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VALIDATION OF A NEW BIOMARKER OF FETAL EXPOSURE TO ALCOHOL

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Abstract

Objective—To test the sensitivity and specificity of fatty acid ethyl esters (FAEEs) extracted from meconium to identify alcohol-using pregnant women with a sensitive and specific methodology, gas chromatography-tandem mass spectroscopy (GC/MS/MS).

Study design—Twenty-seven samples of meconium were obtained from infants from the mixed race community in Cape Town, South Africa, who were enrolled in a longitudinal neurobehavioral study. Maternal alcohol use was reported prospectively during pregnancy. FAEEs were isolated from meconium and quantitated by GC/MS/MS.

Results—Ethyl oleate was the FAEE that correlated most strongly with maternal self-reported drinking, especially with the average ounces of absolute alcohol ingested per drinking day. Ethyl oleate was most strongly related to drinking in the second and third trimesters (Pearson $r = .55$ and $.40$, respectively). At a threshold of 1.5 average ounces of absolute alcohol ingested per drinking day, the area under the receiving operator characteristic curve was .92 (95% confidence interval, 0.74–0.97). Using a cut-off value of 32 ng/g, sensitivity was 84.2% and specificity was 83.3%.

Conclusions—Ethyl oleate concentration in meconium assayed by GC/MS/MS provides a highly sensitive and specific indicator of maternal alcohol use during pregnancy.

Heavy drinking during pregnancy is the cause of fetal alcohol syndrome (FAS), one of the leading known forms of mental retardation.¹ FAS is marked by distinctive craniofacial abnormalities, growth retardation, and central nervous system damage.² Drinking during

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C. F. B. holds a patent for the measurement of fatty acid ethyl ester in meconium. Currently there are no business relationships based on this patent.

pregnancy can also result in a spectrum of effects known as alcohol-related neurodevelopmental disorder (ARND), which range from severe cognitive and behavioral impairment without the classic facial dysmorphism to relatively subtle neurobehavioral deficits.³ It is estimated that 1% of all newborns are affected by prenatal ethanol exposure.⁴ School-age children whose mothers recalled having consumed more than five drinks on at least one occasion during pregnancy had learning problems,⁵ and 6.5-month-old infants whose mothers consumed seven drinks per week on average had measurable deficits in performance on the Fagan Test of Infant Intelligence.⁶ The Centers for Disease Control and Prevention reported an increase in the prevalence of binge drinking (≥ 5 drinks per occasion) among pregnant women from 0.7% in 1991 to 2.7% in 1999.⁷

Because alcohol is metabolized rapidly, there is currently no well validated biological marker of exposure during pregnancy. In one study, the diagnosis of FAS was missed in 100% of newborns who were subsequently diagnosed in childhood.⁸ Two studies have shown a correlation of biological markers with maternal drinking or fetal outcome.⁹ In the first, hemoglobin-acetaldehyde adducts and carbohydrate-deficient transferrin were not associated with the reported level of drinking in pregnant women with alcohol abuse.¹⁰ However, mean corpuscular volume and gamma-glutamyl transferase were significantly higher in women drinking at least eight drinks per week compared with those drinking fewer than eight drinks per week.¹⁰ In another study, tests for carbohydrate-deficient transferrin, gamma-glutamyl transferase, mean corpuscular volume, and hemoglobin-acetaldehyde adducts were combined.¹¹ All women who reported drinking at least 14 drinks per week were positive for one or more markers. Having two or more positive markers was more predictive of infant outcome than any measure of self-reported drinking. Neither of these studies reported sensitivity or specificity of the biomarker. Thus, although statistically significant, the clinical usefulness of these tests is unclear.

A biological marker for risk levels of drinking during pregnancy would permit earlier identification and intervention for affected infants and would facilitate recognition of women at risk for drinking during their next pregnancy. It could also help improve our understanding of the effects of prenatal alcohol exposure on neurobehavioral development. For example, it would provide a long-term, objective measure of fetal exposure that can supplement maternal recall, which is susceptible to social desirability and recall bias. Ethanol is metabolized by both oxidative and nonoxidative pathways. The nonoxidative pathway conjugates ethanol to free fatty acids, forming fatty acid ethyl esters (FAEEs).¹² Compared with ethanol and the oxidative metabolites, FAEEs have a prolonged half-life in tissue and are detected in meconium collected from the human infant within the first 48 to 72 hours postpartum. There is a significant association between maternal self-reported drinking during pregnancy and FAEEs in the infant's meconium.¹³ The FAEEs were identified and quantified in that study by gas chromatography by using a flame ionization detector (FID). With that methodology, the sensitivity to detect any reported maternal drinking during the second or third trimester was in the moderate range (66%–72%), with a specificity of 51%. The aim of this study was to analyze the specificity and sensitivity of meconium FAEEs for detection of alcohol use by using an improved detection technique, gas chromatography-tandem mass spectroscopy (GC/MS/MS), with data from a cohort of infants from Cape Town, South Africa.

METHODS

Participants

Mothers were recruited during pregnancy at an out-patient clinic that serves a predominantly Cape Coloured (mixed race) population to participate in a prospective study on the effects of heavy prenatal alcohol exposure on cognitive and behavioral development.^{14,15} The Cape Coloured are descendants of European, Malaysian, Khoi (Hottentot) aboriginal, and black African ancestors. Very heavy alcohol use is unusually prevalent among women in this community because of their very poor psychosocial and economic circumstances and the historically prevalent but now outlawed practice of paying Coloured workers on wine-producing grape and fruit farms with wine.^{16,17} This article reports the data on the first 27 infants in this cohort for whom meconium samples were collected.

Each mother was interviewed about her alcohol consumption at recruitment by using an interview derived from the timeline follow-back approach developed by Sokol et al.^{18,19} Antenatal care was initiated at a median gestational age of 19 weeks (range = 6–34); median gestational age at recruitment was 25 weeks (range = 8–37). Any woman who reported an average of at least 1.0 oz absolute alcohol (AA) (the equivalent of two standard drinks) per day or at least two incidents of binge drinking (≥ 5 standard drinks) per month during the first trimester of pregnancy was invited to participate in the study. The next woman initiating antenatal care whose gestational age was within 2 weeks of the heavy drinking mother was also invited to participate in the study provided that she drank fewer than seven drinks per week (.5 oz AA per day) and did not binge drink. Women <18 years and those with diabetes, epilepsy, or cardiac problems requiring treatment were not invited to participate. Religiously observant Muslim women were also excluded because their religious practices prohibit alcohol consumption. Infant exclusionary criteria were major chromosomal anomalies, neural tube defects, multiple births, and seizures.

Informed consent was obtained from each mother at the time of recruitment in accordance with the institutional review boards of the University of Witwaterstrand, Wayne State University, Case Western Reserve University, and the Centers for Disease Control and Prevention and in accordance with the Helsinki Declaration of 1975, as revised in 1983. Once the unusually high rates of alcoholism and FAS became evident, a home visitor intervention based on principles of motivational interviewing²⁰ was implemented in collaboration with the Parent Centre, a community-based parenting program run by Stephen Rollnick, PhD, and Mireille Landman, MA. Arrangements were made to refer mothers to an alcohol treatment facility affiliated with the Department of Obstetrics, University of Cape Town School of Medicine.

Procedure

Each mother was interviewed in Afrikaans regarding her alcohol and drug use at recruitment, at a follow-up antenatal visit, and when the infant was 1 month old. During recruitment, the mother was asked about her drinking on a day-by-day basis during a typical 2-week period around the time of conception, with recall linked to specific times of day and activities. She was then asked whether she continued her usual drinking pattern after

becoming pregnant and, if not, when her drinking had changed and what she drank on a day-by-day basis during the past 2 weeks. At the follow-up antenatal visit, the mother was again asked about her drinking during the previous 2 weeks. If there were any weeks since the recruitment visit when she drank greater quantities, she was asked to report her drinking for those weeks as well. At the 1-month postpartum visit, the mother was asked about her drinking during a typical 2-week period during the latter part of pregnancy, as well as her drinking during any weeks during that period when she drank greater quantities. Volume was recorded for each type of alcohol beverage consumed each day and converted to ounces of AA by using multipliers proposed by Bowman et al²¹ (liquor, .4; beer, .04; wine, .2). Three summary measures were constructed for each of five periods: conception; first, second, and third trimesters; and an overall average for the entire pregnancy. The summary measures of alcohol intake were overall average AA per day, ounces AA per drinking day (AADD, quantity per occasion), and frequency (drinking days per week).

Random meconium samples were scraped from the infant's diaper into falcon tubes (15 mL, polypropylene, Becton-Dickinson, Lexington, Ky) within 24 hours after delivery. The samples were refrigerated immediately or within a few hours and then frozen. Most (89%) were frozen at -19°C within 1 day after collection; three were frozen after 2 to 10 days. Toward the end of the data collection, all specimens were transferred to a -70°C freezer and maintained at -70°C until analysis. The length of time between collection and being frozen at -70°C ranged from 0 days to 13.4 months (interquartile range = 14–83 days). When the FAEE concentrations in the three meconium specimens that were frozen at -19°C more than 1 day after collection were compared with those that were frozen immediately, the mean level of one FAEE was lower but two others were higher, indicating that there was no general tendency for the FAEEs to deteriorate over several days of refrigeration. None of the FAEE concentrations were related to the length of time that passed between collection and storage at -70°C (all $P > .25$).

Meconium Analysis

Isolation of FAEEs from meconium was performed as previously described.²² Samples were initially analyzed by gas chromatography/flame ionization detection, which confirmed our earlier finding that the predominant FAEEs in meconium are ethyl palmitate, ethyl oleate, and ethyl linoleate. Samples were subsequently analyzed for these three FAEEs by GC/MS/MS by using a TSQ 7000 mass spectrometer (Thermo Finnigan, San Jose, Calif) interfaced to a Trace GC (Thermo Finnigan) using methane chemical ionization and low energy collisions with argon. Before analysis, ^{13}C -labeled analogues of the three FAEEs were added to the sample extracts to correct for instrument variation and to allow a more accurate quantitation. The two most prevalent fragments of ethyl palmitate, ethyl oleate, and ethyl linoleate were quantitated.

Statistical Analysis

Pearson correlation analyses were used to examine the relation of each of the FAEEs with three self-reported measures of alcohol consumption during pregnancy, as well as the relation of ethyl oleate to alcohol ingested per occasion at conception and during each trimester of pregnancy. A receiver operating characteristic (ROC) curve was constructed to

assess the efficiency of meconium ethyl oleate concentration in identifying mothers who drank at least 1.5 AADD during pregnancy.

RESULTS

The characteristics of the sample are shown in Table I. Seventeen of the women drank heavily during pregnancy (4 drinks per occasion; in most cases, 1–2 days per week), four drank heavily but less frequently (<3 days per month), and six abstained during pregnancy. All 27 samples of meconium contained ethyl linoleate, with 26 containing ethyl palmitate and 25 containing ethyl oleate. The most predominant FAEE was ethyl linoleate, with ethyl palmitate second and ethyl oleate third.

GC/MS/MS analysis yielded two prevalent ions for each FAEE. Because the intercorrelations between these ions for each FAEE were exceptionally high (r ranged from 0.99 to 1.00), subsequent analysis used only the first prevalent ion. The concentrations of these FAEEs in the meconium were highly correlated to each other (r ranged from 0.61 to 0.86, median = .79), particularly ethyl palmitate with ethyl oleate, both on a per wet weight and on a per dry weight basis.

To determine the measure of alcohol consumption most strongly correlated to FAEEs in meconium, FAEE concentrations were examined in relation to three measures averaged over pregnancy: average AA per day, average AADD, and number of drinking days per week (Table II). The highest correlations were found for AADD with ethyl oleate, whether measured on a per gram wet weight or dry weight basis. The rest of the analyses were therefore performed by using ethyl oleate as the biomarker and AADD as the measure of drinking.

Because meconium does not begin formation until the second trimester,²³ we predicted that maternal drinking in the second and third trimester would correlate most highly with the concentration of FAEEs in meconium. Correlations of ethyl oleate concentration to maternal self-reported AADD for the periods at conception, first trimester, second trimester, and third trimester were .29, .38, .52 ($P < .01$), and 0.42 ($P < .05$), respectively, based on wet weight, and 0.32, 0.42 ($P < .05$), 0.55 ($P < .01$), and 0.40 ($P < .05$) based on dry weight, as anticipated.

A scattergram of the relation of log ethyl oleate, micrograms per gram dry weight, with AADD averaged over pregnancy is shown in Figure 1. The general trend is for ethyl oleate concentration to increase with increasing amounts of reported drinking. By observation, higher values of ethyl oleate were seen only at 1.5 AADD or greater. Hence, further analyses were based on a level of 1.5 ounces of AADD.

To assess the efficiency of the meconium test to identify women reporting drinking 1.5 AADD or more, an ROC curve was constructed, as shown in Figure 2. The area under the curve was .92, which was highly significantly different than .5, with a 95% confidence interval of .80 to 1.00.

The positive and negative predictive values of the meconium assay to identify women drinking 1.5 or more AADD were calculated from the ROC curves and are shown in Table III. At a cut-off value of 13 ng/g dry weight ethyl oleate, sensitivity is 100%, specificity is 66.7%, positive predictive value is .91, and negative predictive value is 1.00. At a cut-off value of 77 ng/g dry weight ethyl oleate, sensitivity is 68.4%, specificity is 100%, positive predictive value is 1.00, and negative predictive value is .50.

DISCUSSION

This study evaluated the sensitivity and specificity of FAEE concentrations in meconium in detecting alcohol consumption during pregnancy by using GC/MS/MS technology. FAEEs in postmortem liver and pancreas have been used as a marker of premortem alcohol exposure.²⁴ FAEEs as biomarkers have also been investigated in blood and hair of adults^{25,26} and newborns.²⁷ In newborns, meconium has been used most frequently as the matrix to determine prenatal exposures to drugs such as cocaine.²⁸ There is a large amount of meconium; it is passed during the infant's initial hospitalization, and collection is therefore readily achieved.

The GC/MS/MS methodology identifies peaks by both retention time and mass weight, as compared with the less specific FID method used in our previous study, which identifies eluting peaks solely in terms of retention times. At a specificity of 83%, the sensitivity provided by this methodology was 84%, compared with only 70% to detect heavy drinking (14 drinks per week before pregnancy) using the FID methodology²⁹; at a specificity of 100%, sensitivity was 68%, compared with 52%. Moreover, whereas the FID assays were most strongly related to reported drinking before conception, possibly because of the very low levels of later pregnancy drinking in that cohort,²⁹ the GS/MS/MS assay results were most strongly related to second and third trimester drinking, the period during which the meconium forms in the fetus.

Increased concentrations of ethyl oleate were seen in meconium at 1.5 oz of alcohol (or three standard drinks) per drinking day or higher. Data from several US studies have suggested that five drinks per occasion is the principal level of concern for prenatal alcohol exposure, because it is the critical dose most commonly associated with adverse neurobehavioral outcome.^{30,31} Thus, the ROC analysis suggests that the meconium assay can detect alcohol exposure at levels below which neurobehavioral deficits are generally found. Alternatively, however, the mothers in this cohort were shorter and weighed less than pregnant drinking women in the US.³² It is possible that peak blood alcohol concentration after a three-drink binge in these smaller Cape Coloured women is equivalent to that of an average American woman after a five-drink binge.

The correlation of AADD to ethyl linoleate was not perfect. In some cases, mothers who reported little or no drinking had somewhat higher FAEE concentrations, possibly because of some underreporting of drinking behavior, which is socially stigmatized. In other cases, mothers who reported high levels of drinking had FAEE concentrations that were similar to those of mothers who reported substantially less drinking. The meconium specimens collected were randomly sampled. FAEEs may accumulate unevenly in meconium,

reflecting the time of exposure, and the samples collected may not have formed at the time when the reported drinking behavior occurred. In addition, the genetic polymorphisms in alcohol dehydrogenase and acetaldehyde dehydrogenase in the mother may influence the synthesis of FAEEs. It is also of note that among the six women who reported abstinence, five had detectable ethyl oleate concentrations in their infants' meconium. Because ethanol is a byproduct of human metabolism, some baseline level of FAEEs is to be expected. In addition, there are alternative sources of exposure to ethanol, including elixirs, certain over-the-counter medications, and food additives. Thus, by contrast with the metabolites of drugs of abuse, the mere presence of FAEEs in meconium does not indicate ingestion of alcoholic beverages. Although drugs of abuse have been shown not to influence FAEE levels,¹³ the degree to which maternal illness and medication use influence FAEE concentration has yet to be determined.

The finding that FAEE concentration in meconium is more strongly related to alcohol dose per occasion than to the overall average consumed per day is consistent with data from experimental animal studies. These studies have shown that ingestion of a given dose of alcohol over a short period generates a greater peak blood alcohol concentration and greater neuronal³³ and behavioral impairment³⁴ than when the same dose is ingested gradually over several days. Based on these data, a biomarker of number of drinks per occasion may be more predictive of FAS/ARND than a marker of weekly average alcohol consumption. The association of FAEEs in meconium with alcohol dose per occasion suggests that pregnant women may be able to metabolize a low dose of alcohol relatively rapidly and that FAEE metabolites will be detected in the meconium primarily when alcohol ingestion has exceeded a certain threshold, making the assay particularly useful for identifying women at risk.

The availability of a reliable biomarker of alcohol consumption during pregnancy represents a major advance in the diagnosis and treatment of FAS and ARND. The diagnosis of FAS is difficult because of the extensive training required to identify the relevant craniofacial features, and no reliable behavioral profile has emerged for diagnosing ARND. FAS diagnosis is particularly difficult in the newborn⁸; the facial features are most prominent in the school-age child. It is difficult to ascertain how much alcohol the mother has consumed during pregnancy because of the difficulty of recalling quantities and frequencies of alcohol intake.¹⁹ Moreover, the heavy stigma attached to pregnancy drinking makes obstetricians and midwives reluctant to inquire about drinking and pregnant women even more reluctant to reveal this information. The meconium assay can also facilitate the identification of alcohol-exposed infants, making it possible to intervene with these children earlier in development, when interventions are often most effective. Alcohol-exposed children have fewer secondary disabilities if diagnosed before age 6 years.³⁵ Laboratory experiments with prenatally exposed rats have recently demonstrated the efficacy of at least one form of early intervention,³⁶ suggesting that similar interventions may benefit human beings. A reliable biomarker for alcohol would also improve reliability of measurement in research designed to assess the effects of prenatal alcohol exposure and facilitate statistical control for alcohol exposure in studies of pregnancy smoking, illicit drug use, and other sources of developmental risk. The meconium assay is also potentially useful for evaluating the effectiveness of interventions designed to reduce maternal drinking during pregnancy

because it provides an objective criterion for evaluating the mother's compliance with the intervention protocol.

A major limitation of this study is the small sample size in a very distinct population. Although power was clearly adequate to detect a moderate relationship between prenatal alcohol exposure and meconium ethyl oleate concentration and to suggest impressive sensitivity and specificity, the small sample size renders the reliability of the cut-off derived from the ROC analysis uncertain. A replication study is planned on an additional 68 samples collected from this cohort, and additional replications with infants from other nationalities and ethnic groups will be needed to ascertain the most reliable and useful cut-offs.

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Glossary

AA	Ounces of absolute alcohol
AADD	Ounces of absolute alcohol per drinking day
ARND	Alcohol-related neurodevelopmental disorder
FAEE	Fatty acid ethyl ester
FAS	Fetal alcohol syndrome
FID	Flame ionization detector
GC/MS/MS	Gas chromatography-tandem mass spectroscopy
ROC	Receiver operating characteristic

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Fig 1.
Relation of drinks per occasion during pregnancy to ethyl oleate concentration (dry weight basis) in meconium. One ounce equals two standard drinks.

Fig 2. Receiver operating characteristic curve assessing the sensitivity and specificity of ethyl oleate concentrations in meconium for identifying women who ingested at least 1.5 oz AADD per day during pregnancy.

Table I

Sample characteristics

Characteristic	n	Mean or %	SD	Range
Maternal				
Age at delivery (y)	27	26.1	6.5	17.8–43.8
Education (y)	27	8.6	2.7	.0–12.0
Marital status (% married)	27	48.1	—	—
Parity	27	1.3	1.8	–8
Infant				
Gestational age (wk)	27	39.7	1.8	36.4–43.0
Birth weight (g)	27	2967.2	569.4	1500.0–4240.0
Head circumference (cm)	27	32.8	1.9	29.0–36.0
Sex (% male)	27	66.7	—	—
Self-reported alcohol consumption				
Average AA/d	27	0.9	1.5	.0–7.34
Average AA/drinking day	27	2.6	2.0	.0–7.5
Frequency (d/wk)	27	1.3	1.5	.0–6.7
FAEEs* of alcohol				
Ethyl palmitate	26	6.3	16.5	.0–62.3
Ethyl oleate	25	1.4	5.6	.0–27.7
Ethyl linoleate	27	9.2	25.1	.0–93.9

*
µg/g dry weight (most prevalent ion).

Table II

Correlations of self-reported alcohol consumption averaged over pregnancy with concentrations of FAEEs of alcohol in meconium

FAEE/g	N	AA/d	AADD	Frequency (d/wk)
Adjusted for wet weight				
Ethyl palmitate	26	.25	.34*	.29
Ethyl oleate	25	.34*	.48 [†]	.32
Ethyl linoleate	27	.26	.27	.29
Adjusted for dry weight				
Ethyl palmitate	26	.20	.35*	.24
Ethyl oleate	25	.29	.51 [‡]	.24
Ethyl linoleate	27	.21	.27	.24

* $P < .10$,

[†] $P < .05$,

[‡] $P < .01$.

Table III

ROC curve for ethyl oleate

Cut-off*	Sensitivity (%)	Specificity (%)	Positive predicted value	Negative predicted value
<.006	100.0	.0	.76	—
.006	100.0	16.7	.79	1.00
.009	100.0	33.3	.83	1.00
.012	100.0	50.0	.86	1.00
.013	100.0	66.7	.91	1.00
.016	94.7	66.7	.90	.80
.020	89.5	66.7	.90	.67
.025	84.2	66.7	.89	.57
.032	84.2	83.3	.94	.63
.034	78.9	83.3	.94	.56
.052	73.7	83.3	.93	.50
.061	68.4	83.3	.93	.46
.077	68.4	100.0	1.00	.50
.122	63.2	100.0	1.00	.46
.131	57.9	100.0	1.00	.43
.132	52.6	100.0	1.00	.40
.155	47.4	100.0	1.00	.38
.233	42.1	100.0	1.00	.35
.283	36.8	100.0	1.00	.33
.349	31.6	100.0	1.00	.32
.750	26.3	100.0	1.00	.30
.788	21.1	100.0	1.00	.29
.812	15.8	100.0	1.00	.27
1.778	10.5	100.0	1.00	.26
2.468	5.3	100.0	1.00	.25
27.657	.0	100.0	—	.24

*
µg/g dry weight.