL), and also direct markers. The class of direct markers includes blood ethanol itself, as well as derivatives of ethanol such as fatty acid ethyl esters (FAEE), ethylglucuronide (EtG), ethyl sulphate (EtS), and phosphatidylethanol (PEth). The short half-life of ethanol limits its utility in many situations and the derivatives, having longer half-lives, have therefore received increasing attention.

The identification of alcohol abuse has a great number of applications, medical as well as forensic. The present study is confined to the medical situation, when the degree of alcohol abuse is to be assessed at admittance to treatment programmes. Clinically established biomarkers in blood that indicate alcohol abuse are GGT, CDT, and MCV. Although widely used, none of these established markers is specific and sensitive enough to determine the degree of alcohol abuse or the medical complications of that abuse by themselves (Helander, 2001). Combinations of tests, e.g. CDT and GGT, may increase the diagnostic accuracy, but clinical variables and questionnaires are still needed, especially in situations when problem drinkers are to be identified (Chen *et al*., 2003).

PEth is a phospholipid formed only in the presence of ethanol via the action of phospholipase D (PLD) (Gustavsson and Alling, 1987; Kobayashi and Kanfer, 1987). PEth measured in whole blood has been proposed as a marker of ethanol abuse due to its high specificity and slow degradation rate (Hansson *et al*., 1997; Varga *et al*., 1998). Chronic alcohol-dependent patients have been found to have high PEth levels at admission to a detoxification clinic, and PEth is measurable for up to 14 days after sobriety (Hansson *et al*., 1997). A single binge or repeated intake of <48 g ethanol/day for 3 weeks gave in whole blood a PEth concentration of <0.7 μmol/l, which was the limit of quantification (LOQ) that was possible to achieve in two previous studies (Varga *et al*., 1998; Wurst *et al*., 2003). After repeated intake of 48–102 g ethanol/day for three weeks, the concentration of blood PEth was 1.0–2.1 μmol/l (Varga *et al*., 1998). PEth in blood resides mainly in erythrocytes and the mean half-life ($t_{1/2}$) of PEth in blood from alcoholics was found to be ∼4 days (Varga *et al*., 2000). In a recent study on 18 active alcoholic patients undergoing detoxification, PEth was the only biomarker that detected all subjects; that is, PEth gave no false negatives (Wurst *et al*., 2004). In another study, on 26 sober subjects with a previous history of addiction, no false positive values for PEth were found during a 1-year follow up (Wurst *et al*., 2003).

The aims of the present study were to evaluate whether PEth in blood is better correlated to ethanol consumption during the last 14 days before sampling than the commonly used alcohol markers (CDT, GGT, and MCV) and to compare the diagnostic sensitivities of PEth with that of CDT, GGT, and MCV.

In this study, data from two groups are reported: high alcohol consumers attending a specifically designed outpatient programme and alcoholics who were treated in an inpatient setting.

## SUBJECTS AND METHODS

### *Patients*

Patients were recruited at two different settings, (A) outpatients and (B) inpatients. All patients were diagnosed as

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alcohol dependent according to Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) (American Psychaitric Association, 1997) and The International Statistical Classification of Diseases and Related Health Problems, tenth revision (ICD-10) (World Health Organisation, 1993). In both groups exclusion criteria were illicit drug use and severe medical illness.

(A) A total of 66 actively drinking patients, 55 males and 11 females, attending an outpatient programme for consultation with a doctor about their alcohol consumption. The mean age was 49.1 ± 9.9 years. An interview was performed according to timeline follow-back (Sobell, 1992) to obtain estimations of the alcohol consumption during the previous 14 days. Only one patient reported a non-drinking period; all other patients had been drinking until the day before blood sampling. The daily mean alcohol consumption during 2 weeks before blood testing was 103 ± 64 g ethanol.

(B) A total of 78 patients, 68 males and 10 females, were admitted to the detoxification unit of the Department of Clinical Alcohol Research, Malmö University Hospital, Sweden. The mean age was 52.9 ± 8.5 years. Sampling of blood was carried out within 10 h after the patient had reached zero ethanol in expired air, measured with a breath analyser. The reason for using this procedure was to exclude the possibility of *in vitro* formation of PEth if ethanol is present as described previously (Aradottir *et al*., 2004*b*) An interview was performed according to timeline follow-back (Sobell and Sobell, 1992) to obtain estimations of the alcohol consumption during the previous 14 days. The daily mean alcohol consumption during 2 weeks before blood testing was 204 ± 126 g ethanol.

For both groups 5 ml of whole blood drawn in Na-Heparin tubes was used for the PEth analyses at Institute of Laboratory Medicine in Lund. Other samples were drawn and analysed according to standard procedures in two hospital laboratories for outpatients and inpatients, respectively.

The Ethics Committee of the Medical Faculty at Lund University, Sweden, gave approval for the study (LU146–99 and LU694–03). Patients were given oral and written information about the project and all signed an informed consent.

### *Analyses of phosphatidylethanol*

*Material.* The solvents used for extraction and HPLC analysis (hexane, propan-1-ol, propan-2-ol, and triethylamine) were obtained from Merck (Darmstadt, Germany); all were of HPLC grade except triethylamine, which was of biochemistry grade. Acetic acid was obtained from BDH Laboratory Supplies (Pool, England) and was of HPLC grade. Deionized, sterile filtered water was obtained from a Millipore Milli-Q Plus water purification system and was checked regularly for conductivity. Ethanol was obtained from Kemetyl AB (Haninge, Sweden). Phosphatidylethanol and phosphatidylbutanol were from Avanti Polar Lipids (Alabaster, Alabama, USA). Spray-coated Sodium Heparin tubes (5 ml) for blood withdrawal were of the brand Vacutainer® [Becton Dickinson Vacutainer Systems Europe (UK)].

*Extraction.* The samples were kept at +4°C for up to 7 days, but longer storage up to 3 weeks is possible (Aradottir and Olsson, 2005). Room temperature or freezing was avoided (Aradottir *et al*., 2004*a*). Whole blood was extracted by adding 300 µl of blood to propan-2-ol, containing phosphatidylbutanol (PBut) as the internal standard (IS), and then hexane was added during agitation (Aradottir and Olsson, 2005). After being agitated twice, the samples were centrifuged at 1500 *g* for 10 min and the supernatants were transferred to new tubes.

For samples with PEth concentration below 1.0 µmol/l a new extraction was performed in the same way using 900 µl of whole blood.

*Analysis by HPLC.* The lipid extracts were evaporated under nitrogen and dissolved in 150 µl of hexane:propan-2-ol (3:2 v/v). PEth was analysed by HPLC (Waters Alliance 2690) using an evaporative light-scattering detector (Alltech 500 ELSD). A 250 × 4 mm, Licrosphere 100 DIOL, 5-µm particle size column (Merck, Germany) was used with a tertiary gradient of hexane (A), 1-propanol:water (17:3 v/v) (B), and 1-propanol:acetic acid:triethylamine (316:16:1 v/v) (C). The gradient was run as described earlier (Varga *et al*., 2000). The LOQ for the method was 0.22 µmol PEth/l whole blood and the within-day and between-day coefficients of variation (CV) were <6 and <12%, respectively (Aradottir and Olsson, 2005).

### *Analyses of GGT*

Plasma GGT was measured using an enzymatic colorimetric assay with Roche/Hitachi Modular Analytics (Roche Diagnostics GmbH, Mannheim, Germany) (outpatients) and with Beckman LX20 chemistry analyser (Beckman Coulter Inc, Brea, CA) (inpatients). A comparison of the results for GGT obtained at the two laboratories revealed a difference of <7% during the study time according to External Quality Assurance in Laboratory Medicine in Sweden (EQALIS). Reference values for GGT in the Nordic countries are <1.4 µkat/l for males <40 years and <2.0 µkat/l for males >40 years, and the values for females are <0.8 µkat/l and <1.3 µkat/l, respectively (Rustad *et al*., 2004; Stromme *et al*., 2004). A reference value of 50 U/l for all ages and both genders are also given in order to enable comparison with internationally used and accepted cut-off. (For clarification 1.0 µkat/l = 60 U/l).

### *Analyses of CDT*

CDT in serum was determined using an HPLC method for transferrin glycoforms (Helander *et al*., 2003). The isoforms of transferrin were separated on an anion chromatography column using salt gradient elution. CDT was determined as per cent disialotransferrin of the total amount of transferrin, using baseline integration. A cut-off value for CDT of >1.7% was considered to be above the reference limit according to findings in 132 healthy controls (Helander *et al*., 2003). A total of 17 randomly taken samples were analysed for CDT at both laboratories during the study. Mean values were the same (6.74 CDT%) and the mean difference (± 0.28 CDT%) was independent of the CDT level. A paired students *t*-test of the results gave no indication of a difference between the laboratories ($P = 0.943$). The total imprecision for the CDT method was 9 CV% at the level of 0.9 CDT% and 7 CV% at the level of 1.5 CDT%.

*Analyses of MCV*

MCV was determined by automated techniques on Sysmex® SE-9000 instruments (Toa, Japan) (outpatients) and on Beckman Coulter LH750 (Beckman Coulter Inc, Brea, CA, USA) (inpatients). The reference value used was <98 fl.

*Statistics*

For every parameter, descriptive statistics [mean, standard deviation (SD), and range] were calculated. Correlations between parameters were investigated using Spearman's rank correlation coefficients ($r_s$), calculated using the SPSS 11.5.1 (SPSS Inc. Chicago, IL) statistical software package. Group differences were investigated using Student's t-test. A $P$-value of $\leqslant 0.05$ was considered statistically significant.

## RESULTS

Table 1 compares mean and ranges of values obtained for ethanol intake, PEth, CDT, GGT, and MCV in outpatients and inpatients. The patient groups differed significantly in three categories; ethanol intake, PEth, and CDT, where inpatients had higher mean values than outpatients. The GGT and MCV mean values for inpatients were not significantly higher than for outpatients due to the wide range of values.

Only one patient had no measurable PEth and only one patient had a PEth value of 0.33 μmol/l; both were among the outpatients. In these two patients ethanol intake was estimated at 65 and 39 g/day, respectively, and none of the other markers was positive. All other patients had values >0.7 μmol/l. One outpatient reported no ethanol intake the last 9 days, but before that this patient reported an intake of 440 g ethanol/day for a long time. The PEth value of 0.9 μmol/l and GGT of 2.5 μkat/l (150 U/l) (ref. value <2.0 μkat/l for this patient) probably reflect the earlier high ethanol intake since both PEth and GGT have long half-lives of 3–5 and 14–26 days, respectively (Orrego *et al*., 1985; Varga *et al*., 2000).

Figure 1 shows box-plots of the marker values grouped by the level of ethanol intake. Plots were made for inpatients and outpatients separately and together to show the distribution of data and to demonstrate that the markers react to ethanol intake in the same way in both groups.

In Table 2 the diagnostic sensitivities for detection of excessive alcohol use are given, both for single markers and for combinations of markers. PEth had the highest sensitivity, 98 and 100% in outpatients and inpatients, respectively. If using the cut-off for GGT used in the Nordic countries, CDT as a single marker was more sensitive than GGT or MCV, but if using 50 U/l as cut-off for GGT the sensitivity is almost the same as for CDT or even better, when GGT or MCV were combined with CDT, the sensitivity was improved.

While PEth had sensitivity close to 100% among all patients the sensitivity for CDT and GGT was related to ethanol intake (Fig. 2). In the low intake group (<40 g/day) CDT or GGT had a sensitivity of ∼40%, in the intermediate intake group (40–60 g/day) CDT or GGT had a sensitivity of ∼60%, in the 80–120 g/day intake group CDT and GGT had a sensitivity of 80%, and only in the very high intake group (>200 g/day) the sensitivity of CDT or GGT approached 90%.

The correlation between levels of alcohol markers and reported ethanol intake is shown in Table 3. A significant correlation was found between ethanol intake and PEth in both inpatients and outpatients, and when the groups were combined the correlation became stronger.

No significant correlation was found between CDT and ethanol intake in the inpatient group while significance was achieved in the outpatient group. When both groups were combined there was a significant correlation between CDT and ethanol intake, although the correlation was weaker than for PEth. A few patients with a very high reported ethanol intake had CDT levels below the cut-off level (Fig.1). Nearly all patients with CDT below the cut-off limit had high levels of PEth (Fig. 3).

GGT was correlated to ethanol intake in inpatients, but no significant correlation was found in outpatients. When the groups were combined a statistically significant correlation was found. No significant correlation was found between ethanol intake and MCV for either of the groups.

PEth correlated significantly to CDT and GGT in both in-and outpatients, but not to MCV (Table 3). No correlation was found between CDT and GGT or between CDT and MCV.

## DISCUSSION

Biochemical markers are widely used and are needed in a number of situations where alcohol abuse may occur and should be avoided. Detection in medical practices, during

Table 1. Reported ethanol intake and marker levels

| | Outpatients ($n$ = 66) | | | | Inpatients ($n$ = 78) | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean | Median | Range | SD | Mean | Median | Range | SD |
| Ethanol intake (g/day) | | | | | | | | |
| All patients*** | 102.8 | 84.5 | 0–275 | 64.3 | 204.3 | 173 | 11–600 | 126 |
| Males | 110 ($n$ = 55) | 99 | 0–275 | 68 | 213 ($n$ = 68) | 195 | 11–600 | 130 |
| Females | 66 ($n$ = 11) | 63 | 13–154 | 39 | 149 ($n$ = 10) | 141 | 58–335 | 81 |
| PEth***(μmol/l) | 3.4 | 2.9 | 0–13.1 | 2.6 | 7.7 | 7.5 | 1.9–15.9 | 3.2 |
| CDT disialo%** | 3.9 | 2.3 | 0.6–17.6 | 4.0 | 6.0 | 4.3 | 1.5–21.5 | 4.7 |
| GGT (μkat/l) | 3.3 | 1.3 | 0.2–54.0 | 8.1 | 5.1 | 1.7 | 0.2–47.1 | 8.2 |
| GGT (U/l) | 199 | 78 | 13–3240 | 489 | 307 | 102 | 11–2826 | 493 |
| MCV (fl) | 85.5 | 95.1 | 68–122 | 7.9 | 96.8 | 97.0 | 84–112 | 6.3 |

Using student's $t$-test:
**The difference between the groups is significant at the 0.01 level.
***The difference between the groups is significant at the 0.001 level.

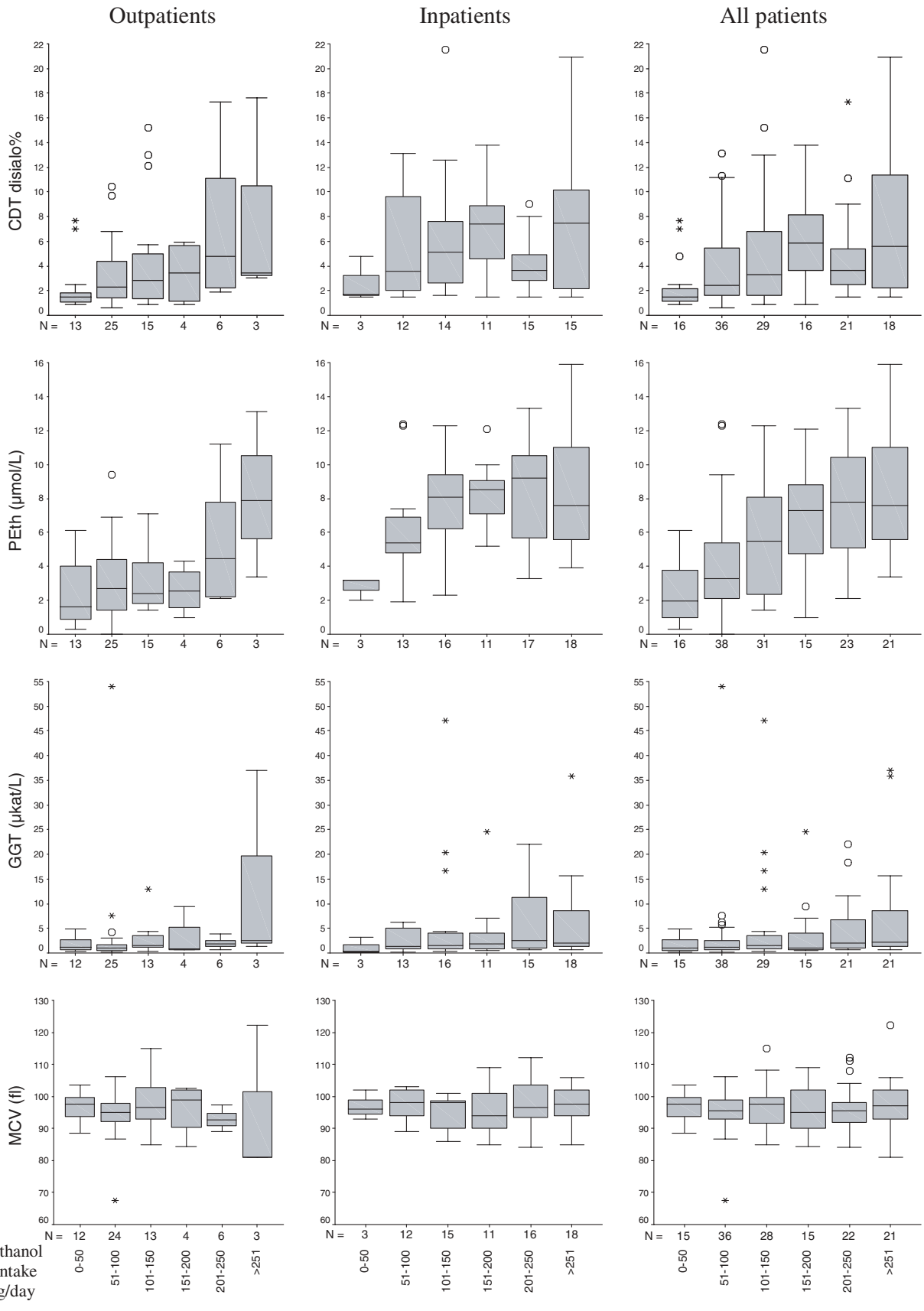


Fig. 1. Box-plot of the alcohol markers grouped after reported ethanol intake (g/day). The box-plot identifies the median, the middle 50% of the data, the range, the outliers (open circle), and the extreme points (asterisk).

Table 2. Sensitivity of markers and combination of markers in the detection of excessive alcohol use

| Test | Cut-off | Outpatients sensitivity (%) | *n* | Inpatients sensitivity (%) | *n* | All patients sensitivity (%) | *n* |
|---|---|---|---|---|---|---|---|
| PEth (μmol/l) | >0.22 | 98 | 66 | 100 | 78 | 99 | 144 |
| CDT disialo% | >1.7 | 64 | 66 | 89 | 70 | 77 | 136 |
| GGT (μkat/l) | [a] | 35 | 63 | 49 | 76 | 44 | 139 |
| GGT (U/l) | >50 | 67 | 63 | 80 | 76 | 74 | 139 |
| MCV (fl) | >98 | 32 | 62 | 47 | 75 | 40 | 137 |
| CDT+GGT (μkat/l) | | 78 | 63 | 94 | 68 | 85 | 131 |
| CDT+GGT (U/l) | | 90 | 63 | 97 | 68 | 94 | 131 |
| CDT+MCV | | 71 | 63 | 88 | 68 | 78 | 130 |
| MCV+GGT (μkat/l) | | 56 | 61 | 65 | 68 | 61 | 129 |
| MCV+GGT (U/l) | | 74 | 61 | 78 | 68 | 76 | 129 |

For GGT figures are shown for two different cut-offs.
[a]females: <40 years, <0.8 μkat/l; >40 years, <1.3 μkat/l; males: <40 years, <1.4 μkat/l; >40 years, <2.0 μkat/l.

Table 3. Spearman's rank correlation ($r_s$) between biological state alcohol markers and total grams of ethanol consumed the last 14 days

| | Outpatients | | | | Inpatients | | | | All patients | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PEth | CDT | GGT | MCV | PEth | CDT | GGT | MCV | PEth | CDT | GGT | MCV |
| Ethanol intake | 0.3483** | 0.303* | 0.215 | –0.72 | 0.289* | 0.104 | 0.231* | 0.060 | 0.568*** | 0.347*** | 0.288** | 0.031 |
| CDT | 0.543*** | 1 | | | 0.433*** | 1 | | | 0.589*** | 1 | | |
| GGT | 0.333** | –0.150 | 1 | | 0.231* | –0.157 | 1 | | 0.326*** | –0.086 | 1 | |
| MCV | 0.200 | 0.074 | 0.192 | 1 | –0.030 | 0.219 | 0.485*** | 1 | 0.088 | 0.113 | 0.377*** | 1 |

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

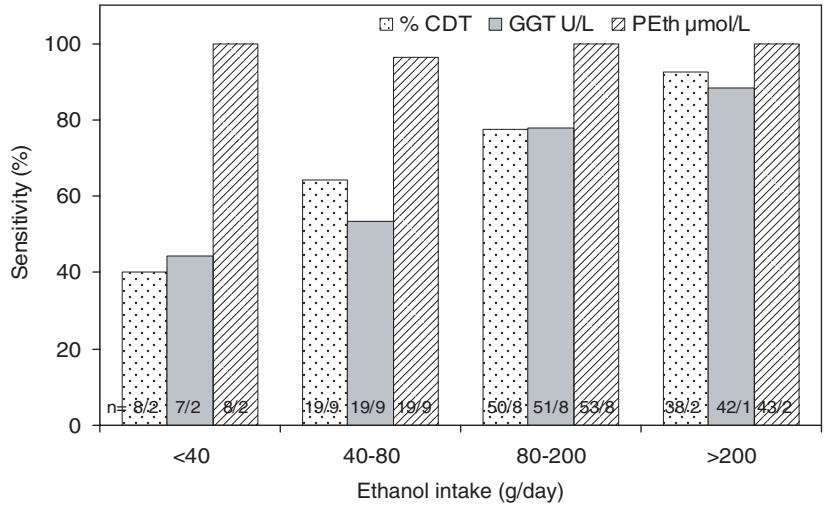


Fig. 2. The sensitivity (%) of CDT, GGT, and PEth is shown in relation to ethanol intake. The groups are ordered in four levels after ethanol intake, low consumption (<40 g/day), intermediate consumption (40–80 g/day), high consumption (80–200 g/day), and very high consumption (>200 g/day). Number of persons included in each group is shown in the bars as males/females.

treatment of recovering alcoholics, during pregnancy, and also for forensic purposes are examples of such situations. Conversely, there is usually no need to confirm the alcohol abuse by a marker in a treatment-seeking population. The reason for approaching such populations in the present study was the need to study very well-characterized patients in order to evaluate and compare markers in relation to alcohol consumption.

Three major findings emerged from this study: (i) PEth has better sensitivity than the other markers; (ii) PEth has the best correlation to reported alcohol consumption; and (iii) the findings were the same in inpatient and in an outpatient setting. Taken together, our results suggest that PEth is a superior biomarker for ethanol abuse than CDT, GGT, or MCV.

Although the sensitivity of biological markers varies depending on the group studied, the sensitivities of CDT, GGT, and MCV in the present study are in accordance with earlier reports on these markers, as reviewed by Musshoff (Musshoff and Daldrup, 1998). When patients in the present study were grouped together in four classes according to daily ethanol intake it became very obvious that sensitivity of CDT or GGT varied from 40% to ~90% (Figure 2). In the WHO/ISBRA collaborative project it is concluded that neither CDT nor GGT perform well in the early detection of

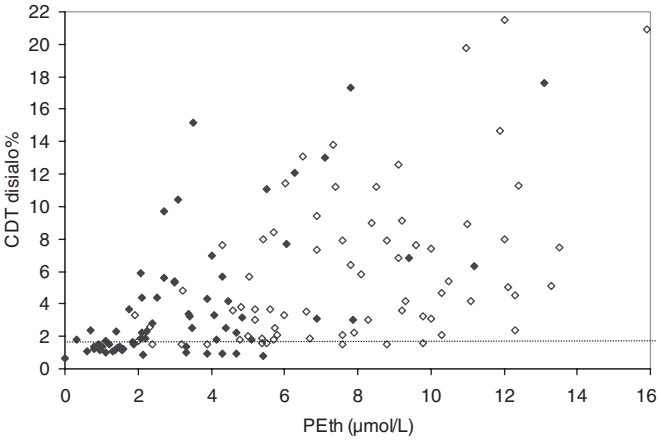


**Fig. 3.** Correlation between PEth and CDT values in all patients analysed for both markers ($n$ = 136). The dotted line at 1.7% CDT represents the cut-off line for CDT. Filled squares indicate outpatients; open squares indicate inpatients. The Spearman rank correlation coefficient, $r_s = 0.589$ ($P < 0.0001$).

hazardous alcohol consumption; this is particularly evident in females (Conigrave *et al*., 2002). A recent multi-centre study concluded that CDT and GGT were comparable in identifying heavy alcohol consumption in males, but GGT appeared to perform better in females (Anton *et al*., 2002). In the present study, CDT performed as well or slightly better than GGT amongst males, whereas, for females, GGT appeared to perform better than CDT. The number of females in the study is too small to draw any firm conclusions regarding the possible effect of gender, especially in view of the findings that mean ethanol consumption was lower in females than in males (Table 1).

CDT and GGT are independently associated to ethanol abuse and therefore the combination of these two markers is proposed to increase diagnostic accuracy (Sillanaukee and Olsson, 2001; Sillanaukee *et al*., 2000*a*, 2000*b*; Chen *et al*., 2003). Consistent with this, also in this study CDT and GGT were not significantly correlated and the combination of these two markers increased the sensitivity in all patients from 77% for CDT and 44/74% for GGT up to 85/94% for the combination.

CDT and PEth were correlated (Fig. 3 and Table 3), but PEth showed higher sensitivity than CDT, 99% compared with 77%. The diagnostic specificity of PEth is theoretically very high since PEth is only formed in the presence of ethanol, giving a biological specificity of 100%. Provided that analytical errors are excluded (Varga *et al*., 1998; Aradottir and Olsson, 2005) the overall specificity should be close to 100%.

No false positive PEth values have been recorded in blood from humans, neither as a consequence of endogenous molecules nor as a consequence of drugs (Varga *et al*., 1998; Wurst *et al*., 2003). One might speculate that in populations with low prevalence of alcohol abuse or with less extensive abuse, PEth is a better marker of drinkers at risk than CDT and GGT, since the prevalence of false positives is much lower.

The correlation between reported ethanol intake and measured marker levels showed that PEth was the only marker that was significantly correlated to ethanol intake when in-and outpatients were analysed separately (Table 3 and Fig. 1). GGT was significantly correlated to ethanol intake only in inpatients ($r_s = 0.231$), who were more severely injured by alcohol abuse and had higher ethanol consumption than outpatients. CDT was significantly correlated to ethanol intake in outpatients ($r_s = 0.303$) but not in inpatients. This anomaly may be explained by the fact that some individuals show no or low increase in CDT (non- or low responders) despite excessive ethanol intake, which has been found also in other studies. (Helander *et al*., 1996; Salmela *et al*., 1994). Furthermore, reported ethanol intake is a more reliable index of actual intake in outpatients because they perform better in a timeline follow-back interview depending on them, being less affected of their alcoholism. When all patients from the in- and outpatient groups were combined, it was found that PEth, CDT, and GGT significantly correlated to ethanol intake, but the strongest correlation found was for PEth. MCV did not correlate to ethanol intake in this study.

In an earlier study on healthy volunteers, quantifiable levels (>0.7 µmol/l) of PEth were obtained after ethanol intake of 48–102 g/day for 18 days, while subjects with lower ethanol intake did not show measurable PEth levels (Varga *et al*., 1998). In that study the measured PEth level was on average 1.88 ± 0.37 µmol/l, which is lower than the levels measured for the outpatients (3.40 ± 2.6 µmol/l) and inpatients (7.7 ± 3.2 µmol/l) in the present study. There is thus a rank order correlation between average alcohol intake and PEth levels also when different groups of alcohol consumers are compared.

Further studies are needed to establish the cut-off values for PEth concentrations valid for social drinkers, for persons with at-risk alcohol consumption, and for apparently healthy persons with hazardous drinking.

In conclusion, PEth is highly correlated to ethanol intake and the present results indicate that its sensitivity is higher than that of previously established alcohol markers. PEth seems to be a good candidate as a new alcohol marker due to the high sensitivity and high specificity.

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