Alcoholism: Clinical and Experimental Research

Vol. 45, No. 4
April 2021

Phosphatidylethanol as Blood Biomarker of Alcohol Consumption in Early Pregnancy: An Observational Study in 4,067 Pregnant Women

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Background: The teratogenic effects of alcohol are well documented, but there is a lack of screening methods to detect alcohol use during pregnancy. Phosphatidylethanol 16:0/18:1 (PEth) is a specific and sensitive biomarker reflecting alcohol intake up to several weeks after consumption. The aim of this study was to investigate the prevalence of positive PEth values as an indicator of early prenatal alcohol exposure in a general population of pregnant women.

Methods: Rhesus typing is routinely performed in Norway in all pregnancies around gestational week 12. Rhesus-negative women have an additional test taken around week 24. Blood samples submitted to St. Olav University Hospital in Trøndelag, Norway, for Rhesus typing during the period September 2017 to October 2018 were collected. A total of 4,533 whole blood samples from 4,067 women were analyzed for PEth (limit of quantification of 0.003 μM).

Results: Fifty-eight women had a positive PEth sample. Of these, 50 women were positive around gestational week 12, 3 women were positive around week 24, and in 5 cases, the timing was unknown. There were no significant differences in proportions of women with positive PEth values related to age, or rural versus urban residency.

Conclusion: In an unselected pregnant population in Norway, 1.4% had a positive PEth sample around gestational week 12, whereas 0.4% had a positive sample around week 24. The use of PEth as an alcohol biomarker should be further investigated as a diagnostic tool in the antenatal setting.

Key Words: Pregnancy, PEth, Alcohol Biomarkers, Fetal Alcohol Syndrome, Fetal Alcohol Spectrum Disorders.

IT IS WELL documented that alcohol has teratogenic effects, and there is no known safe level of alcohol consumption in pregnancy (Flak et al., 2014; Polanska et al., 2015). Clinical guidelines in many countries, including Norway, therefore recommend to completely abstain from alcohol use in pregnancy (Norwegian Directorate of Health, 2017). Despite this advice, it is estimated that 25% of European and 10% of women worldwide use alcohol during pregnancy (Popova et al., 2017). Prenatal alcohol exposure may cause fetal alcohol spectrum disorder (FASD), a preventable cause of mental retardation with lifelong implications for exposed children. The most severe form of FASD is the fetal alcohol syndrome (FAS), which includes a triad of growth restriction, abnormal facial features, and mental retardation. It is estimated that globally, about 119,000 children are born with FAS and more than 1,000,000 children with FASD every year (Popova et al., 2017). It can be difficult to identify FASD postnatally because the condition can be confused with a number of other diseases and syndromes (Williams and Smith, 2015). Most affected children therefore remain undiagnosed (Chudley, 2008; Popova et al., 2017). In addition, lack of appropriate screening methods and diagnostic possibilities makes prevalence calculations challenging (May et al., 2009). The incidence of FAS in Norway has been

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Received for publication June 23, 2020; accepted February 8, 2021.

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DOI: 10.1111/acer.14577

estimated to be 1 to 2 children per 1,000 births (Løhaugen et al., 2015), but no exact frequency of the more invisible FASD diagnosis was provided. In general, it has been suggested that the rate of FAS to FASD is 1 to 9 or 1 to 10 (Popova et al., 2017). Diagnosing FASD in a newborn child or at an early age is of major importance for successful intervention (Alvik et al., 2013; Popova et al., 2016). Children diagnosed with FASD after the age of 12 years have a 2- to 4-fold increased risk of substance and alcohol abuse, criminal involvement, and school disruption compared with children diagnosed at an earlier age (Streissguth et al., 2004).

Confirmed prenatal alcohol use can strengthen the FAS/FASD diagnosis; therefore, reliable information on alcohol exposure in utero will be a valuable tool for correct diagnosis. The World Health Organization (WHO) recommends screening all pregnant women for alcohol use as early as possible (World Health Organization, 2014). The WHO does not provide detailed advice on how to screen pregnant women, but screening methods based on questionnaires are mentioned as an alternative. However, self-reporting has been shown to underestimate alcohol use and to be unreliable among pregnant women due to poor memory, social stigma, and fear of child welfare interventions (Ferraguti et al., 2017; Lange et al., 2017; Raggio et al., 2019).

More objective methods of detecting alcohol exposure include the analyses of biomarkers of alcohol consumption. Indirect biomarkers such as gamma-glutamyl transferase (GGT), mean red blood cell corpuscular volume (MCV), and carbohydrate deficient transferrin (CDT), however, have a low sensitivity and specificity (Howlett et al., 2017). In addition, CDT might be falsely elevated in pregnancy (Joya et al., 2012). Direct biomarkers of ethanol (EtOH) intake such as EtOH itself or the EtOH metabolites ethyl glucuronide (EtG) and ethyl sulfate (EtS) have very short detection times (Ulwelling and Smith, 2018). In contrast, phosphatidylethanol (PEth) is a specific direct biomarker only produced with the existence of EtOH (Stewart et al., 2014; Varga et al., 1998). It has a high sensitivity in detecting moderate and high alcohol intake (Aradottir et al., 2006; Gustavsson and Alling, 1987; Helander et al., 2019a; Kechagias et al., 2015; Kwak et al., 2012), including women of reproductive age (Stewart et al., 2010). PEth can be analyzed in whole blood or dried blood spots and may reflect alcohol consumption more than a month prior to sampling, depending on the limit of detection of the analytical method used (Helander et al., 2019a; Kechagias et al., 2015; Viel et al., 2012).

Studies on PEth as a biomarker for alcohol consumption in pregnancy have mostly been carried out during late pregnancy or with newborn children (Bakhireva et al., 2014; Bakhireva et al., 2017; Bracero et al., 2017; Comasco et al., 2012; Howlett et al., 2017; Maxwell et al., 2019; May et al., 2018; Naik et al., 2020). To our knowledge, only 2 studies have investigated its use in early pregnancy (Kwak et al., 2012; Kwak et al., 2014). However, these studies were small and conducted on selected populations. The aim of the present study was to investigate the prevalence of positive PEth samples as an indicator of early prenatal alcohol exposure in a general population of pregnant women.

MATERIALS AND METHODS

As a part of the Norwegian prenatal care program, Rhesus typing and antibody screening are performed in all pregnant women around gestational week 12. Rhesus-negative women have a second blood sample taken for noninvasive prenatal testing (NIPT) for Rhesus and autoantibodies around gestational week 24. Blood samples sent to the Department of Immunology and Transfusion Medicine at St. Olav University Hospital between September 15, 2017, and October 4, 2018, were included in the present study. The Department receives samples from the majority of pregnant women in the county of Trøndelag, comprising 8.7% of the Norwegian population. Samples with insufficient material remaining after the routine examinations, or where further investigations due to alloimmunization were indicated, were not included. Due to technical difficulties, the collection was paused for approximately 3 weeks during July 2018. Thus, the inclusion period lasted 365 days. Due to the fact that the antenatal care program in Norway is free of charge, the attendance rate is close to 100%.

The Regional Committee for Medical and Health Research Ethics in Central Norway approved the study (folder No. 2017/581). Individual active informed consent was not required; instead, all participants were informed in general terms by mail after sampling that their biological material could be used in medical research. The aim of the study and the specific substance to be tested were not presented. The subjects were also informed about the possibility of reserving themselves against such use in the Norwegian Registry of Withdrawal from Biological Research Consent (Norwegian Institute of Public Health, 2017). By March 1, 2019, only 0.2‰, that is, 1,294 of Norway's population of 5.4 million, were included in this register. As the aim of the study was not specified in the information letter, we have no reason to believe that alcohol consumption would have constituted a bias in the reservations.

During the study period, a total of 5,526 samples from 4,755 women were received for analysis at the Department of Immunology and Transfusion Medicine, St. Olav University Hospital (Fig. S1). We excluded 75 samples from 65 women listed in the reservation register and 877 samples due to insufficient amount of blood or technical factors. Seven samples from women below the age of 18 were excluded from the study on the basis that individuals in Norway below this age cannot legally provide informed consent by themselves. As patients above 50 years of age were considered not to be of a reproductive age, samples from these women were also excluded from the study; hence, 1 sample from a 74-year-old woman was removed, assuming that the Rhesus test had been ordered by mistake. In addition, 32 samples from 31 women were excluded because they were duplicates obtained on the same date. Finally, 1 sample was excluded due to an analytical error. Thus, 4,533 samples from 4,067 women were included (Fig. S1), corresponding to 82.0% of all samples (89.8% of all samples from week 12 and 62.8% of all samples from week 24).

As part of the RhD and autoantibody screening, the collected 6ml samples were centrifuged, and 410 µl plasma and 25 µl red blood cells were pipetted off and used for these analyses. Thereafter, the remainder of the sample was thoroughly mixed and stored at −80°C, as this temperature has been shown to maintain stable concentrations for at least 90 days (Skråstad et al., 2020). The PEth analog 16:0/18:1 was analyzed with a validated routine ultraperformance liquid chromatography tandem mass spectrometry (UPLC®-MSMS) method, described in detail previously (Andreassen et al., 2018). The extraction method was modified to lower the limit of quantification to 0.003 µM. The modifications were as follows:

increasing the sample volume from 150 µl to 300 µl blood; increasing the total protein precipitation solvent (2-propanol) from 450 µl to 1,050 µl; and, adding an evaporation (50°C, 60 minutes under stream of air) and resolving (2-propanol, 100 µl) step to the sample preparation. Chromatographic separation was achieved with a Waters Acquity BEH-Phenyl column (2.1 × 30 mm, 1.7 µm) with precolumn using a gradient elution starting with 40% ammonium formate (5 mM, pH 10.1, mobile phase A) in combination with 60% acetonitrile (mobile phase B). During the first 90 seconds, the gradient went from 60% to 95% B, then remained at 95% B for 6 seconds. Forty-two seconds with 60% B was found sufficient to equilibrate the column before next injection. The flow rate was 0.5 ml/min and the run time 2.3 minutes. The injection volume was 2 µl.

For detection and quantification of PEth 16:0/18:1 in negative ionization mode, the m/z 701.7 > 255.2 and m/z 701.7 > 281.3 transitions were used. The m/z 706.7 > 281.3 transition was used for the internal standard PEth 16:0/18:1-d_5. The 5-point calibration curve went from 0.003 µM to 0.50 µM with a quadratic curve fit and a coefficient of determination, R^2, >0.9999. Coefficients of variance for between-assay precision (n = 5) were ≤ 7.4% with inaccuracies ≤ 2.4% at the 0.003 (Standard 1), 0.008 (Quality control [QC] 1), 0.004 (QC 2), and 0.4 (QC 3) µM levels.

SPSS Statistics, version 25.0. (Armonk, NY) was used for statistical analyses. Independent sample t-tests were used to compare means, and chi-square tests were used to compare proportions. p Values of <0.05 were considered statistically significant.

RESULTS

In total, 4,533 samples from 4,067 women were included in the final analysis. The age and residency of the women are presented in Table 1. Of the women, 3,652 (89.8%) contributed with 1 sample, 369 (9.1%) with 2 samples, 41 (1.0%) with 3 samples, and 5 (0.1%) with 4 samples. A total of 3,451 samples (76.1%) were from the first trimester and 830 (18.3%) from the second trimester, whereas sampling time was unknown for 252 samples (5.6%) (Table 2).

Overall, 58 of the 4,533 samples (1.3%) were positive for PEth 16:0/18:1 when using a limit of quantification of 0.003 µM. All were from different women; that is, 1.4% of the 4,067 women had a positive PEth sample. In the first trimester, 1.4% of the samples were positive, and in the second trimester, 0.4% were positive (Table 2). Among the 58 positive women, 46 had only 1 registered blood sample, 11 women had 2 samples, and 1 woman had 3 samples. For PEth-positive women with multiple blood samples, only 1 had a first sample that was negative and a second (taken 8 days later) that was positive. Otherwise, all women had their positive sample as the first 1 taken. There were no significant differences between the proportions of positive samples when comparing women from rural municipalities (1.4%) to women from urban areas (1.5%) defined as having more than 100 000 inhabitants (Table 1). The same applied to women with positive or negative samples that both had an average age of just over 30 years (Table 1).

Seasonal variations in detectable PEth values are presented in Fig. S2, which shows that during spring, there were 0.5% (6 cases), and during fall, there were 1.9% (25 cases) of positive cases (p = 0.015).

The distribution of PEth concentrations among the 58 positive samples is displayed in Fig. 1. The mean and the median PEth concentrations of the positive samples were 0.026 and 0.010 µM respectively, and the highest PEth concentration measured was 0.287 µM. Individual PEth concentrations in the 58 positive samples are listed in Table S1.

DISCUSSION

The principal finding from the study was that 1.4% of the pregnant women included had a positive PEth sample around gestational week 12 whereas 0.4% had a positive PEth sample around gestational week 24.

The population in Trøndelag represents approximately 9% of the total population in Norway and has been shown to be representative of the general Norwegian population across different health factors (Holmen et al., 2003). In terms of the consumption of alcohol, the population in Trøndelag is representative of the general population, although it has been established that residents in the urban county Oslo drink alcohol more often, and Trøndelag has a lower percentage of alcohol abstinence and a higher percentage of

Table 1. Age and Residence Characteristics Among Those With Positive and Negative Phosphatidylethanol 16:0/18:1 (PEth) Samples

	Women with a positive PEth sample (n = 58)	Women with no positive PEth samples (n = 4009)	p-Value
Mean age ± SD (years)	30.3 ± 5.5	30.2 ± 4.7	0.88
Residency—urban (n = 3,479)	50 (1.5%)	3,429 (98.5%)	0.89
Residency—rural (n = 588)	8 (1.4%)	580 (98.6%)	

Table 2. Number of Samples Positive and Negative for Phosphatidylethanol 16:0/18:1 (PEth) Separated by Trimester of Sampling

	PEth-positive samples	PEth-negative samples	Total
First trimester	50 (1.4%)	3,401 (98.6%)	3,451 (76.1%)
Second trimester	3 (0.4%)	827 (99.6%)	830 (18.3%)
Time of sampling unknown	5 (2.0%)	247 (98.0%)	252 (5.6%)
Total	58 (1.3%)	4,475 (98.7%)	4,533 (100%)

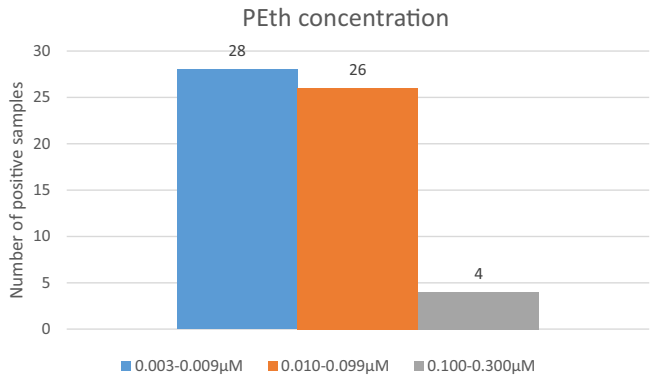

Fig. 1. Distribution of PEth concentration in the 58 positive samples. Conversion factor: PEth (µM) × 703 = PEth (ng/ml).

binge drinking (Norwegian Institute of Public Health, 2018). Thus, even though there are some differences in drinking patterns across the country, we are confident that our findings at large are representative of the overall pregnant population in Norway.

This study has limitations that need to be acknowledged. One limitation was the lack of anamnestic and/or self-reported information on alcohol consumption, as we were not given permission to collect such data by the ethics committee. Moreover, this information is not collected in a thorough and standardized manner in the Norwegian prenatal care program. Consequently, we were unable to correlate the measured PEth concentrations to a specific level of alcohol consumption. Another limitation was that we were unable to link the PEth results to perinatal outcomes, as it would have been particularly useful to identify PEth concentrations that represent a definitely harmful level. In any case, pregnant women should ideally abstain from alcohol and none should receive a positive PEth value at the end of the first trimester (Centers for Disease Control and Prevention, 2020; Norwegian Directorate of Health, 2017).

In our study, neither age (mean 30.3 vs. 30.2 years) nor rural living conditions (1.4% positive) vs. urban living conditions (1.5% positive) influenced the proportion of women with positive PEth values. In comparison, a recent European study found that pregnant alcohol consumers were more likely to be older, and also more highly educated and more often employed (Mardby et al., 2017). This aligns with Norwegian statistics on public health highlighting that higher education and higher income increase the frequency of alcohol consumption within the general population (Norwegian Institute of Public Health, 2018). We also know from previous studies that late recognition of pregnancy, high consumption of alcohol before pregnancy, and having a partner who consumes more alcohol are risk factors for giving birth to a child with FASD (Alvik et al., 2006; May et al., 2014). Unfortunately, we were unable to include those variables in this study. If, on the other hand, we look at the seasonal variation, where fall had the highest proportion of PEth positive samples, this is in line with what has been found in the general population where summer is the seasonal period with the highest reported alcohol use (Knudsen and Skogen, 2015; Uitenbroek, 1996).

Due to the heterogeneous design across studies and large differences between countries, it is challenging to compare our current results with previously reported incidences of alcohol consumption during pregnancy. The results from the Norwegian Mother, Father and Child Cohort Study, conducted between 1999 and 2008, showed that 31.8% of pregnant women reported drinking alcohol in the first trimester (Magnus et al., 2014). This is in line with findings from another Norwegian study from 2000 to 2001 where 44.4% of women reported alcohol use early in pregnancy (Alvik et al., 2006). The most up-to-date information on alcohol consumption among pregnant women in Norway is from a questionnaire study carried out during 2011/2012, comparing alcohol consumption among pregnant women across Europe. Women in Norway and Sweden had the lowest rate of alcohol use, 4.1% and 7.2%, respectively, after being aware of their pregnancy. In comparison, the frequency was 28.5% in the United Kingdom (Mardby et al., 2017). It is important to note that the blood samples in the present study were drawn toward the end of the first trimester, and the elimination half-life of PEth is 4 to 12 days (Helander et al., 2019a; Kechagias et al., 2015). Thus, with a maximum detection period of approximately 4 to 6 weeks, alcohol consumption during the first few weeks of pregnancy (or a small amount later in the first trimester) will probably not result in a positive PEth test taken toward the end of the first trimester. As expected, we found a lower number of positives than in questionnaire studies, where alcohol consumption during the entire pregnancy is considered. Thus, in our opinion a PEth analysis may identify at-risk patients to a larger extent that questionnaire studies, by indicating a more significant intake. Also, one can assume that women with a high level of alcohol consumption to a greater extent have an addiction disorder and may want to withhold information about their alcohol consumption.

It is relevant comparing the PEth concentrations in the 58 positive samples in our study to the proposed guidelines for evaluating PEth results. In a review by Ulwelling and Smith, PEth concentrations > 20 ng/ml (0.028 µM) are suggested as a limit of significant consumption while PEth concentrations > 200 ng/ml (0.28 µM) are regarded as indicative for heavy consumption (Ulwelling and Smith, 2018). According to this information, 9 women in our study would have a significant alcohol intake and 1 would have a heavy alcohol consumption. It is estimated that 2 to 4 drinks a day up to several days a week are required to be included in the significant group (Ulwelling and Smith, 2018). So despite the fact that the largest proportion of pregnant women had a PEth value below 0.28 µM in our study, such values are not necessarily harmless. Our study had 14 positive cases per 1,000 births, which would have resulted in approximately 770

children vulnerable for FAS or FASD at a national level, given the Norwegian birth rate of 55,000 annually (Statistics Norway, 2019). The estimated number of children with FAS born annually in Norway is 60 to 120 (Løhaugen et al., 2015). If we assume that the ratio of FAS to FASD is 1 to 10, our results are in line with previous assumptions (Chudley, 2008; Popova et al., 2017).

In nonpregnant populations, PEth concentrations show a relatively high correlation to a given level of alcohol consumption at a group level, albeit with a considerable interindividual variability (Helander et al., 2019b). This has also been shown in the first trimester of pregnancy, where a positive correlation between the self-reported number of alcohol units consumed and the PEth concentration was found (Kwak et al., 2014). PEth as a biomarker of alcohol consumption in pregnancy has been evaluated in several previous studies (Bakhireva et al., 2014; Bakhireva et al., 2017; Bracero et al., 2017; Comasco et al., 2012; Howlett et al., 2017; Kwak et al., 2012; Kwak et al., 2014; Maxwell et al., 2019; May et al., 2018; Naik et al., 2020; Yang et al., 2015). PEth was shown to have high sensitivity for recent heavy drinking, but lower sensitivity for moderate or low consumption (Kwak et al., 2014; Stewart et al., 2010). In contrast, a study from South Korea found detectable PEth concentrations 4 to 6 weeks after the last alcohol intake in 13 women in the first trimester, with a self-reported low-to-moderate alcohol consumption (Kwak et al., 2012). Another study by the same authors revealed that 17% had a positive PEth sample after 3 to 4 weeks of abstinence when using a limit of quantification of 0.0031 μM (Kwak et al., 2014), that is, similar to our study. Studies with a larger number of participants have been carried out immediately after birth, where PEth has been measured in dried blood spots or umbilical cord blood (Bakhireva et al., 2014; Bakhireva et al., 2017; Baldwin et al., 2020; Bracero et al., 2017; Maxwell et al., 2019). These studies have shown a significantly higher proportion of alcohol-exposed pregnancies than in our study. This difference may be accounted for by inclusion bias, but most likely reflects how the general population of pregnant women in Norway consume less alcohol.

It seems reasonable to suggest that women with a positive PEth test taken at a routine control at the end of the first trimester, constitute a potential risk group that should be targeted for healthcare follow-up regarding alcohol use. A positive test could open up a dialogue about alcohol consumption and its consequences during pregnancy, and ensure best practice treatment and maternal education from the earliest stage. In addition, a positive PEth value will increase the reliability of a FAS/FASD diagnosis in the infant.

Further research is needed to clarify the relationship between a given PEth value measured in pregnancy and alcohol consumption, including marker sensitivity. Additionally, studies linking PEth results to perinatal outcomes would be particularly useful for identifying harmful levels of alcohol consumption in pregnancy.

CONCLUSION

This study has shown that analyzing PEth during an early antenatal healthcare visit is a feasible procedure that may help to identify pregnant women who are potentially in need of intervention. The fact that 1.4% of pregnant Norwegian women, who are among the lowest alcohol consumers in Europe, tested positive during the latter part of the first trimester indicates that there remains a need to promote abstinence during pregnancy.

FUNDING

This project was funded by Norwegian Women's Public Health Association, and the Department of Clinical Pharmacology and Clinic of Substance Use and Addiction Medicine, St. Olav University Hospital, Trondheim, Norway. The funding sources had no involvement in the study design, writing, or submission of the article.

ACKNOWLEDGMENTS

The authors would like to thank the staff at the Department of Immunology and Transfusion Medicine, the Department of Clinical Pharmacology, St. Olav University Hospital, and the Norwegian Women's Public Health Association.

CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Number of samples excluded from analysis and reasons for exclusion.

Fig. S2. Seasonal distribution of 58 positive PEth samples.

Table S1. PEth concentrations in the 58 positive samples.