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The Validity of Phosphatidylethanol in Dried Blood Spots of Newborns for the Identification of Prenatal Alcohol Exposure

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Abstract

Background—Accurate identification of prenatal alcohol exposure (PAE) in the newborn period offers an opportunity for early identification of children at risk for future neurocognitive problems and the implementation of interventional approaches earlier in life. PAE newborn screening by measuring phosphatidylethanol in dried blood spot (PEth-DBS) cards is feasible, logistically easier, and more cost-efficient compared to other biomarkers. However, the sensitivity and specificity of this method have yet to be established.

Methods—This prospective cohort study examined validity of PEth-DBS among 28 infants with PAE and 32 controls relative to maternal self-report and other biomarkers. Pregnant women were recruited from a University of New Mexico clinic and followed to early postpartum period. The composite index, which was based on self-reported measures of alcohol use and allowed to classify subjects into PAE and control groups, was the criterion measure used to estimate sensitivity and specificity of PEth-DBS.

Results—The study included large proportions of patients representing ethnic minorities (7.4% American Indian, 81.7% Hispanic/Latina), low education (54.2% <high school), and unplanned pregnancy (90.0%). No differences in socio-demographic characteristics, smoking, or illicit drug use were observed among the study groups. The sensitivity of maternal biomarkers (GGT, %CDT, UEtG, UEtS) was low (<15%) reflecting a moderate chronic or intermittent binge pattern of drinking in this cohort. PEth-DBS demonstrated 100% specificity and the highest sensitivity (32.1%) compared to other biomarkers. A battery consisting of maternal direct ethanol metabolites

(UEtG, UEtS, PEth) and newborn PEth-DBS increased sensitivity to 50% without a substantial drop in specificity (93.8%).

Conclusions—Newborn PEth-DBS is a highly specific biomarker and can facilitate accurate detection of PAE in conjunction with other biomarkers. Minimal invasiveness, ease of storage and transportation of DBS cards, absence of post-collection synthesis, cost savings, and potential integration with routine newborn screening are all unique advantages of this method.

INTRODUCTION

Despite the Healthy People 2020 goals for abstinence from alcohol use and binge drinking among pregnant women, roughly 10% of pregnant women in the U.S. continue to drink (Anderson et al., 2006, MMWR, 2012). Prenatal alcohol exposure (PAE) can cause birth defects and lifelong neurocognitive deficits in affected children collectively termed Fetal Alcohol Spectrum Disorder (FASD). While the prevalence of the entire spectrum of FASD is difficult to estimate, some studies have suggested that 1–5% of young school-aged children in the U.S. are affected (May and Gossage, 2001, May et al., 2009). The prevalence may be as high as 13.6–20.9% in high-risk populations in other countries (May et al., 2013).

A substantial barrier to the early diagnosis of FASD is a requirement for a confirmation of maternal drinking. Prenatal records often lack information on alcohol use, and maternal self-report can be unreliable due to the social stigma associated with drinking during pregnancy. In addition, a biological mother might not be available to provide information about alcohol use if a child is adopted or is in foster care. Thus, there has been a long-standing need to develop reliable laboratory tests to confirm PAE by measuring direct (ethanol metabolites/conjugation products) or indirect (ethanol-induced protein alterations) alcohol biomarkers in either maternal or infant biological samples (reviewed by (Bakhireva and Savage, 2011). Unfortunately, the sensitivity of indirect biomarkers, such as gamma glutamyltranspeptidase (GGT), mean corpuscular volume (MCV), and carbohydrate-deficient transferrin (% disialotransferrin from total transferrin or %dCDT), often does not exceed 50% in pregnant women (reviewed by (Bakhireva and Savage, 2011). Direct ethanol metabolites, such as fatty acid ethyl esters (FAEEs), phosphatidylethanol (PEth), ethyl glucuronide (EtG) and ethyl sulfate (EtS), have generated increased interest due to their higher sensitivity and specificity.

PEth has emerged as a highly promising novel biomarker with a relatively broad detection window of 3–4 weeks (Hannuksela et al., 2007, Isaksson et al., 2011). The sensitivity of PEth in liquid blood specimens varies from 53% to 100% (reviewed by (Viel et al., 2012) likely due to variations in alcohol consumption patterns between the studies and the methods for estimation of alcohol use. To date, only one small-scale study has been conducted in pregnant women (Kwak et al., 2012). A study conducted among women of reproductive age demonstrated that PEth was detectable in 93% of subjects averaging >2 drinks/day, but only in 53% of subjects drinking 1 drink/day (Steward et al., 2010). The specificity of PEth is thought to be superior to other biomarkers (Hartmann et al., 2007, Comasco et al., 2009) and is not influenced by the most common maternal chronic conditions or co-exposures (Stewart et al., 2009).

The U.S. Drug Testing Lab (USDTL) recently developed and validated a high pressure liquid chromatography-tandem mass spectrometry (LC/MS/MS) method for detection of PEth in dried blood spots (PEth-DBS) collected on filter paper (Jones et al., 2011). DBS cards are routinely collected from more than 95% of the newborns in the United States to screen for metabolic genetic disorders. The analysis of capillary blood in DBS specimens offers numerous advantages compared to liquid specimens obtained by phlebotomy, including minimal invasiveness and ease of collection, storage, and transportation. In addition, while liquid blood specimens are susceptible to false positive results due to post-collection synthesis of PEth if ethanol is present in blood (Aradottir et al., 2004, Helander and Zheng, 2009, Jones et al., 2011, Varga and Alling, 2002), post-collection synthesis does not occur on DBS cards (Jones et al., 2011).

We recently demonstrated that the collection of an additional DBS card for PEth analysis during routine newborn screening is feasible for the majority of infants and provides a lower cost alternative to PEth analysis in liquid blood specimens or meconium FAEE (Bakhireva et al., 2013). To date, there have been no published studies validating PEth as a marker of PAE in DBS specimens from a newborn. The objective of this current study was to examine validity of newborn PEth-DBS relative to more established direct and indirect ethanol biomarkers and maternal self-report in a prospective cohort of 60 maternal-infant pairs.

MATERIALS AND METHODS

Study design and recruitment of patients

Subjects were participants of the Biomarkers in Pregnancy Study (P.I.: Bakhireva) – a prospective cohort study at the University of New Mexico (UNM) described elsewhere (Bakhireva et al., 2012b). In brief, patients were recruited from the UNM specialty clinic during one of the first prenatal care visits. Inclusion criteria for BIPS were the ability to give consent in English or Spanish, a singleton pregnancy, and gestational age < 32 weeks. Patients who agreed to participate went through an informed consent process and participated in a baseline interview on site. Subjects had to meet criteria for the PAE or control groups based on self-reported measures of alcohol use, as described below. During the same visit, patients participated in the collection of biospecimens for analysis of ethanol biomarkers.

Upon admission to the UNM hospital for labor and delivery, patients' electronic research orders for collection of biospecimens from the mother (blood, urine) and child (meconium, DBS card) were activated. A follow-up interview with the mother captured any changes in alcohol and drug use patterns since the baseline interview. The study was approved by the UNM Human Research Review Committee (HRRC: 09-004); all study participants provided written consent.

Self-reported alcohol use and group allocation

The baseline interview ascertained maternal alcohol consumption during the previous 2 weeks and the month around their last menstrual period (LMP) by administering the Timeline Follow-back Procedure (TLFB). In TLFB, patients are asked to report quantities of

specific alcoholic beverages consumed each day during a specific timeframe, which are then converted into absolute ounces of alcohol per day (AAD) and per drinking day (AADD) (Jacobson et al., 2002). A 14-day recall period, instead of a more traditional 30-day TLFB was chosen to minimize recall bias and improve the accuracy of the report, as previously demonstrated (Hoeppe et al., 2010). Average alcohol consumption measures were estimated for the periconceptional period (AAD0 and AADD0) and the 2-week period before enrollment (AAD1 and AADD1), similar to other studies (Burden et al., 2005, Chiodo et al., 2009).

To be classified into the PAE group, a patient had to report average ounces of absolute alcohol per day at enrollment (AAD1) ≥ 0.21 (equivalent to ≥ 3 drinks/week) or ounces of absolute alcohol per drinking day (AADD1) ≥ 2.0 (equivalent to at least one episode of binge drinking). To be classified into the control group, a patient had to meet all of the following criteria: a) no binge drinking episodes in the periconceptional period; b) ounces of absolute alcohol per day in periconceptional period (AAD0) ≤ 0.14 (equivalent to ≤ 2 drinks/week); c) ounces of absolute alcohol per day (AAD1) and per drinking day (AADD1) at enrollment equal to zero. During the baseline interview, patients were also administered a standard AUDIT questionnaire. At the follow-up interview during the hospital stay after labor and delivery, another TLFB interview was administered to estimate alcohol use per day and per drinking day (AAD2 and AADD2) during late pregnancy.

Laboratory measures

At the baseline visit, maternal specimens were analyzed for a battery of biomarkers, including: serum GGT₁ and %dCDT₁, urine EtG and EtS (UEtG₁, UEtS₁), and whole blood PEth₁. At the follow-up visit, maternal serum and urine were collected and analyzed again for GGT₂, %dCDT₂, UEtG₂, and UEtS₂. Meconium samples were collected at the newborn nursery and placed in a -80°C freezer immediately after collection. An additional DBS card with at least one blood spot was collected from newborns during a routine heel puncture for genetic screening, as previously described (Bakhireva et al., 2013). Maternal serum specimens were sent to the TriCore Reference Laboratory (Albuquerque, NM) and the Medical University of South Carolina (Charleston, SC) for GGT and %dCDT analyses, respectively. GGT was analyzed as a part of a liver panel by the enzymatic rate method for GGT activity on a Beckman Synchron LX analyzer. CDT was analyzed in serum by an internationally-validated HPLC and spectrophotometric detection method (Anton and Youngblood, 2006, Bakhireva et al., 2012b). Maternal urine and whole blood specimens were sent to a contract laboratory (USDTL, Des Plaines, IL, USA) for analysis of UEtG, UEtS, and PEth by LC/MS/MS (Jones et al., 2011).

The analysis of FAEEs from meconium was conducted at Emory University following an established protocol (Bearer et al., 2003, Bearer et al., 2005). Briefly, meconium samples were homogenized and FAEEs extracted into methanol. FAEEs from the sample extracts were separated using a FactorFour Capillary column ($30\text{ m} \times 0.25\text{ mm} \times 0.25\text{ }\mu\text{m}$; Varian, Palo Alto, CA) with headspace-solid phase microextraction followed by gas chromatography–mass spectrometry (Agilent 5890) GC/MS) as outlined by Gareri et al (Gareri et al., 2008). The method was validated at the Children's Pediatric Research Center

Biomarkers Core at Emory University, and data on linearity, imprecision, recovery, analytical sensitivity, as well as detection and quantification limits were similar. Briefly, individual FAEEs were quantified using their corresponding d5-ethyl esters as internal standards and compared to the peak area ratios of each FAEE to the appropriate internal standard on a calibration curve. The concentrations of nine individual FAEEs (lauric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, and arachidonic) and their cumulative value were reported in relation to the dry weight of the meconium sample. The limit of detection (LOD) for each analyte was 50 ng/g and the additive limit of quantitation (LOQ) was 200 ng/g.

The analysis of newborn PETH-DBS was conducted by LC/MS/MS, as previously described (Jones et al., 2011, Bakhireva et al., 2013). In brief, three punches from each DBS were obtained, extracted with methanol, reconstituted into a mobile phase, analyzed by LC/MS/MS, and compared to a standard curve of known amounts of PETH in blood spots. Separation was achieved using an Agilent Zorbax Eclipse Plus C-8 column with an Agilent Technologies 6460 tandem mass spectrometer detector using electro-spray ionization (ESI) in the negative mode. The LOD was 2.0 ng/mL, the LOQ was 8.0 ng/mL, all intra- and inter-assay accuracy determinations within 10.8% of target concentration, and the intra- and inter-assay precision calculations were less than 8.7% (Jones et al., 2011). All contract laboratories were blinded to the patients' exposure status.

Other measures

Demographic information (age, marital status, education, race/ethnicity, employment) and medical/reproductive health characteristics (gravidity, parity, pregnancy dates, complications during pregnancy, chronic conditions, use of medications) were collected. Patients were asked to report their use of recreational drugs and smoking habits (lifetime, around LMP, and during pregnancy). Self-reported drug use was confirmed by urine drug screens conducted, on average, once a month for all patients. In addition, information on pregnancy and newborn outcomes, including any diagnosis of major structural anomalies identified in the newborn period, was abstracted from electronic medical records.

Statistical analysis

All analyses were conducted using SAS 9.3 (SAS Institute Inc., Cary, NC). Summary statistics were estimated to describe the study population. The alcohol-consumption pattern before pregnancy, during the periconceptional period and during pregnancy, as well as illicit drug use and smoking were compared among PAE and control patients. A Chi-square and t-test were used to compare proportions and means, respectively. The Fisher exact test was used instead of a Chi-square whenever more than 20% of cells had expected counts less than 5. For the biomarkers analyses, previously reported cutoff concentrations were used to determine 'positivity' for each biomarker, specifically %dCDT > 2.0%; PETH > 8 ng/mL, GGT > 40 U/L, UETG > 25 ng/mL, UETS > 7 ng/mL. Given that FAEE is not a single-molecule biomarker and there is no consensus in the literature on which specific FAEEs or what cutoff concentrations should be used for identification of PAE (Joya et al., 2012), sensitivity and specificity were evaluated at different cutoff values and FAEE composition. In addition to previously proposed cutoff concentrations of 10,000 ng/g for five FAEEs

(Moore et al., 2003) and 600 ng/g (2 nmol/g) for four FAEEs (Chan et al., 2003, Chan et al., 2004), a higher cutoff of 100,000 ng/g for all nine FAEE molecular species was used in order to maximize specificity and make these results more comparable to the other biomarkers. Sensitivity and specificity of ethyl oleate above the LOD (>50 ng/g) was also ascertained given that it has been highly correlated with self-reported maternal drinking in some prior studies (Bearer et al., 2003).

The composite index, which was based on self-reported measures of alcohol use and allowed us to classify subjects into PAE and control groups, was the criterion measure to estimate sensitivity and specificity. A test battery was considered 'positive' if at least one biomarker in that battery was above the cutoff. A test battery was considered 'negative' if all biomarkers were negative. In addition, correlation analysis between the two newborn biomarkers was conducted; Spearman correlation coefficient was estimated. Meconium FAEE and newborn PEth-DBS concentrations were transformed using $\log_{10}$ plus 1 (a constant value of 1 was added to allow for log-transformation of values below LOD) to normalize the data for correlation analysis.

RESULTS

A description of the study population is provided in Table 1. Most women were recruited during the second trimester of pregnancy (mean gestational age at recruitment 20.5 ± 7.1 weeks) and the mean age at recruitment was 26.6 ± 5.2 years. The sample included a large percentage of ethnic minorities (7.4% American Indian, 81.7% Hispanic/Latina), single (55.0%) or separated/divorced (8.3%) women, and patients with low education level (54.2% < high school). The vast majority of the patients had public health insurance (96.7%) and reported an unplanned pregnancy (90.0%). There was a slightly higher proportion of Hispanic/Latina patients in the control group (90.6%) compared to alcohol-exposed group (71.4%); this difference was of a borderline statistical significance ($p=0.055$). No other differences in socio-demographic characteristics were observed (all $p>0.05$).

As shown in Table 2, the mean AUDIT score was substantially higher among PAE patients (13.7 ± 9.2) compared to controls (2.7 ± 4.3 ; $p<0.001$). Binge drinking in the periconceptional period was prevalent among PAE group: 39.3% reported binge drinking 2 times per week and 21.4% reported 5 binge episodes per week. None of the controls reported binge drinking around the LMP or any alcohol use during pregnancy, consistent with the eligibility criteria. Among PAE subjects, the average alcohol consumption per day in the periconceptional period was high (1.86 ± 3.03 oz of AA), but it dramatically decreased during pregnancy. While 100% of alcohol-exposed women reported at least one binge episode sometime between the LMP and enrollment, the average daily consumption was moderate (0.15 ± 0.44 oz of AA). At the follow-up interview, only one patient in the PAE group admitted alcohol use during the 2 weeks before delivery.

Given that the study population was recruited from a specialty clinic providing care to pregnant women with a history of substance abuse, 83.3% of all patients were exposed to opioids (heroin or recreational use of prescription opioids) and/or received opioid-maintenance therapy (OMT) (Table 2). Use of other drugs of abuse and tobacco products

was also prevalent in both groups. Despite the high prevalence, no differences in the prevalence of tobacco use or other classes of drugs of abuse were noted between the study groups (all p -values >0.05).

The prevalence for testing positive for maternal biomarkers at each time point and biomarkers of the newborn is presented in Table 3. Statistically significant differences between the PAE and control groups were observed for maternal GGT, UEtG, and PEth at the baseline visit (all $p<0.05$). With respect to biomarkers in the newborn, 32.1% of patients in the PAE group and none of controls tested positive for newborn PEth-DBS ($p<0.001$).

The sensitivity and specificity of all biomarkers are presented in Table 4. Newborn PEth-DBS demonstrated 100% specificity. In this group of patients with moderate chronic or intermittent binge drinking pattern, the sensitivity of PEth-DBS only reached 32.1%, but was higher compared to other biomarkers. PEth in maternal samples at baseline (mid-gestation) was 22.2% with 100% specificity. The sensitivity of other maternal biomarkers was below 15%. The sensitivity of meconium FAEEs was similar to newborn PEth-DBS (28.6% vs. 32.1%, respectively) but had a lower specificity (81.3% vs. 100%) at 100,000 ng/g cutoff. When lower 10,000 ng/g and 600 ng/g cutoff concentrations were applied, the sensitivity of meconium FAEE increased to 85.7% and 100%, respectively. However, specificity dropped substantially to 12.5% reflecting a high false positive rate among controls. In addition, no significant correlation between meconium FAEE and PEth-DBS was observed (Spearman coefficient = 0.07; $p=0.617$).

The sensitivity of newborn PEth-DBS improved to 50% when it was combined with other maternal biomarkers (GGT₂, %dCDT₂, UEtG₂, UEtS₂) at delivery or with meconium FAEE with a small drop in specificity from 100% to 81.3% (Table 4). A battery consisting of newborn PEth-DBS and maternal direct ethanol metabolites (UEtG, UEtS, and PEth) measured at either baseline (mid-gestation) or at delivery resulted in the highest sensitivity and specificity (50% and 93.8%, respectively). A newborn with the highest PEth-DBS concentration (406 ng/mL) was also positive for four maternal biomarkers (UEtS₁, UEtG₁, PEth₁, and GGT₂). One patient in the study who was diagnosed with Fetal Alcohol Syndrome (FAS) in the newborn period was positive for PEth-DBS (concentration 51.8 ng/mL) and three maternal direct biomarkers (UEtS₂, UEtG₂, PEth₁). Interestingly, indirect maternal biomarkers (GGT, %CDT) for this subject were within the normal range at both baseline and at delivery (data not shown).

DISCUSSION

The findings suggest that a PEth-DBS collected from a newborn is a highly specific biomarker that can facilitate accurate detection of PAE in conjunction with other biomarkers. However, while the specificity of PEth-DBS was 100% in the study, sensitivity was only 32.1%. Given that the sensitivity of other biomarkers was even lower, we believe that this is a reflection of the moderate alcohol consumption pattern in the study. It should be also noted that one limitation of newborn PEth-DBS compared to meconium biomarkers is inability to detect PAE beyond the 2–3 weeks prior to delivery. A battery consisting of PEth-DBS and meconium FAEE or PEth-DBS and maternal biomarkers can improve

sensitivity at a small loss of specificity, as demonstrated in our study. A battery consisting of maternal direct ethanol biomarkers measured during pregnancy and at delivery and a newborn PEth-DBS resulted in the highest sensitivity and specificity compared to other combinations of biomarkers.

The American College of Obstetricians and Gynecologists (ACOG) defines ‘at risk’ drinking as more than 7 drinks per week (equivalent to $> 0.5\text{oz AA}$) or more than one binge episode in 90 days; however, any amount of drinking during pregnancy is considered harmful (American College of Obstetricians and Gynecologists, 2011). While the majority of alcohol-exposed subjects in our study reported heavy binge-like drinking pattern around conception (18% reported 5 binge drinking episodes per week), alcohol use during the 2-week intervals before enrollment and delivery was in the moderate-to-light range. This could be due to an actual decrease in alcohol consumption after pregnancy recognition or under-reporting. Under-reporting is very common among pregnant women, particularly among problem drinkers (Ernhart et al., 1988). It should be noted that the reporting timeframe of alcohol use during the first part of pregnancy, which was used to classify patients into the study groups, and the newborn PEth-DBS detection window do not overlap. However, some prior studies suggest that self-reported alcohol consumption around conception or before pregnancy recognition might be less affected by self-report bias, and thus might be more accurate estimates of alcohol use during pregnancy (Chang, 2001, Day et al., 1993). If self-report in early postpartum period was used to ascertain PAE in our study, 28.6% of infants with PAE (assuming 100% specificity of PEth-DBS) would have been missed. Similar to our results, other studies also suggested that direct ethanol metabolites can identify additional patients who would have been missed by self-report (Wurst et al., 2008).

The episodic binge drinking pattern could also lower the sensitivity of biomarkers if the binge episode occurred outside the detection window for a particular biomarker. Among biomarkers examined, meconium FAEE and GGT have the widest detection windows (about 4 months and 1 month, respectively), while UEtG and UEtS have the shortest (about 5 days and 30 hours, respectively). PEth is known to be detected in peripheral blood for up to a month after the last drinking episode (Viel et al., 2012); however, the sensitivity is known to decrease after 2 weeks since the last drinking episode (Wurst et al., 2010). The elimination window of PEth in newborn children with PAE has not been studied yet. We have previously discussed (Bakhireva et al., 2013) that at least theoretically, the elimination window can be either shorter (due to shorter lifespan of erythrocytes in the fetus and a newborn child) or longer (assuming that fetus/newborn has a lower capacity to metabolize PEth than moderate alcohol consuming adults). Future prospective studies with multiple repeated maternal blood alcohol concentration (BAC) tests in the third trimester need to address this question.

While only one woman classified into the PAE group (based on alcohol use in early pregnancy) admitted alcohol use during the 2 weeks before delivery, 9 newborns (32.1%) tested positive for PEth-DBS. Thus, this can suggest that newborn PEth-DBS is more reliable than self-report for detecting PAE. Conversely, the question of potential false positive results needs to be addressed. In liquid blood specimens, neo-formation of PEth *in vitro* at the room temperature or at -20°C is known to occur in subjects with a BAC higher

than 100 mg/dL (Aradottir et al., 2004, Jones et al., 2011, Varga and Alling, 2002). However, vapor exposure of DBS cards to ethanol for 3 days at room temperature did not result in post-collection synthesis of PEth (Jones et al., 2011). In our study, the lack of positive newborn PEth-DBS tests in the control group further highlights the high specificity of this test.

Meconium FAEE provided a comparable sensitivity to PEth-DBS; however, the specificity of FAEE was much lower (81.3% vs.100%) even at the highest cutoff of 100,000 ng/g. The lower specificity of meconium FAEE in our study is a reflection of a high false positive rate, which was also demonstrated by other groups (Zelner et al., 2012). Meconium specimens collected later in postpartum period are especially prone to false-positive results due to contamination with transitional stool and ethanol-producing microorganisms (Zelner et al., 2012). Given that newborn PEth-DBS has much shorter detection window than meconium FAEE, but yielded comparable sensitivity, further highlights that PEth-DBS can potentially detect lower levels of PAE than meconium FAEE. Other disadvantages of meconium FAEE analysis include difficulty of collection and storage, potential contamination with transitional stool, light- and temperature degradation, and difficulties with extraction of FAEEs from a complex media. DBS cards, on the other hand, can be stored at a room temperature and shipped without dry ice. Our recent study demonstrated that 87.4% of infants admitted to the newborn nursery had enough blood after a routine heel prick to fill at least one full blood spot for PAE screening and PEth-DBS analysis was more cost-efficient than meconium FAEE or analysis of PEth in liquid blood specimens (Bakhireva et al., 2013).

In this study, a composite self-reported measure was used to categorize subjects into the study groups and to estimate validity of biomarkers. It should be acknowledged that there is no 'gold standard' to ascertain PAE. Prior studies examining the validity of biomarkers used different 'gold standards' including diagnosis of alcohol dependence (Aradottir et al., 2006, Wurst et al., 2010, Hartmann et al., 2007, Neumann et al., 2009), data obtained by in-depth maternal interviews (Chen et al., 2003, Comasco et al., 2009), neurobehavioral delays later in life or other adverse infant outcomes (Stoler et al., 1998, Peterson et al., 2008), and screening questionnaires (Neumann et al., 2008, Wurst et al., 2008). We used a composite index based on self-reported measures of alcohol use in the periconceptional period and the first part of pregnancy to classify subjects into study groups. A similar index was used by other groups and was more predictive of future alcohol-related neurobehavioral deficits in affected children than individual measures of self-report (Chiodo et al., 2009). Compared with Chiodo's "at risk" alcohol exposure (ARAE) metric, our composite measure did not include screening questionnaires (i.e., MAST, CAGE, T-ACE). While we collected and reported AUDIT scores in both study groups, we felt that since the AUDIT captures a 12-month period, it might not reflect drinking during pregnancy as accurately. We also used slightly lower AA per day during pregnancy measures to include moderate drinkers in the sample.

It should also be noted that there is no established cutoff concentration for PEth for the assessment of PAE in either DBS or liquid blood specimens (Viel et al., 2012), thus any values above the limit of detection (LOD) were considered 'positive' in this report. The

USDTL has proposed > 20 ng/mL as a possible cutoff and another study used 0.10 μmol/L (roughly equivalent to 28 ng/mL) as a cutoff for identification of 'any regular drinking' in liquid blood specimens (Helander et al., 2012). In our study, however, none of the controls had positive PEth results even with the lower cutoff concentration. Similarly, LOD cutoffs were used for UEtG and UEtS to ensure that lower levels of drinking are captured. While a traditional cutoff for %dCDT is 1.7%, recent reports (Kenan et al., 2011, Bianchi et al., 2011, Bakhireva et al., 2012a) indicate that a cutoff of 2.0 is more appropriate for pregnant women. Given that there is no uniformly accepted cutoff concentration for meconium FAEE, several cutoffs were used in this study. In our study population, co-exposure with other substances was very prevalent given that the patients were recruited from a specialty clinic providing prenatal care to women with a history of substance abuse. However, drug use was similar in both study groups. In addition, given that PEth is a direct ethanol metabolite, a potential confounding effect by other substances is unlikely.

Several issues remain to be investigated before PEth-DBS can be recommended as a reliable method for PAE assessment in the newborn period. Several recent studies (Mizejewski, 2010, Zizzo et al., 2013), including ours (Bakhireva et al., 2013), have brought up ethical issues of newborn testing for PAE, acceptability by patients, and conditions for ethical implementation of such testing, including maternal consent. Second, the stability of PEth-DBS is largely unknown. Whether or not banked DBS cards can be analyzed years after collection to confirm PAE in a child suspected to have FASD is unclear. Current studies in our laboratory have begun to address this question. Third, currently there is no agreement regarding the PEth cutoff concentration that should be used for assessing PAE in newborns. Fourth, the validity of newborn PEth-DBS needs to be compared to other direct ethanol metabolites examined in more established (e.g. EtG/EtS in meconium) or novel biological media (e.g. finger nails, newborn hair, umbilical cord tissue). Lastly, in addition to the utility of PEth-DBS in the identification of PAE, it would be important to determine its predictive ability to identify future neurodevelopmental deficits in affected children.

In summary, this is the first report examining the validity of PEth-DBS for assessing PAE in newborn children. Two prior reports which analyzed PEth-DBS were conducted among adults (Faller et al., 2011, Jones et al., 2011). In our study, newborn PEth-DBS demonstrated 100% specificity and at least comparable sensitivity to other biomarkers measured in the same cohort. Moreover, the sensitivity of PEth-DBS improved when it was used in combination with other biomarkers. While no single measure may be sufficient to accurately characterize PAE, a combination of clinical and laboratory measures and/or a battery of biomarkers and maternal interview could substantially improve clinical utility. Clearly, larger multi-site studies are warranted to further examine the utility of newborn PEth-DBS in identification of PAE and children at risk for FASD.

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Table 1

Demographic Characteristics of the Study Sample (N=60)

Characteristics	Controls (N=32)	PAE (N=28)	p-value
	Mean±s.d.	Mean±s.d	
Maternal age	26.3±4.5	27.0±5.9	0.647
Gestational age at enrollment	20.5±6.3	20.5±8.1	0.990
	%	%	
Ethnicity: Hispanic	90.6	71.4	0.055
Race:			
White	90.6	78.6	0.281 *
American Indian	6.3	7.1	1.000 *
Marital status:			0.496
Single/ separated/divorced	59.4	67.9	
Married/co-habiting	40.6	32.1	
Health insurance:			0.214 *
No insurance	0.0	7.1	
Any other insurance	100.0	92.9	
Education			0.328
Less than high school	53.1	53.6	
High school or equivalent	31.3	17.9	
College, vocational school or higher	15.6	28.6	
Primigravida	15.6	10.7	0.712 *
Unplanned pregnancy	84.4	96.4	0.201 *

PAE, prenatal alcohol exposure

* Fisher's exact test

Table 2

Use of Alcohol & Other Substances by Study Group (N=60)

Substances of abuse	Controls N=32	PAE N=28	p- value
<u>Alcohol Use</u>			
<i>Past 12 months</i>			
AUDIT (Mean±s.d.)	2.7±4.3	13.6±9.2	<.001
AUDIT 8 (%)	9.4	60.7	<.001
<i>Alcohol use in periconceptional period</i>			
Frequency of binge (4 drinks)			<.001
No binge episodes (%)	100.0	7.1	
Once/week or less (%)	0.00	53.6	
2–4 times a week (%)	0.00	21.4	
5 times/week (%)	0.00	17.9	
Average AA per day (AAD0; Mean±s.d.)	0.01±0.02	1.86±3.03	<.001
Average AA per drinking day (AADD0; Mean±s.d.) ^a	0.89±0.51	4.14±3.10	<.001
<i>Alcohol use at enrollment</i>			
At least one episode of binge (%)	0.0	100.0	<.001
Average AA per day (AAD1; Mean±s.d.)	0.0	0.15±0.44	<.001
Average AA per drinking day (AADD1; Mean±s.d.) ^a	0.0	1.6±0.8	<.001
<i>Alcohol use 2 weeks before delivery</i>			
Average AA per day (AAD2; Mean±s.d.))	0.0	0.001±0.007	<.001
Average AA per drinking day (AADD2; Mean±s.d.) ^a	0.0	0.50	--
<u>Illicit Drugs and Tobacco</u>			
Marijuana (%)	53.1	64.3	0.382
Cocaine/crack-cocaine (%)	34.4	39.3	0.694
Opioids/opioid-maintenance therapy (%)	90.6	75.0	0.166 ^b
Benzodiazepines (%)	28.1	35.7	0.528
Amphetamines (%)	31.3	25.0	0.592
Tobacco (%)	84.4	67.9	0.131

^a Only among patients who reported any drinking during that time period^b Fisher's exact test

AA = ounces of absolute alcohol.

Table 3

Prevalence of Ethanol Biomarkers (% positive) by Study Group (N=60).

Biomarkers	Controls (%)	PAE (%)	p-value
<u>Maternal Biomarkers:</u>			
GGT ₁ > 40 U/L	0	14.8	0.026
GGT ₂ > 40 U/L	13.8	10.7	0.723
%dCDT ₁ > 2.0%	0	3.7	0.272
%dCDT ₂ > 2.0%	3.3	10.7	0.268
UEtG ₁ 25 ng/mL	3.1	14.8	0.108
UEtG ₂ 25 ng/mL	3.1	7.4	0.476
UEtS ₁ 7 ng/mL	0	14.8	0.024
UEtS ₂ 7 ng/mL	3.1	7.4	0.476
PEth ₁ > 8 ng/mL	0	22.2	0.006
<u>Biomarkers in the Newborn:</u>			
PEth_DBs > 8 ng/mL	0	32.1	<0.001
Meconium FAEE:			
Sum of 9 FAEEs ^a >100,000 ng/g	18.7	28.6	0.370
Sum of 6 FAEEs ^b > 10,000 ng/g	87.5	85.7	0.839
Sum of 4 FAEEs ^c > 600 ng/g	87.5	100	0.053
Ethyl oleate > 50 ng/g	75.0	85.7	0.301

Subscripted numbers “1” and “2” refer to maternal biomarkers measured at the baseline (mid-gestation) and follow-up (at delivery), respectively.

^a Lauric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, arachidonic

^b Palmitic, palmitoleic, stearic, oleic, linoleic, arachidonic

^c Palmitic, stearic, oleic, linoleic

Table 4

The Validity of Traditional and Novel Biomarkers and Their Combination (N=60)

Biomarkers	Sensitivity (%)	Specificity (%)
<u>Maternal Biomarkers:</u>		
GGT ₁ > 40 U/L	14.8	100.0
GGT ₂ > 40 U/L	10.7	86.2
%dCDT ₁ > 2.0%	3.7	100
%dCDT ₂ > 2.0%	10.7	96.7
UEtG ₁ 25 ng/mL	14.8	96.9
UEtG ₂ 25 ng/mL	7.4	96.9
UEtS ₁ 7 ng/mL	14.8	100.0
UEtS ₂ 7 ng/mL	7.4	96.9
PEth ₁ > 8 ng/mL	22.2	100.0
<u>Biomarkers of a Newborn:</u>		
PEth-DBS > 8 ng/mL	32.1	100.0
Meconium FAEE:		
Sum of 9 FAEEs ^a >100,000 ng/g	28.6	81.3
Sum of 6 FAEEs ^b > 10,000 ng/g	85.7	12.5
Sum of 4 FAEEs ^c > 600 ng/g	100	12.5
Ethyl oleate > 50 ng/g	85.7	25.0
<u>Combination of Biomarkers^d:</u>		
Maternal biomarkers at baseline	32.1	96.9
Maternal biomarkers at delivery + PEth-DBS	50.0	81.3
Meconium FAEE >100,000 ng/g + PEth-DBS	50.0	81.3
Maternal direct biomarkers ^e + PEth-DBS	50.0	93.8

Subscripted numbers “1” and “2” refer to maternal biomarkers measured at the baseline (mid-gestation) and follow-up (at delivery), respectively.

^a Lauric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, arachidonic

^b Palmitic, palmitoleic, stearic, oleic, linoleic, arachidonic

^c Palmitic, stearic, oleic, linoleic

^d A battery was considered ‘positive’ if at least one of biomarkers in a battery was positive

^e UEtG, UEtS, PEth assessed at either baseline or at delivery