

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

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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.

Key Words: 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.

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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 $\mu\text{g/g}$ 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 μL of acetone, and vortexed. Samples (1 μL) 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 μm) was utilized and the

oven temperature was programmed: initial temperature of 100°C , increased at a rate of $25^\circ\text{C}/\text{min}$ to 200°C , then $5^\circ\text{C}/\text{min}$ 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 $\mu\text{g/g}$ FAEEs (ethyl esters of laurate, myristate, palmitate, linoleate, oleate, α -linolenate, stearate, AA, and DHA). For the recovery study, FAEEs were spiked in meconium at 1, 4, and 8 $\mu\text{g/g}$ concentrations with ethyl heptadecanoate as an internal standard.

At 1 to 8 $\mu\text{g/g}$ concentrations of the different FAEEs 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 FAEEs 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 $\mu\text{g/g}$ for ethyl laurate, ethyl myristate, ethyl palmitate, ethyl linoleate, and ethyl oleate; 0.10 $\mu\text{g/g}$ for ethyl α -linolenate and ethyl stearate; 0.20 $\mu\text{g/g}$ for ethyl AA; and 1 $\mu\text{g/g}$ for ethyl DHA. For categorical values (positive/negative groupings) or actual concentrations, FAEEs were considered as "negative or zero concentration: if the actual concentration was below the LOD." The calibration curve of each FAEE 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).

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

Characteristics	Control ($n = 31$)	Alcohol-exposed ($n = 93$)	<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

^aMean (SD).

^bStatistical significance based on *t*-test.

^cStatistical significance based on chi-square test.

^dSample size for control group; single $n = 25$, SES $n = 24$.

^eStatistical significance based on Fisher's exact test.

^fSES scores of < 29.5 and ≥ 7.5 (Hollingshead, 1975).

^gSample size for control $n = 15$; for exposed $n = 35$.

^hNo statistics are computed. All screens for barbiturates were negative.

ⁱSample size for control $n = 14$; for exposed $n = 36$.

^jMedian.

^kStatistical significance based on Mann–Whitney test. SES, socioeconomic status.

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

	Percentile						<i>p</i>
	Control ($n = 31$) ^a			Alcohol-exposed ($n = 93$)			
	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.

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

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**

^aAAD, AADD, and PD at time of conception.

^bAAD, 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 (n = 31)	Alcohol-exposed (n = 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.

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

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

	FAEE Concentration ($\mu\text{g/g}$ meconium) ^a						<i>p</i> ^b
	Percentile						
	Control (n = 31)			Alcohol-exposed (n = 93)			
	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.

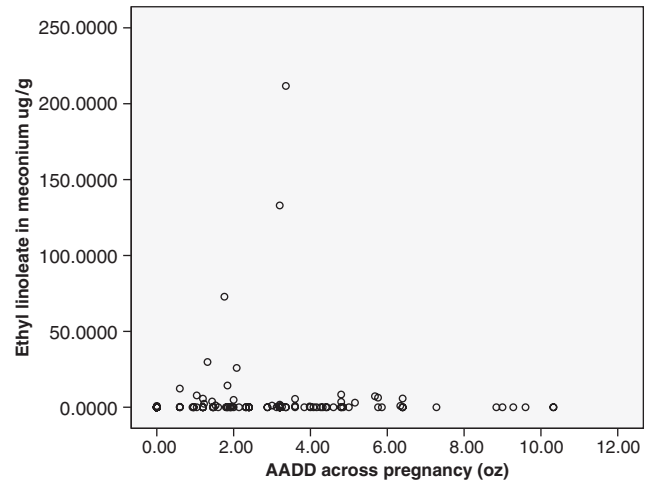


Fig. 1. Scatter plot of amount (oz) of absolute alcohol consumed per drinking day (AADD) across pregnancy versus ethyl linoleate concentration in meconium ($\mu\text{g/g}$).

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.

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

^aValues 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 .

* $p \leq 0.05$, ** $p \leq 0.01$.

Combinations of FAEEs (ethyl palmitate, stearate, and oleate) did not show any significant association between the FAEEs and alcohol exposure.

Of the polyunsaturated long-chain FAEEs, 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 acyl-coenzyme, ethanol-*O*-acyltransferase (Diczfalusy et al., 2001). A number of enzymes have fatty acid synthase activity, including lipoprotein lipase, carboxylesterase, and carboxyl ester lipase (Best and Laposata, 2003; Laposata, 1999). In adults, FAEEs 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 FAEE is also proportional to the amount of alcohol intake (Laposata, 1997). Different types or species of FAEEs 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, FAEEs 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 FAEEs 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 FAEE that predominate. The FAEEs 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.

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