Published in final edited form as:

Alcohol Clin Exp Res. 2006 July ; 30(7): 1152–1159. doi:10.1111/j.1530-0277.2006.00131.x.

Fatty Acid Ethyl Esters in Meconium: Are They Biomarkers of Fetal Alcohol Exposure and Effect?

Enrique M. Ostrea Jr., Joel D. Hernandez, Dawn M. Bielawski, Jack M. Kan, Gregorio M. Leonardo, Michelle Buda Abela, Michael W. Church, John H. Hannigan, James J. Janisse, Joel W. Ager, and Robert J. Sokol

Department of Pediatrics, Hutzel Women's Hospital and the Mott Center for Human Growth and Development (EMO, JDH, DMB, JMK, GML); and the Department of Obstetrics, (MBA, MWC, JHH, RJS); and the Center for Healthcare Effectiveness Research, Wayne State University, Detroit, Michigan (JJJ, JWA).

Abstract

Background—Biomarkers of fetal exposure to alcohol are important to establish so that early detection and intervention can be made on these infants to prevent undesirable outcomes. The aim of this study was to analyze long-chain fatty acid ethyl esters (FAEEs) in meconium as potential biomarkers of fetal alcohol exposure and effect.

Methods—Fatty acid ethyl esters were analyzed in the meconium of 124 singleton infants by positive chemical ionization gas chromatography/mass spectrometry (GC/MS) and correlated to maternal ethanol use.

Results—A total of 124 mother/infant dyads were enrolled in the study: 31 were in the control group and 93 were in the alcohol-exposed group. The incidence (28% vs 9.7%, $p=0.037$) of ethyl linoleate detected in meconium was significantly higher in the alcohol-exposed groups than the control groups. Similarly, when the concentrations of ethyl linoleate in meconium were grouped (trichotomized), there was a significant linear by linear association between alcohol exposure and group concentrations of ethyl linoleate ($p=0.013$). Furthermore, only alcohol-exposed infants were found in the group with the highest ethyl linoleate concentration. The sensitivity of ethyl linoleate in detecting prenatal alcohol exposure was only 26.9%, and its specificity and positive predictive value were 96.8 and 96.2%, respectively. There was no significant correlation between the concentration of ethyl linoleate in meconium and absolute alcohol consumed (oz) per drinking day across pregnancy, although a trend toward a positive correlation is seen at lower amounts of alcohol consumed. Among the polyunsaturated, long-chain FAEEs, there was weak evidence that the incidence (21.5% vs 6.5%, $p=0.057$) and concentration ($p=0.064$) of ethyl arachidonate (AA) were significantly higher in the alcohol-exposed groups than the control groups. Ethyl linolenate and ethyl docosahexanoate (DHA) in meconium were found only in the alcohol group, although not at statistically significant levels. Highly significant correlations were found among the concentrations of ethyl linoleate, ethyl linolenate, ethyl AA, and ethyl DHA in meconium (correlations ranged between $r_s=0.203$, $p=0.024$; and $r_s=0.594$, $p<0.001$).

Conclusion—We conclude that FAEEs in meconium, particularly ethyl linoleate and ethyl AA, are biomarkers of high specificity for prenatal exposure to alcohol in newborn infants. We also propose that ethyl AA and DHA could be potential biomarkers of fetal alcohol effects on the developing fetal brain and should be investigated further.

Keywords

Gas Chromatography/Mass Spectroscopy; Fetal Alcohol Syndrome; Maternal Alcoholism; Fatty Acid Ethyl Esters; Ethyl Laurate; Ethyl Palmitate; Ethyl Oleate; Ethyl Linoleate; Ethyl Myristate; Ethyl Linolenate; Ethyl Arachidonate; Ethyl Docosahexanoate

Early detection of infants who have been exposed to alcohol during pregnancy is important so that early intervention can be instituted to prevent the emergence of further undesirable outcomes (Bearer, 2001; Van Der Leeden et al., 2001). Although a number of alcohol biomarkers have been identified in pregnant woman, only a few have been reported in infants. These include urinary dolichol (Wisniewski et al., 1983), serum γ -glutamyl transferase activity (Leonardo et al., 2002; Mirlesse et al., 1996), and certain isoforms of alcohol dehydrogenase and α 1-antitrypsin (Robinson et al., 1995). Fatty acid ethyl esters (FAEEs) are formed principally from the enzymatic esterification of serum fatty acids with ethanol, and their increase in the serum of adult alcoholics is proportional to alcohol intake (Laposata, 1997). In animal studies, mouse heart, liver, placenta, and fetal tissues were shown to accumulate significant amounts of FAEE after maternal ethanol exposure (Bearer et al., 1992). The presence of FAEEs in meconium was initially reported by Mac et al. (1994) and subsequently by Klein et al. (1999) and Bearer et al. (1999). These studies have suggested that FAEEs in meconium may be a useful biomarker of prenatal alcohol exposure in the newborn infant.

Meconium is an ideal matrix to analyze for fetal exposure to many xenobiotic agents for several reasons. Meconium is a repository of many agents to which the fetus is exposed during pregnancy, as both the drugs themselves and their metabolites are deposited in meconium through bile secretion or fetal swallowing of amniotic fluid. Also, meconium is formed early in gestation (starting at the 12th week of pregnancy), continues to be formed until birth, and is not normally excreted by the fetus until after birth. These features indicate that meconium provides a wide window to detect fetal exposure to various agents. To date, meconium has been analyzed to detect fetal exposure to illicit drugs (Bibb et al., 1995; Ostrea et al., 1988, 1989, 1992, 2001; Ryan et al., 1994), nicotine metabolites (Ostrea et al., 1994), many prescribed and over-the-counter medications, food additives (Ostrea et al., 1998), and environmental toxicants (Ostrea et al., 2002; Whitehall et al., 2000; Whyatt and Barr, 2001).

We report on the analysis of FAEEs in meconium of infants born to a cohort of pregnant women for whom levels of alcohol consumption at periconception and during pregnancy are reported. This report includes an extensive analysis of long-chain saturated and polyunsaturated FAEEs, including ethyl esters of arachidonate (AA) and, for the first time, ethyl DHA.

METHODS

Fatty acid ethyl esters were analyzed in meconium by positive chemical ionization (PCI) gas chromatography/mass spectrometry (GC/MS) (Bielawski et al., 2003). Briefly, meconium (0.5 g) was weighed and placed in a Sarstedt tube, 3.5 mL of distilled water was added, and the mixture was vortexed until homogeneous. An internal standard (ethyl heptadecanoate) was added (to make a concentration of 8 μ g/g meconium) plus 3.5 mL of hexane. The mixture was placed in a rotary mixer on medium speed for 30 minutes, centrifuged at 2,800 r.p.m. (1,600 $\times$ g) for 30 minutes, and then frozen for several hours at -20°C to solidify the free lipids in the hexane layer. The hexane layer was aspirated and added to a solid-phase extraction column (CUNAX153 columns, United Chemical Technologies, Bristol, PA) that

had been preconditioned with 4 mL of methylene chloride and 4 mL of hexane. The column was eluted twice with 4 mL each of hexane and methylene chloride. All eluates were collected, evaporated under nitrogen, reconstituted with 100 μ L of acetone, and vortexed. Samples (1 μ L) were injected in splitless mode using an autosampler (Agilent 7683 Series, Agilent Technologies, Wilmington, DE). The GC/MS was an Agilent GC6890/MS5973N using PCI in selected ion monitoring (SIM) mode with helium carrier gas at 11.15 psi constant pressure and a flow rate of 1.0 mL/min. Inlet temperature was 250 °C and detector temperature was 280 °C. An HP-5MS column (0.25 mm $\times$ 30 m $\times$ 0.25 μ m) was utilized and the oven temperature was programmed: initial temperature of 100 °C, increased at a rate of 25 °C/min to 200 °C, then 5 °C/min to a final temperature of 300 °C for 5 minutes. Methane was used as the reagent gas.

Matrix-spiked calibrators, also used for determining the limits of detection (LODs), ranged from 0.05 to 8 μ g/g FAEs (ethyl esters of laurate, myristate, palmitate, linoleate, oleate, α -linolenate, stearate, AA, and DHA). For the recovery study, FAEs were spiked in meconium at 1, 4, and 8 μ g/g concentrations with ethyl heptadecanoate as an internal standard.

At 1 to 8 μ g/g concentrations of the different FAEs in spiked meconium, the mean ($\pm$ SD) recovery rate was $101.0 \pm 4.6\%$ and the mean ($\pm$ SD) interassay and intraassay coefficients of variation were 12.1 ± 5.3 and $4.6 \pm 1.1\%$, respectively. The LODs for the individual FAEs were determined using the empirical approach (Armbruster et al., 1994) with decreasing concentrations of matrix-spiked calibrators. Limit of detection was defined as the lowest concentration where (a) the quantitation and qualifier(s) peaks were present and (b) the mass ratios of quantitation versus qualifier(s) were within $\pm 20\%$ range of uncertainty. Target and qualifier ions for SIM were calculated using the molecular weights of the compounds. The empirical method of LOD determination consists of measuring progressively more dilute concentrations of analyte and LOD represents the lowest concentration at which the results still satisfy our predetermined criteria (Armbruster et al., 1994). The empirical LOD method was chosen as it provides a value that represents the actual limit of the feasibility of our assay, a value that meets all analytical acceptance criteria (Lawson, 1994). The LODs were 0.05 μ g/g for ethyl laurate, ethyl myristate, ethyl palmitate, ethyl linoleate, and ethyl oleate; 0.10 μ g/g for ethyl α -linolenate and ethyl stearate; 0.20 μ g/g for ethyl AA; and 1 μ g/g for ethyl DHA. For categorical values (positive/negative groupings) or actual concentrations, FAEs were considered as “negative or zero concentration: if the actual concentration was below the LOD.” The calibration curve of each FAE displayed a linear fit and coefficients of determination (r^2) ranged from 0.96 to 0.98.

Clinical Study

Pregnant women ($n=124$) were prospectively interviewed for their alcohol use at the time of conception (periconception) and within pregnancy. Measures of alcohol use included (1) average amount (oz) of absolute alcohol consumed per day or AAD, (2) average amount (oz) of absolute alcohol consumed per drinking day or AADD, and (3) proportion of 7 days in which alcohol was used or PD. Alcohol consumption at the time of conception was used based on the findings of Streissguth et al. (1980) that outcome measures of alcohol effect are more significantly associated with self-reported drinking before recognition of pregnancy, as it is free from the stigma of drinking around pregnancy. The control group consisted of those mothers who reported no alcohol intake around the time of conception or in pregnancy whereas the alcohol-exposed group consisted of those who used alcohol at the time of conception and/or any time during pregnancy. Standard questionnaires to assess alcohol use such as the Michigan Alcohol Screening Test (MAST), TACE, and CAGE screens were also utilized (Russell et al., 1996). The study was approved by the Human Investigation

Committee of Wayne State University and informed consent was obtained from all subjects. After delivery, meconium was collected from the diapers of the infants, pooled into 1 container per infant, and frozen at -20°C until being analyzed for FAEEs.

Statistical Analysis

Comparisons between the alcohol-exposed and control groups were performed. For comparison of categorical data, Pearson's chi-square or Fisher's exact test was used. For normally distributed continuous data, *t*-tests were performed. For continuous data that did not meet the normality assumption, Mann-Whitney tests were performed. Associations between variables were based on the Pearson's chi-square test for categorical data and Spearman's ρ correlation for continuous data. To calculate the sensitivity and specificity of the FAEE in detecting alcohol exposure, a receiving operator curve (ROC) analysis was performed. In all of the statistical analyses, the level of significance was taken as $p \leq 0.05$.

RESULTS

A total of 124 mother/infant dyads were included in the study: 31 were in the control group and 93 were in the alcohol-exposed group. There was no significant difference in maternal age, gravidity, parity, marital status, socioeconomic status (Hollingshead, 1975), or race between the 2 groups based on alcohol use during pregnancy (Table 1). There was also no significant difference in the rate of positive drug screen for amphetamine, barbiturates, cocaine, or opiates between the 2 groups. However, the rate of positive test for cannabinoids (0% vs 25%, $p=0.047$) and the number of cigarettes per day (median of 0 vs 6, $p=0.01$) were both significantly higher in the alcohol-exposed group than the control group. There were no significant differences in mean gestational age, birth weight, length, or head circumference between the 2 groups (Table 1).

There were significant differences in the MAST, CAGE, and TACE scores between the control and the alcohol-exposed groups (Table 2) and AAD, AADD, and PD were significantly correlated to each other, both at the time of conception and in pregnancy, as well as to MAST, CAGE, and TACE scores (Table 3).

The detection (any amount) and the concentrations ($\mu\text{g/g}$) of FAEEs in meconium in the control and alcohol-exposed groups are shown in Tables 4 and 5. The incidence (28% vs 9.7%, $p=0.037$ by chi-square analysis) and concentrations of ethyl linoleate were significantly higher in the alcohol-exposed groups than the control groups. Specifically, the 3 control subjects who had concentrations above 0 had values ranging from 0.15 to 0.82 $\mu\text{g/g}$ and the 26 alcohol-exposed subjects who had concentrations above 0 had ethyl linoleate values ranging from 0.25 to 211.72 ($p=0.016$ by the Mann-Whitney test). An ROC was used to calculate sensitivity and specificity for no exposure versus some exposure. The area under the curve was 0.60 for ethyl linoleate with a 96% confidence interval of 0.51 to 0.69. The cut point that maximized sensitivity and specificity was 0.25. At this level the sensitivity was 26.9%, the specificity was 96.8%, and the positive predictive value was 96.2%. Although the area under the curve is not very large, it is significantly different from 0.5. The low area under the curve is mainly due to the poor sensitivity of the test even though the specificity is very high.

The scatter plot of ethyl linoleate concentration in relation to average amount (oz) of absolute alcohol consumed on actual drinking days (AADD) across pregnancy is shown in Fig. 1. For this scatter plot an extreme outlier with an AADD value of 20.0 has been "winsorized" to a value of 10.33, which is just above the next highest value. At low amounts of AADD, there appears to be a positive relationship between concentration of ethyl linoleate and amount of alcohol exposure. At high amounts of AADD, this relationship

disappears. Overall there was no significant relationship between AADD and ethyl linoleate levels ($r_s=0.126$, $p<0.17$). When the concentrations of ethyl linoleate in meconium were grouped (trichotomized), there was a significant association (Table 6) between alcohol exposure and increasing concentrations of ethyl linoleate (linear-by-linear association, $p=0.019$). Furthermore, only alcohol-exposed infants were found in group C, the group with the highest ethyl linoleate concentration. Combinations of FAEs (ethyl palmitate, stearate, and oleate) did not show any significant association between the FAEs and alcohol exposure.

Of the polyunsaturated long-chain FAEs, the incidence (21.5% vs 6.5%, $p=0.057$ by chi-square analysis) and concentration (2 control subjects had concentrations above 0 with values of 0.90 and 3.60 and 20 alcohol-exposed subjects had concentrations above 0 with values ranging from 0.81 to 3.19, $p=0.064$ by the Mann–Whitney test) of ethyl AA showed weak evidence of being higher in the alcohol-exposed groups than the control groups (Tables 4 and 5). An ROC was used to calculate sensitivity and specificity from no exposure versus some exposure. The area under the curve was 0.57 with a 95% confidence interval of 0.48 to 0.66. The cut point that maximized sensitivity and specificity was 0.902. At this level, the sensitivity was 18.3% and the specificity was 96.8%. The positive predictive value was 94.4%. When the concentrations of ethyl AA in meconium were grouped (trichotomized), there was no significant association between alcohol exposure and group concentrations of ethyl AA (linear-by-linear association, $p=0.076$). Ethyl linolenate and ethyl DHA were only found in the meconium of the alcohol-exposed group and not in the control group (Table 4).

As shown in Table 7, the correlations between the concentrations of ethyl linoleate, ethyl linolenate, ethyl AA, and ethyl DHA were all significant. Based on Spearman's ρ , the correlations ranged between $r_s=0.203$ ($p=0.024$) and $r_s=0.594$ ($p<0.001$).

DISCUSSION

Fatty acid ethyl esters are formed by the nonoxidative esterification of ethanol with free fatty acids through the action of fatty acid synthase and acylcoenzyme, ethanol-*O*-acyltransferase (Diczfalusy et al., 2001). A number of enzymes have fatty acid synthase activity, including lipo-protein lipase, carboxylesterase, and carboxyl ester lipase (Best and Laposata, 2003; Laposata, 1999). In adults, FAEs have been validated as a biomarker of alcohol use and have been found in the blood, adipose tissue, liver, heart, brain, and hair of alcoholics (Best et al., 2003; Calabrese et al., 2001; Hartwig et al., 2003; Laposata et al., 2002; Refaai et al., 2002; Salem et al., 2001). The amount of FAE is also proportional to the amount of alcohol intake (Laposata, 1997). Different types or species of FAEs have been identified based on the tissue examined and the acuteness of alcohol intake, i.e., binge consumption versus chronic alcoholism (Laposata et al., 2000; Refaai et al., 2002). Fatty acid ethyl esters are toxic in the brain, liver, and heart of alcoholics through their disruption of mitochondrial and other cell functions (Bora and Lange, 1993; Beckemeier and Bora, 1998). In pregnancy, FAEs have been found in the placenta of infants whose mothers used alcohol during pregnancy (Bearer et al., 1992). Fatty acid ethyl esters have been detected in the meconium of newborn infants and suggested as a potential biomarker of fetal alcohol exposure (Bearer et al., 1999, 2003, 2005; Klein et al., 1999; Mac et al., 1994).

Our study shows that the FAEs in meconium are significantly associated with fetal alcohol exposure, consistent with previously reported observations (Bearer et al., 1999, 2003, 2005; Klein et al., 1999; Mac et al., 1994; Moore and Lewis, 2001). However, these 6 studies differ in the types of FAE that predominate. The FAEs detected in these studies included ethyl laurate, ethyl palmitate, ethyl stearate (Klein et al., 1999; Mac et al., 1994), ethyl

linoleate (Bearer et al., 1999; Moore and Lewis, 2001), and ethyl oleate (Bearer et al., 2003; Moore and Lewis, 2001). There is no clear explanation for the differences among the various studies and our current findings. It is likely that the maternal diet, which influences the composition of serum fatty acids may be an important factor (Stark et al., 2005a, 2005b). Two different populations that were studied by Bearer et al. (1999) showed ethyl linoleate as the predominant FAEE in meconium in a cohort from Cleveland, Ohio, in contrast to ethyl oleate in a cohort from Cape Town, South Africa (Bearer et al., 2003). In vitro studies, using Hep G2 cells, have suggested that the concentration of fatty acids in the extracellular medium may be an important factor for the synthesis of FAEEs (Dan and Laposata, 1997). Ethanol showed the synthesis of ethyl palmitate and oleate over other FAEEs in the presence of higher concentrations of palmitate and oleate in the extracellular medium. In another study, FAEEs were found in meconium even in a nondrinking population, although the FAEE concentrations were much lower than those in the alcohol-exposed group (Chan et al., 2003). Small quantities of ethanol that are present in certain medications or food additives were thought to explain these findings (Chan et al., 2004). Lastly, the difference in the types of FAEE that were noted in the various studies may also be attributed to the difference in techniques used in measuring FAEE (Bearer et al., 1999, 2003, 2005; Chan et al., 2003; Mac et al., 1994).

Our study has shown significant association in both incidence (Table 4) and concentrations of ethyl linoleate in meconium with fetal exposure to alcohol (Tables 5 and 6). Although the sensitivity of ethyl linoleate as a biomarker of exposure was low (26.9%), its specificity and positive predictive value were >96%. This implies that if ethyl linoleate is found in meconium, it is highly likely that the fetus was exposed to alcohol. Consistent with the report by Bearer et al. (2001), we found significant correlation between the concentrations of ethyl linoleate and measures of alcohol consumption (AADD) across pregnancy, but only when linoleate concentration was trichotomized. The scatter plot (Fig. 1) showed a positive relationship between ethyl linoleate concentration and increasing AADD across pregnancy (up to 2.3 oz/d). However, beyond an AADD of 3.5 oz, there was an abrupt fall or absence in the concentration of ethyl linoleate in meconium. We cannot ascertain the cause of this phenomenon and propose a number of possibilities. First, it is possible that there may be 2 subsets of pregnant mothers: one that produces FAEEs and one that does not upon exposure to ethanol during pregnancy. Second, we strongly feel that this phenomenon may be related to maternal nutrition. At high amounts of alcohol intake, maternal nutritional intake may be significantly reduced and intake of nutrients, specifically fats and fatty acids, may also be diminished (Stark et al., 2005a, 2005b), thus limiting the formation of ethyl esters from essential fatty acids. Future studies, therefore, detailing nutritional intake in pregnant women who abuse alcohol may be an important factor when assessing FAEEs as biomarkers of prenatal exposure in infants.

As with ethyl linoleate, the sensitivities of ethyl linolenate, ethyl AA, or ethyl DHA as biomarkers of fetal alcohol exposure were also low (range=3.2% to 21.5%); however, their specificities ranged between 93.5 and 100%. Thus, the presence of ethyl linoleate, ethyl linolenate, ethyl AA, or ethyl DHA in meconium is highly indicative of prenatal alcohol exposure.

Of the polyunsaturated long-chain FAEEs, the incidence and concentration of ethyl AA showed weak evidence of being higher in the alcohol-exposed group than the control group (Tables 4 and 5). However, we feel that this may represent a type II error due to a small sample size. Enrollment therefore of more subjects to achieve adequate statistical power may help determine whether ethyl AA is also a good biomarker of alcohol exposure in newborn infants.

This is the first study to report on ethyl DHA in meconium. Gas chromatography/mass spectrometry analysis by PCI, in contrast to electron impact ionization, preserves high-molecular-weight ions better and allows for the identification of the very long-chain, polyunsaturated fatty acids of AA and DHA. Our findings, particularly with ethyl DHA, are of great interest as this FAEE was only found in the alcohol-exposed group (Table 4). We are intrigued by the possibility that ethyl DHA in meconium may also be a biomarker of fetal alcohol effect, besides fetal alcohol exposure. This hypothesis is derived from several observations. First, DHA is an important fatty acid for normal fetal brain and retinal development and α -linolenic acid is a precursor of DHA (Carlson, 2001; Uauy-Dagach and Mena, 1995). In the present study, ethyl linolenate and ethyl DHA were found only in the alcohol-exposed infants (Table 4). We therefore propose that the presence of ethyl DHA and ethyl linolenate in meconium may be significant because the esterification of DHA acid and α -linolenic acid by alcohol may promote the excretion of these important fatty acids as soluble ethyl esters, ultimately limiting the availability of DHA for fetal brain development and therefore acting as a potential mechanism of the fetal alcohol syndrome or other fetal alcohol spectrum disorders. The latter is a concept that has been previously suggested in a number of reports (Beblo et al., 2005; Denkins et al., 2000; Horrocks and Yeo, 1999; Pawlosky et al., 2001; Stark et al., 2005a, 2005b). Another possibility is that ethyl DHA may be directly toxic to the developing fetal brain. Fatty acid ethyl esters are considered potentially toxic in the brain, liver, and heart of alcoholics through their disruption of mitochondrial and other cell functions (Bora and Lange, 1993; Beckemeier and Bora, 1998). There are also several factors that influence the formation of ethyl DHA, and the low occurrence of ethyl DHA detection even among alcohol-exposed infants (unlike ethyl linoleate) may reflect differences in nutrition (Beblo et al., 2005; Denkins et al., 2000; Stark et al., 2005a, 2005b) or may be secondary to its higher limit of detection compared with other FAEEs. Of interest, however, is a relatively low incidence of diagnosis of FAS among alcohol-exposed infants (Abel, 1998). It would be instructive to test how ethyl DHA levels in meconium parallel the low incidence of alcohol-related complications in infants, reflecting a possible nutritional risk factor for alcohol teratogenesis (Abel and Hannigan, 1995). Conversely, our hypothesis is not supported by the fact that none of the 5 infants who were positive for ethyl DHA in meconium showed features of FAS at birth. Nonetheless, alcohol-exposed infants may only manifest subtle fetal alcohol effects at birth but show neurodevelopmental characteristics at a later time (Bertrand et al., 2004; Little et al., 1990). What is therefore needed is a prospective, case control study of alcohol-exposed infants, with meconium analyzed at birth for FAEEs, and with follow-up of the infants for at least 1 year to determine the presence of neurodevelopmental delays that are suggestive of FAS or fetal alcohol effects. Such a clinical study will help establish whether FAEEs can be used not only as a biomarker of alcohol exposure but also as a biomarker of fetal alcohol effect. These intriguing questions require further investigations, and some of that research is under way in our group.

We conclude that FAEEs in meconium, particularly ethyl linoleate and ethyl AA, are biomarkers of high specificity for prenatal exposure to alcohol in newborn infants. We also propose that ethyl AA and DHA could be potential biomarkers of fetal alcohol effects on the developing fetal brain and should be investigated further.

Acknowledgments

Supported in part by National Institutes of Health Grants: NIAAA Grant R01AA10941 (MWC—principal investigator) and N01AA83019 (JHH—principal investigator).

REFERENCES

- Abel EL. Fetal alcohol syndrome: the 'American Paradox'. *Alcohol Alcohol*. 1998; 33:195–201. [PubMed: 9632044]
- Abel EL, Hannigan JH. Maternal risk factors in fetal alcohol syndrome: provocative and permissive influences. *Neurotoxicol Teratol*. 1995; 17:445–462. [PubMed: 7565491]
- Armbruster D, Tillman M, Hubbs L. Limit of detection (LOD)/limit of quantitation (LOQ): comparison of the empirical and the statistical methods exemplified with GCMS assays of abused drugs. *Clin Chem*. 1994; 40:1233–1238. [PubMed: 8013092]
- Bearer C. Markers to detect drinking during pregnancy. *Alcohol Res Health*. 2001; 25:210–218. [PubMed: 11810960]
- Bearer CF, Gould S, Emerson R, Kinnunen P, Cook CS. Fetal alcohol syndrome and fatty acid ethyl esters. *Pediatr Res*. 1992; 31:492–495. [PubMed: 1603626]
- Bearer CF, Jacobson JL, Jacobson SW, Barr D, Croxford J, Molteno CD, Viljoen DL, Marais AS, Chiodo LM, Cwik AS. Validation of a new biomarker of fetal exposure to alcohol. *J Pediatr*. 2003; 143:463–469. [PubMed: 14571221]
- Bearer CF, Lee S, Salvator AE, Minnes S, Swick A, Yamashita T, Singer LT. Ethyl linoleate in meconium: a biomarker for prenatal ethanol exposure. *Alcohol Clin Exp Res*. 1999; 23:487–493. [PubMed: 10195823]
- Bearer CF, Santiago LM, O'Riordan MA, Buck K, Lee SC, Singer LT. Fatty acid ethyl esters: quantitative biomarkers for maternal alcohol consumption. *J Pediatr*. 2005; 146:824–830. [PubMed: 15973326]
- Beblo S, Stark K, Murthy M, Janisse J, Rockett H, Whitty JE, Buda-Abela M, Martier SS, Sokol RJ, Hannigan JH, Salem N Jr. Effects of alcohol intake during pregnancy on docosahexaenoic acid and arachidonic acid in umbilical cord vessels of Black women. *Pediatrics*. 2005; 115:194–203. [PubMed: 15630008]
- Beckemeier ME, Bora PS. Fatty acid ethyl esters: potentially toxic products of myocardial ethanol metabolism. *J Mol Cell Cardiol*. 1998; 30:2487–2494. [PubMed: 9925383]
- Bertrand, J.; Floyd, RL.; Weber, MK.; O'Connor, M.; Riley, EP.; Johnson, KA.; Cohen, DE. National Task Force on FAS/FAE Fetal Alcohol Syndrome: Guidelines for Referral and Diagnosis. Centers for Disease Control and Prevention; Atlanta, GA: 2004.
- Best CA, Cluette-Brown JE, Teruya M, Teruya A, Laposata M. Red blood cell fatty acid ethyl esters: a significant component of fatty acid ethyl esters in the blood. *J Lipid Res*. 2003; 44:612–620. [PubMed: 12562827]
- Best CA, Laposata M. Fatty acid ethyl esters: toxic non-oxidative metabolites of ethanol and makers of ethanol intake. *Front Biosci*. 2003; 8:202–217.
- Bibb KW, Stewart DL, Walker JR, Cook VD, Wagener RE. Drug screening in newborns and mothers using meconium samples, paired urine samples, and interviews. *J Perinat Med*. 1995; 15:199–202.
- Bielawski DM, Ostrea EM, Hernandez JD, Abela MB, Church MW, Hannigan JH, Sokol RJ. Detection of fatty acid ethyl esters in meconium by positive chemical ionization gas chromatography/mass spectrometry (GC/MS). *Alcohol Clin Exp Res*. 2003; 27:39A.
- Bora PS, Lange LG. Molecular mechanism of ethanol metabolism by human brain to fatty acid ethyl esters. *Alcohol Clin Exp Res*. 1993; 17:28–30. [PubMed: 8452205]
- Calabrese V, Scapagnini G, Catalano C, Dinotta F, Bates TE, Calvani M, Stella AM. Effects of acetyl-L-carnitine on the formation of fatty acid ethyl esters in brain and peripheral organs after short term ethanol administration in rat. *Neurochem Res*. 2001; 26:167–174. [PubMed: 11478744]
- Carlson SE. Docosahexaenoic acid and arachidonic acid in infant development. *Semin Neonatol*. 2001; 6:437–449. [PubMed: 11988033]
- Chan D, Bar-Oz B, Pellerin B, Paciorek C, Klein J, Kapur B, Farine D, Koren G. Population baseline of meconium fatty acid ethyl esters among infants of nondrinking women in Jerusalem and Toronto. *Ther Drug Monit*. 2003; 25:271–278. [PubMed: 12766552]
- Chan D, Caprara D, Blanchette P, Klein J, Koren G. Recent developments in meconium and hair testing methods for the confirmation of gestational exposures to alcohol and tobacco smoke. *Clin Biochem*. 2004; 37:429–428. [PubMed: 15183290]

- Dan L, Laposata M. Ethyl palmitate and ethyl oleate are the predominant fatty acid ethyl esters in the blood after ethanol ingestion and their synthesis is differentially influenced by the extracellular concentrations of their corresponding fatty acids. *Alcohol Clin Exp Res.* 1997; 21:286–292. [PubMed: 9113265]
- Denkins YM, Woods J, Whitty JE, Hannigan JH, Martier SS, Sokol RJ, Salem NE. Effects of gestational alcohol exposure on the fatty acid composition of umbilical cord serum in humans. *Am J Clin Nutr.* 2000; 71:300s–306s. [PubMed: 10617986]
- Diczfalussy MA, Bjorkhem I, Einarsson C, Hillebrant CG, Alexson SE. Characterization of enzymes involved in formation of ethyl esters of long chain fatty acids in humans. *J Lipid Res.* 2001; 42:1025–1032. [PubMed: 11441128]
- Hartwig S, Auwarter V, Pragst F. Fatty acid ethyl esters in scalp, pubic, axillary, beard and body hair as markers for alcohol misuse. *Alcohol Alcohol.* 2003; 38:163–167. [PubMed: 12634265]
- Hollingshead, AB. Four factor index of social status. Department of Social Work, Yale University; New Haven, CT: 1975. Unpublished paper
- Horrocks LA, Yeo YK. Health benefits of docosahexaenoic acid (DHA). *Pharmacol Res.* 1999; 40:211–225. [PubMed: 10479465]
- Klein J, Karaskov T, Koren G. Fatty acid ethyl esters: a novel biologic marker for heavy in utero ethanol exposure: a case report. *Therap Drug Monit.* 1999; 21:644–646. [PubMed: 10604826]
- Laposata M. Fatty acid ethyl esters: short-term and long-term serum markers of ethanol intake. *Clin Chem.* 1997; 43:1527–1534. [PubMed: 9265904]
- Laposata M. Fatty acid ethyl esters: current facts and speculations. *Prostaglandins Leukot Essent Fatty Acids.* 1999; 60:313–315. [PubMed: 10471114]
- Laposata M, Hasaba A, Best CA, Yoerger DM, McQuillan BM, Salem RO, Refaai MA, Laposata M, Kabakibi A, Walden MP, Cluette-Brown JE, Nanji AA, Refaai MA, Werner J. Differences in the fatty acid composition in fatty acid ethyl esters in organs and their secretions. *Alcohol Clin Exp Res.* 2000; 24:1488–1491. [PubMed: 11045855]
- Laposata M, Hasaba A, Best CA, Yoerger DM, McQuillan BM, Salem RO, Refaai MA, Soderberg BL. Fatty acid ethyl esters: recent observations [review]. *Prostaglandins Leukot Essent Fatty Acids.* 2002; 67:193–196. [PubMed: 12324241]
- Lawson GM. Defining limit of detection and limit of quantitation as applied to drug of abuse testing: striving for a consensus. *Clin Chem.* 1994; 40:1218–1219. [PubMed: 8013089]
- Leonardo GM, Hernandez JD, Bielawski D, Kan JM, Ostrea EM Jr. Abella MB, Church M, Sokol R. Umbilical cord γ -glutamyl transferase: a potential biomarker of fetal exposure to alcohol. *Pediatr Res.* 2002; 51:360A.
- Little BB, Snell LM, Rosenfeld CR, Gilstrap LC, Gant NF. Failure to recognize fetal alcohol syndrome in newborn infants. *Am J Dis Child.* 1990; 144:1142–1146. [PubMed: 2403097]
- Mac E, Pacis M, Garcia G, Ostrea EM Jr. A marker of fetal exposure to alcohol by meconium analysis. *Pediatr Res.* 1994; 35:238A. [PubMed: 8165060]
- Mirlesse B, Jacquemard F, Daffos F, Forestier F. Fetal gamma-glutamyl transferase activity: clinical implications in fetal medicine. *Biol Neonat.* 1996; 70:193–198.
- Moore CM, Lewis D. Fatty acid ethyl esters in meconium: biomarkers for the detection of alcohol exposure in neonates. *Clin Chim Acta.* 2001; 312:235–237. [PubMed: 11706815]
- Ostrea EM Jr. Brady M, Gause S, Raymundo AL. Drug screening of newborns by meconium analysis: a large-scale, prospective, epidemiologic study. *Pediatrics.* 1992; 89:107–113. [PubMed: 1727992]
- Ostrea EM Jr. Brady M, Parks PM, Asensio DC, Naluz A. Drug screening of meconium in infants of drug dependent mothers: an alternative to urine testing. *J Pediatr.* 1989; 115:474–477. [PubMed: 2769510]
- Ostrea EM Jr. Knapp DK, Romero A, Montes M, Ostrea AR. Meconium analysis to assess fetal exposure to active and passive maternal smoking. *J Pediatr.* 1994; 15:471–476. [PubMed: 8120724]
- Ostrea EM Jr. Knapp K, Tannenbaum L, Ostrea AR, Romero A, Salari V, Ager J. Estimates of illicit drug use during pregnancy by maternal interview, hair analysis, and meconium analysis. *J Pediatr.* 2001; 138:344–348. [PubMed: 11241040]

- Ostrea EM Jr, Matias O, Keane C, Mac E, Utarnachitt R, Ostrea A, Mazhar M. Spectrum of gestational exposure to illicit drugs and other xenobiotic agents in newborn infants by meconium analysis. *J Pediatr*. 1998; 133:513–515. [PubMed: 9787689]
- Ostrea EM Jr, Morales V, Ngoumgna E, Prescilla R, Tan E, Hernandez E, Ramirez GB, Manlapaz M. Prevalence of fetal exposure to environmental toxins as determined by meconium analysis. *Neurotoxicology*. 2002; 23:329–339. [PubMed: 12389578]
- Ostrea EM Jr, Parks P, Brady M. Rapid isolation and detection of drugs in meconium of infants of drug dependent mothers. *Clin Chem*. 1988; 34:2372–2373. [PubMed: 3180439]
- Pawlosky RJ, Bacher J, Salem N Jr. Ethanol consumption alters electroretinograms and depletes neural tissues of docosahexaenoic acid in rhesus monkeys: nutritional consequences of a low n-3 fatty acid diet. *Alcohol Clin Exp Res*. 2001; 25:1758–1765. [PubMed: 11781509]
- Refaai MA, Nguyen PN, Steffensen TS, Evans RJ, Cluette-Brown JE, Laposata M. Liver and adipose tissue fatty acid ethyl esters obtained at autopsy are postmortem markers of premortem ethanol intake. *Clin Chem*. 2002; 48:77–83. [PubMed: 11751541]
- Robinson MK, Myrick JE, Henderson LO, Coles CD, Powell MK, Orr GA, Lemkin PF. Two dimensional protein electrophoresis and multiple hypothesis testing to detect potential serum protein biomarkers in children with fetal alcohol syndrome. *Electrophoresis*. 1995; 16:1176–1183. [PubMed: 7498162]
- Russell M, Martier SS, Sokol RJ, Mudar P, Jacobson S, Jacobson J. Detecting risk drinking during pregnancy: a comparison of four screening questionnaires. *Am J Public Health*. 1996; 86:1435–1439. [PubMed: 8876514]
- Ryan RM, Wagner CL, Schultz JM, Varley J, DiPreta J, Sherer DM, Phelps DL, Kwong T. Meconium analysis for improved identification of infants exposed to cocaine in utero. *J Pediatr*. 1994; 125:435–440. [PubMed: 8071754]
- Salem RO, Refaai MA, Cluette-Brown JE, Russo JW, Laposata M. Fatty acid ethyl esters in liver and adipose tissues as postmortem markers of ethanol intake. *Clin Chem*. 2001; 47:722–725. [PubMed: 11274023]
- Stark KD, Beblo S, Murthy M, Buda-Abela M, Janisse J, Rockett H, Whitty JE, Martier SS, Sokol RJ, Hannigan JH, Salem N Jr. Comparison of plasma and erythrocyte total fatty acid composition from African-American women at 24 weeks gestation, infant delivery and 3 months postpartum. *J Lipid Res*. 2005a; 46:516–525. [PubMed: 15604519]
- Stark K, Beblo S, Murthy M, Whitty JE, Buda-Abela M, Janisse J, Rockett H, Martier SS, Sokol RJ, Hannigan JH, Salem N Jr. Alcohol consumption in pregnant, African-American women is associated with decreased plasma and erythrocyte docosahexaenoic acid. *Alcohol Clin Exp Res*. 2005b; 29:130–140. [PubMed: 15654301]
- Streissguth AP, Barr HM, Martin DC, Henman CS. Effect of maternal alcohol, nicotine and caffeine use during pregnancy on infant mental and motor development at 8 months. *Alcohol Clin Exp Res*. 1980; 4:152–164. [PubMed: 6990818]
- Uauy-Dagach R, Mena P. Nutritional role of omega-3 fatty acids during the perinatal period. *Clin Perinatol*. 1995; 22:157–175. [PubMed: 7781250]
- Van Der Leeden M, Van Dongen K, Kleinhout M, Phaff J, De Groot CJ, De Groot L, Hesselings PB. Infants exposed to alcohol prenatally: outcome at 3 and 7 months of age. *Ann Trop Paediatr*. 2001; 21:127–134. [PubMed: 11471255]
- Whitehall J, Ostrea EM, Patole S. Fetal exposure to pollutants in Townesville, Australia as detected in meconium. *Pediatr Res*. 2000; 47:299A.
- Whyatt RM, Barr DB. Measurement of organophosphate metabolites in postpartum meconium as a potential biomarker of prenatal exposure: a validation study. *Environ Health Perspect*. 2001; 109:417–420. [PubMed: 11335191]
- Wisniewski KE, Pullarkat RJ, Harin A, Vartolo M. Increased urinary dolichol in newborns whose mothers were heavy alcohol users. *Ann Neurol*. 1983; 14:382A.

Fig. 1. Scatter plot of amount (oz) of absolute alcohol consumed per drinking day (AADD) across pregnancy versus ethyl linoleate concentration in meconium ($\mu\text{g/g}$).

Table 1Maternal and Neonatal Characteristics of the Study Population (*n*=124)

Characteristics	Control (<i>n</i> =31)	Alcohol- exposed (<i>n</i> =93)	<i>p</i>
Maternal age ^a	27.7 (7.2)	26.4 (5.9)	0.318 ^b
Gravidity ≥ 4	48.4%	53.8%	0.604 ^c
Parity ≥ 4	25.8%	16.1%	0.230 ^c
Single ^d	80%	87.1%	0.353 ^e
Black	77.4%	88.2%	0.151 ^e
Socioeconomic status ^{d,f}	75%	73.1%	0.852 ^c
Positive amphetamine screen ^g	6.7%	0%	0.300 ^e
Positive barbiturates screen ^{g,h}	0%	0%	
Positive opiate screen ^g	6.7%	0%	0.300 ^e
Positive cocaine screen ^g	6.7%	17.1%	0.659 ^e
Positive cannabinoid screen ⁱ	0%	25.0%	0.047 ^e
Cigarette (sticks per day) ^j	0.0	6.0	0.010 ^k
Infant gestation (wk) ^a	38.7 (2.3)	38.7 (2.7)	0.873 ^b
Birth weight (g) ^a	3164.8 (649.8)	3087.2 (585.4)	0.535 ^b
Length (cm) ^a	49.5 (2.6)	49.5 (3.0)	0.929 ^b
Head circumference (cm) ^a	33.7 (1.8)	33.3 (1.9)	0.214 ^b

^a Mean (SD).^b Statistical significance based on *t*-test.^c Statistical significance based on chi-square test.^d Sample size for control group; single *n*=25, SES *n*=24.^e Statistical significance based on Fisher's exact test.^f SES scores of <29.5 and ≥ 7.5 (Hollingshead, 1975).^g Sample size for control *n*=15; for exposed *n*=35.^h No statistics are computed. All screens for barbiturates were negative.ⁱ Sample size for control *n*=14; for exposed *n*=36.^j Median.^k Statistical significance based on Mann-Whitney test.

SES, socioeconomic status.

Table 2
Measures of Alcohol Use, at Each Percentile Group, Among 124 Pregnant Women

	Percentile						
	Control (n=31) ^a			Alcohol-exposed (n=93)			p
	10	50	90	10	50	90	
MAST score ^{a,b}	0	0	4.0	0	0	13.8	0.015
CAGE score ^{a,b}	0	0	1.6	0	0	3.0	0.013
TACE score ^{a,b}	0	0	2.7	0	2.0	4.0	0.001
AAD at time of conception ^c				0.17	0.91	3.47	
AADD at time of conception ^c				0.94	3.20	7.06	
PD at time of conception ^c				0.14	0.29	1.00	
AAD across pregnancy ^{c,d}				0.04	0.18	0.69	
AADD across pregnancy ^{c,d}				1.20	3.20	6.40	
PD across pregnancy ^{c,d}				0.02	0.06	0.16	

^aFor control group, MAST (n=24), CAGE (n=23), and TACE (n=22).

^bStatistical significance based on Mann-Whitney test.

^cGroups were created based on these alcohol measures. Therefore, no significance test is performed.

^dFor exposed group across pregnancy AAD, AADD, and PD (n=89).

MAST, Michigan Alcohol Screening Test; AAD, average amount (oz) of absolute alcohol consumed per day; AADD, average amount (oz) of absolute alcohol consumed per actual drinking day; PD, proportion of 7 days in which alcohol was used.

Table 3
Correlations Among Measures of Alcohol Exposure and MAST, TACE, and CAGE

	AADD.0 ^a	PD.0 ^a	MAST	CAGE	TACE
AAD.0 ^a	0.892**	0.838**	0.526**	0.430**	0.583**
AADD.0 ^a		0.616**	0.384**	0.305**	0.485**
PD.0 ^a			0.458**	0.408**	0.503**
MAST				0.707**	0.576**
CAGE					0.727**
	AADD.XP ^b	PD.XP ^b	MAST	CAGE	TACE
AAD.XP ^b	0.860**	0.900**	0.527**	0.363**	0.541**
AADD.XP ^b		0.620**	0.340**	0.271**	0.460**
PD.XP ^b			0.476**	0.321**	0.451**

^a AAD, AADD, and PD at time of conception.

^b AAD, AADD, and PD across pregnancy.

**
 $p < 0.01$.

Correlations were based on Spearman's ρ .

Sample size ranges between 111 and 124.

MAST, Michigan Alcohol Screening Test; AAD, average amount (oz) of absolute alcohol consumed per day; AADD, average amount (oz) of absolute alcohol consumed per actual drinking day; PD, proportion of 7 days in which alcohol was used.

Table 4

Incidence (% Positive) of FAEEs in Meconium of Alcohol-Exposed and Control Infants

	% Positive		<i>p</i> ^a
	Control (<i>n</i> =31)	Alcohol- exposed (<i>n</i> =93)	
Ethyl laurate ^a	19.4	19.4	1.000
Ethyl myristate ^a	71.0	67.7	0.738
Ethyl palmitate ^a	58.1	58.1	1.000
Ethyl stearate ^a	12.9	19.4	0.415
Ethyl oleate ^a	41.9	49.5	0.467
Ethyl linoleate ^a	9.7	28.0	0.037
Ethyl α -linolenate ^b	0	3.2	0.572
Ethyl arachidonate ^a	6.5	21.5	0.057
Ethyl docosahexanoate ^b	0	4.3	0.571

^aComparison of % positive between control and alcohol-exposed infants based on chi-square test.^bComparison of % positive between control and alcohol-exposed infants based on Fisher's exact test.

FAEE, fatty acid ethyl esters.

Table 5
Concentrations of FAEs in Meconium of Alcohol-Exposed and Control Infants

	FAEE Concentration ($\mu\text{g/g}$ meconium) ^a										<i>p</i> ^b
	Percentile						Alcohol-exposed (<i>n</i> =93)				
	Control (<i>n</i> =31)										
	10	50	90	10	50	90	10	50	90		
Ethyl laurate	0	0	0.110	0	0	0.429	0.840				
Ethyl myristate	0	0.114	0.874	0	0.106	0.794	0.749				
Ethyl palmitate	0.00	0.382	2.402	0	0.402	1.746	0.483				
Ethyl stearate	0	0	0.837	0	0	0.934	0.391				
Ethyl oleate	0	0	5.547	0	0	16.58	0.379				
Ethyl linoleate	0	0	0.123	0	0	5.715	0.021				
Ethyl α -linolenate ^c	0	0	0	0	0	0	0.313				
Ethyl arachidonate	0	0	0	0	0	1.168	0.064				
Ethyl docosahexanoate ^d	0	0	0	0	0	0	0.242				

^aFAEE concentration levels are given at the 10th, 50th, and 90th percentiles of their distributions.

^bStatistical significance based on Mann–Whitney test.

^c98% of alcohol-exposed subjects have no ethyl α -linolenate.

^d96% of alcohol-exposed subjects have no ethyl docosahexanoate.
FAEE, fatty acid ethyl esters.

Table 6

Association Between Antenatal Alcohol Exposure in the Infant and Trichotomized Ethyl Linoleate Concentration in Meconium Based on Their Actual Concentrations

Alcohol Exposed	Trichotomized ethyl linoleate ($\mu\text{g/g}$) ^a			Total
	Group A	Group B	Group C	
Non-exposed	28 (90.3%)	3 (3.7%)	0 (0%)	31
Exposed	67 (72%)	13 (14%)	13 (14%)	93
	95	16	13	124

^a Values for trichotomized ethyl linoleate were set by inspection of the distribution of the concentrations of ethyl linoleate and determining the limits that were most appropriate.

Group A: ethyl linoleate=0 $\mu\text{g/g}$ meconium.

Group B: 0<ethyl linoleate $\leq$ 3.456 $\mu\text{g/g}$ meconium.

Group C: ethyl linoleate>3.456 $\mu\text{g/g}$ meconium.

Table 7

Correlations Among Concentrations of Ethyl Linoleate, Ethyl Linolenate, Ethyl Arachidonate, and Ethyl Docosahexanoate in Meconium ($n=124$)

	Ethyl α -linolenate ($\mu\text{g/g}$)	Ethyl arachidonate ($\mu\text{g/g}$)	Ethyl docosahexanoate ($\mu\text{g/g}$)
Ethyl linoleate ($\mu\text{g/g}$)	0.203 [*]	0.594 ^{**}	0.320 ^{**}
Ethyl α -linolenate ($\mu\text{g/g}$)		0.369 ^{**}	0.280 ^{**}
Ethyl arachidonate ($\mu\text{g/g}$)			0.289 ^{**}

Correlation based on Spearman's ρ .

*
 $p \leq 0.05$

**
 $p \leq 0.01$.