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LINEAR VERSUS NON-LINEAR DOSE-RESPONSE RELATIONSHIP BETWEEN PRENATAL ALCOHOL EXPOSURE AND MECONIUM CONCENTRATION OF NINE DIFFERENT FATTY ACID ETHYL ESTERS

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□ Presence of individual fatty acid ethyl esters (FAEEs) in meconium is considered to be a reliable biomarker of prenatal alcohol exposure, and their concentration has been found to be linearly associated with poor postnatal development, supporting the widely extended idea that ethanol is a non-threshold teratogen. However, a growing number of epidemiological studies have consistently found a lack of adverse short- and long-term fetal outcomes at low exposure levels. We therefore aimed to investigate the relationship between the concentration of individual FAEEs and prenatal alcohol exposure in meconium samples collected within the first 6 to 12?h after birth from 182 babies born to abstainer mothers and from 54 babies born to women who self-reported either light or moderate alcohol ingestion in the second or third trimester of pregnancy. In most cases, the individual FAEE concentrations were negligible and not significantly different ($P > 0.05$) between exposed and control babies. The concentrations appeared to increase linearly with the dose only in the few babies born to mothers who reported >3 drinks/week. These results provide evidence that the correlation between prenatal alcohol exposure and individual FAEE concentrations in meconium is non-linear shape, with a threshold probably at 3 drinks/week.

Keywords: Biochemical markers, Prenatal alcohol exposure, Pregnancy outcomes, Threshold level

INTRODUCTION

The association between prenatal exposure to risky levels of ethanol (alcohol) and the increased risk of devastating developmental consequences in the exposed offspring is unquestionable. It is therefore comprehensible that there is an intensive search for a reliable biomarker that

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can help to identify those babies who were prenatally exposed to such risky levels. Of the potential candidates, the presence of fatty acid ethyl esters (FAEEs) in meconium, particularly ethyl linoleate and ethyl oleate, has been proposed to be reliable biomarkers of prenatal alcohol exposure (PAE) (Bearer *et al.* 1999, 2003, 2005).

Since ethanol is widely considered a non-threshold teratogen, it is not surprising that the increasing meconium concentrations of ethyl myristate, ethyl oleate, ethyl linoleate, ethyl linolenate, and ethyl arachidonate, individually, were found to be linearly related to poorer neurodevelopmental outcomes at the age of 2 years among the in utero exposed offspring (Peterson *et al.* 2008). In contrast, a growing number of epidemiological studies have consistently found a lack of short- and long-term adverse maternal and fetal outcomes when exposure occurred at low or very low levels (Kelly *et al.* 2013, Han *et al.* 2012, O'Leary *et al.* 2013, Robinson *et al.* 2010). Aligned with these findings, our group recently reported a study where the sum of nine different FAEEs was found to be an unreliable composed biomarker of low prenatal alcohol exposure (Kwak *et al.* 2014a). However, the possibility that at least one of the FAEEs remained linearly correlated to the intensity of alcohol ingestion was not explored.

Therefore, the present study aimed to investigate if the relationship between the concentration of nine individual FAEEs and prenatal alcohol exposure was linear or nonlinear at low dose levels.

METHODS

Participants

The participants included in the present study were part of a larger prospective cohort study of Korean women previously described elsewhere (Kwak *et al.* 2014a). The study was approved by the institutional review board at the Cheil General Hospital and Women's Healthcare Center, Seoul, Republic of Korea, and was conducted in compliance of the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects.

Briefly, we invited 410 consecutive singleton pregnant women to participate in the study of FAEEs in meconium as biomarkers of PAE. Participants self-reporting alcohol exposure during pregnancy had to be exposed specifically during either the first, second or third trimester of pregnancy. They also had to be receiving prenatal obstetric care at this hospital, and be scheduled to deliver vaginally. Invitation to participate in the study, recruitment and collection of the information on PAE were performed during the prenatal assessment at 34 weeks of gestation. Pregnant women reporting ethanol consumption within the 72 hours prior to the study assessment, suffering from psychiatric problems, or physically or

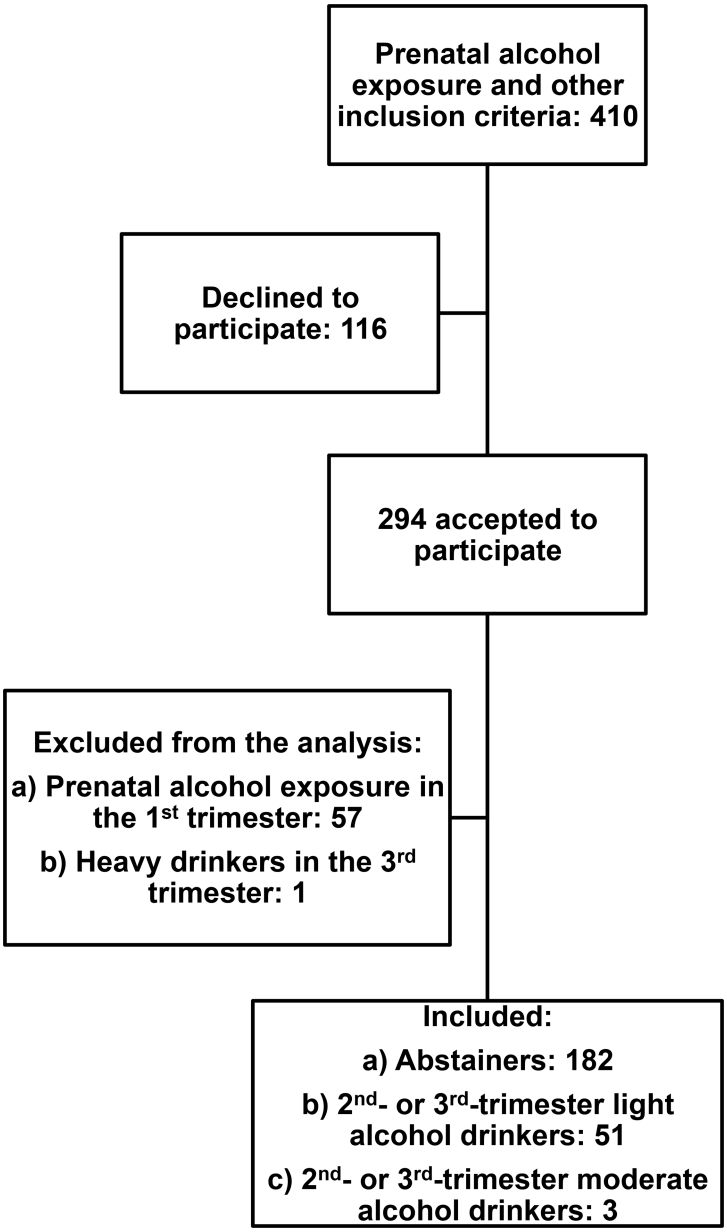

FIGURE 1. Study flow chart.

cognitively impaired, were not considered as eligible since these factors have been shown to decrease the accuracy of self-reports (Connors and Volk 2003).

Of the invited participants, 294 consented to participate in the study. Of them, 182 were abstainers and 54 self-reported light or moderate alcohol ingestion in either the second or third trimester of pregnancy

(Figure!1). PAE was classified as light if participants self-reported the ingestion of <3 drinks/week (average) during the exposure period, and moderate if 3-7 drinks/week (1 standard drink= 0.6 ounces, or 14 g of ethanol). These grouping criteria were based on the categories described by the United States National Institute on Alcohol Abuse and Alcoholism (US NIAAA 2010), which were slightly modified by our group in order to avoid the overlapping between the maximum number of drinks in the light exposure group and the minimum in the moderate exposure group (Kwak et al. 2014b).

Details of PAE were collected on an electronic form especially designed for the purpose of the study. As previously suggested elsewhere by Connors and Volk (2003), in order to minimize response bias and maximize validity of self-reports, properly trained investigators using electronic files collected the information and stored it immediately in a password-protected system with restricted-access privileges.

Individual FAEEs in meconium

Quantification of FAEEs in meconium was performed by a liquid chromatography/tandem mass spectrometry (LC-MS/MS) method previously described in detail elsewhere by our group (Kwak *et al.* 2010). The nine FAEEs quantified included ethyl laurate (E12:0), ethyl myristate (E14:0), ethyl palmitate (E16:0), ethyl palmitoleate (E16:1), ethyl stearate (E18:0), ethyl oleate (E18:1), ethyl linoleate (E18:2), ethyl linolenate (E18:3), and ethyl arachidonate (E20:4). The limit of detection and quantification varied from 0.01 to 0.08 nmol/g and from 0.02 to 0.27 nmol/g, respectively. The intra- and interassay precision varied from 7 to 21% and from 10 to 17%, respectively. The intra- and inter-assay accuracy ranged from -17 to 15% and from -4 to 14%, respectively.

Data analysis

The age of participants in each group was summarized as mean ± SD and compared between the two study groups by means of an unpaired Student t test. Gravidity, parity and subjects concomitantly reporting cigarette smoking were reported as counts and percentages, and compared between groups by means of Fisher's exact test.

In relation to the intensity of PAE, four characteristics were collected and summarized as mean ± SD: average of drinks ingested per week (drinks/week), average of drinks ingested per occasion (drinks/occasion), average of days drinking per week (days drinking/week), and times binge drinking. Individual FAEE concentrations in each study group were summarized using median values followed by the 25%-75% quartiles, and the minimum and maximum values, and compared among groups by means of the Mann-Whitney U test.

In order to characterize how well the concentration of individual FAEEs in meconium could differentiate abstainers from women with PAE, we conducted a receiver operating characteristic analysis and computed the area under the curve (AUC) and the sensitivity and specificity values. In order to investigate a dose-response relationship between FAEE concentrations and each of the three study indicators of PAE, we conducted linear regression analyzes between the meconium concentrations of each FAEE and either drinks/week, days drinking/week, and drinks/occasion. In those cases where the correlation was statistically significant, we added the FAEE concentrations from the abstainer group and repeated the linear regression analysis. This procedure simulated a scenario where the history of PAE is unknown or unreliable.

Finally, in order to qualitatively assess the presence of potential outliers that could be affecting the linear correlation analyzes, we conducted visual inspections of the linear regressions plotted with SigmaPlot v. 10.0 (Systat Software Inc., Chicago, IL, USA). All the statistical analyzes were performed using SPSS v. 18 (IBM, Chicago, IL, USA), and a two-tailed $P<0.05$ was considered as the significance limit for each of the different tests.

RESULTS

Demographic data and baseline characteristics

Age of participants self-reporting second-third trimester, light-to-moderate PAE was 32.3 years in average, and was not significantly different from the control group (Table!1). Similarly, gravidity, parity, and the proportion of participants who reported positive cigarette smoking during pregnancy were not statistically different between the two study groups.

TABLE 1. Demographic and baseline characteristics of participants

	Abstainers (n= 182)	Drinkers (n= 54)	P-value
Age (years; mean ± SD)	32.3 ± 3.7	32.0 ± 4.2	0.55[a]
Gravidity [n; median (range)]	2 (1-6)	2 (1-8)	0.083[a]
Parity [n; median (range)]	0 (0-3)	1 (0-2)	0.087[a]
Smoking during pregnancy [n(%)]	1 (0.5%)	2 (3.7%)	0.13[b]
Intensity of ethanol ingestion during pregnancy			
Drinks/week [median (range)]	-	0.5 (0.05-8.0)	-
Drinks/occasion [median (range)]	-	1.0 (0.5-4.0)	-
Days drinking/week [median (range)]	-	0.25 (0.1-3.0)	-
Binge drinking[c] [n(%)]	-	2 (3.7%)	-

[a] Mann-Whitney U test
[b] Fisher's exact test
[c] ≥4 alcoholic drinks/occasion

Meconium concentration of individual FAEEs

The meconium concentrations of each of the nine FAEEs were, in general, at negligible levels (Table!2). No significant differences were detected when comparing the FAEE concentrations in babies born from exposed women to those in babies born from abstainers. For example, the median (and 25th and 75th quartiles) of the meconium concentration of ethyl laurate was <LOQ (<LOQ-0.23) in the abstainer group, and 0.02 (<LOQ-0.23) in the exposed group (P= 0.45).

Receiver operating characteristic analysis

The largest AUCs were obtained with ethyl arachidonate followed by ethyl myristate and ethyl palmitate, i.e. 0.57, 0.53 and 0.52, respectively (Table!3, and Figure!2). However, none of them reached a significant level (P >0.05), and therefore no further analysis was conducted to identify a cutoff concentration for discriminating between positive (low-to-moderate) and negative PAE.

Dose-response analysis

In the dose-response analysis, drinks/week was consistently correlated with meconium concentrations of each of the nine FAEEs, with correlation coefficients >0.70 observed with ethyl laurate, ethyl myristate, ethyl oleate, ethyl linoleate, and ethyl linolenate (Table!4). Adding data from abstainers to the linear regression analyzes consistently decreased the coefficients of correlations, although this procedure improved the *P* value of the correlations. The only exception was observed with ethyl arachido-

TABLE 2. FAEE concentrations (nmol/g) in in the two study groups.

	Self-reported drinking behaviour			
FAEEs	Abstainers n= 182	Drinkers n= 54	95%CI for the difference	*P* value*
Ethyl laurate	<LOQ (<LOQ-0.23) [<LOQ-2.11]	0.02 (<LOQ-0.23) [<LOQ-13.4]	-1.15 to 0.21	0.45
Ethyl myristate	0.02 (<LOQ-0.31) [<LOQ-5.21]	0.01 (<LOQ-0.25) [<LOQ-17.5]	-0.94 to 0.39	0.54
Ethyl palmitate	0.02 (<LOQ-0.29) [<LOQ-9.71]	0.02 (<LOQ-0.19) [<LOQ-11.0]	-0.35 to 0.35	0.61
Ethyl palmitoleate	<LOQ (<LOQ-0.19) [<LOQ-3.83]	<LOQ (<LOQ-0.18) [<LOQ-1.74]	-1.00 to 0.20	0.95
Ethyl stearate	0.01 (<LOQ-0.22) [<LOQ-3.99]	0.02 (<LOQ-0.22) [<LOQ-5.03]	-0.20 to 0.09	0.88
Ethyl oleate	0.04 (<LOQ-0.32) (<LOQ-9.53)	0.04 (<LOQ-0.24) [<LOQ-28.7]	-1.62 to 0.65	0.80
Ethyl linoleate	0.03 (<LOQ-0.14) [<LOQ-6.20]	0.01 (<LOQ-0.16) [<LOQ-30.6]	-1.75 to 0.60	0.83
Ethyl linolenate	<LOQ (<LOQ-0.03) [<LOQ-3.12]	<LOQ (<LOQ-0.01) [<LOQ-10.2]	-0.60 to 0.19	0.88
Ethyl arachidonate	0.01 (<LOQ-0.15) [<LOQ-2.33]	<LOQ (<LOQ-0.09) [<LOQ-0.47]	-0.01 to 0.15	0.60

*Mann-Whitney U test.

Concentrations are summarized as median together with the 25th and 75th quartiles in parenthesis, and the minimum and maximum values in square brackets. Alcohol exposure among drinkers was classified as light or moderate and occurred in the 2nd or 3rd trimester of gestation.

TABLE 3. Receiver operating characteristic analysis of FAEEs in meconium

FAEEs analyzed	AUC	95% CI	*P* value
Ethyl laurate	0.47	0.78 – 0.56	0.48
Ethyl myristate	0.53	0.44 – 0.61	0.56
Ethyl palmitate	0.52	0.44 – 0.61	0.63
Ethyl palmitoleate	0.50	0.41 – 0.59	0.96
Ethyl stearate	0.49	0.41 – 0.58	0.89
Ethyl oleate	0.51	0.43 – 0.60	0.81
Ethyl linoleate	0.51	0.42 – 0.60	0.84
Ethyl linolenate	0.51	0.42 – 0.59	0.90
Ethyl arachidonate	0.57	0.48 – 0.65	0.12

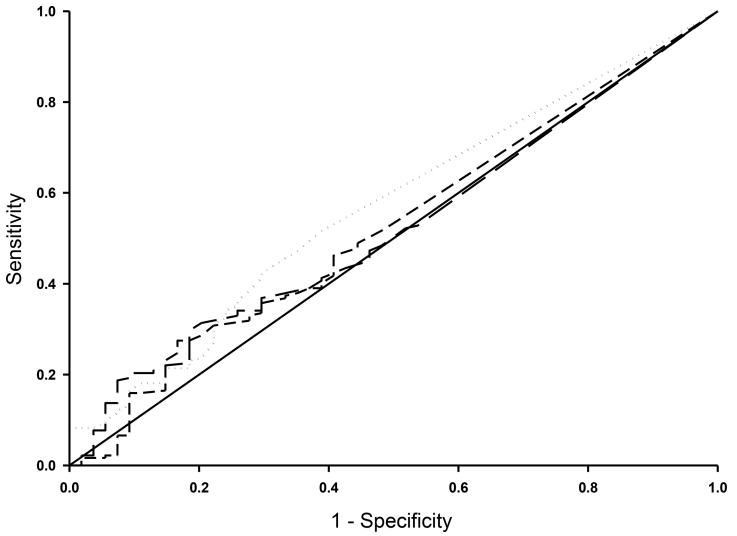

FIGURE 2. ROC curves for ethyl arachidonate (dotted line), ethyl myristate (short-dash line), and ethyl palmitate (long-dash line). The AUCs were 0.57, 0.53, and 0.52, respectively.

nate, which was marginally (P= 0.05) correlated to drinks/week in babies born to women who self-reported PAE, and the correlation coefficient dropped close to zero when data from abstainers were included.

Visual inspection of the linear regression plots

Since drinks/week consistently correlated with meconium concentrations of the nine FAEEs studied, we conducted a visual inspection of the linear regression plots constructed for this indicator of PAE. During this process, we identified two FAEE concentrations that had been collected from babies born to mothers exposed to >3 drinks/week, and that were clearly higher than the rest of the data set. These FAEE concentrations clearly influenced the correlation coefficients, as illustrated with the meconium concentrations of ethyl oleate in Figure 3.

TABLE 4. Pearson's correlation analysis between the study indicators of prenatal alcohol exposure and FAEEs in meconium

	Drinkers only			Drinkers and abstainers		
FAEEs analyzed	Linear equation (mx +b)	r value (95% CI)	P value	Linear equation (mx +b)	r value (95% CI)	P value
Ethyl laurate	1.85 (±0.21) x_1 - 0.88	0.77 (0.64;0.86)	<0.001	1.43 (±0.10) x_1 + 0.01	0.70 (0.63;0.76)	<0.001
	1.80 (±0.50) x_2 - 0.36	0.45 (0.21;0.64)	<0.001	1.44 (±0.19) x_2 + 0.10	0.44 (0.33;0.54)	<0.001
	1.47 (±0.44) x_3 - 1.47	0.42 (0.17;0.62)	0.002	0.60 (±0.11) x_3 + 0.08	0.34 (0.22;0.45)	<0.001
Ethyl myristate	1.69 (±0.22) x_1 - 0.84	0.73 (0.57;0.83)	<0.001	1.24 (±0.11) x_1 + 0.11	0.58 (0.49;0.66)	<0.001
	2.10 (±0.46) x_2 - 0.61	0.54 (0.32;0.70)	<0.001	1.48 (±0.20) x_2 + 0.16	0.43 (0.32;0.53)	<0.001
Ethyl palmitate	0.99 (±0.15) x_1 - 0.43	0.67 (0.48;0.79)	<0.001	0.67 (±0.12) x_1 + 0.26	0.35 (0.24;0.46)	<0.001
	1.26 (±0.30) x_2 - 0.31	0.50 (0.27;0.68)	<0.001	0.78 (±0.19) x_2 + 0.29	0.26 (0.13;0.37)	<0.001
Ethyl stearate	0.46 (±0.07) x_1 - 0.17	0.68 (0.50;0.80)	<0.001	0.33 (±0.05) x_1 + 0.10	0.43 (0.31;0.52)	<0.001
	0.60 (±0.13) x_2 -0.13	0.53 (0.31;0.70)	<0.001	0.41 (±0.08) x_2 + 0.11	0.33 (0.21;0.44)	<0.001
Ethyl oleate	3.09 (±0.34) x_1 - 1.56	0.78 (0.65;0.87)	<0.001	2.23 (±0.19) x_1 + 0.18	0.62 (0.53;0.69)	<0.001
	3.58 (±0.78) x_2 - 1.01	0.54 (0.32;0.71)	<0.001	2.54 (±0.35) x_2 + 0.28	0.43 (0.32;0.53)	<0.001
	1.70 (±0.76) x_3 - 1.46	0.30 (0.03;0.52)	0.03	0.67 (±0.21) x_3 + 0.39	0.21 (0.08;0.33)	0.001
Ethyl linoleate	3.15 (±0.36) x_1 -1.72	0.77 (0.63;0.86)	<0.001	2.34 (±0.18) x_1 - 0.014	0.65 (0.57;0.72)	<0.001
	3.78 (±0.80) x_2 - 1.23	0.55 (0.33;0.71)	<0.001	2.72 (±0.33) x_2 + 0.082	0.47 (0.36;0.56)	<0.001
Ethyl linolenate	1.07 (±0.12) x_1 - 0.60	0.78 (0.64;0.87)	<0.001	0.80 (±0.06) x_1 - 0.030	0.67 (0.59;0.73)	<0.001
	1.28 (±0.27) x_2 - 0.43	0.55 (0.33;0.71)	<0.001	0.93 (±0.11) x_2 + 0.003	0.48 (0.34;0.57)	<0.001
Ethyl arachidonate	0.033 (±0.02) x_1 + 0.045	0.27 (-0.002;0.50)	0.05	-	-0.03	0.59

m= slope; b= intercept; r= the Pearson's correlation coefficient; x_1= drinks/week; x_2= days drinking/week; x_3= drinks/occasion

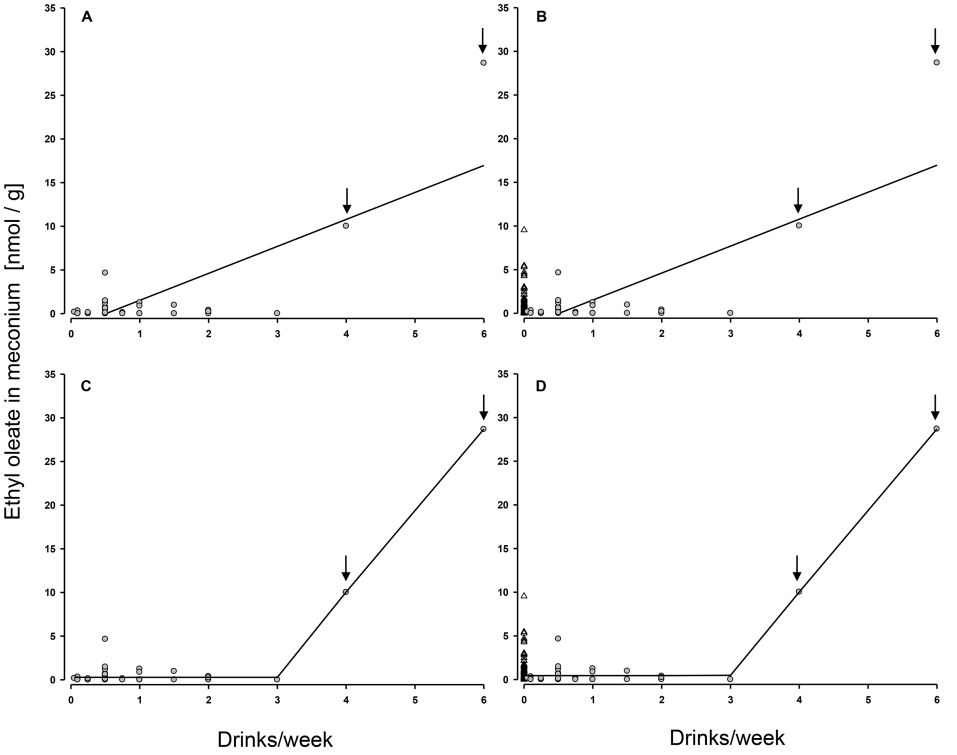

FIGURE 3. Linear correlation analysis between the concentration of ethyl oleate in meconium from babies born to women with PAE (circles) was slightly modified when data from abstainers (triangles) were added (panels A and B, respectively). The FAEE concentrations in meconium from two babies born to participants with history of PAE >3 drinks/week (arrows), suggest that the FAEEs could be more useful to identify moderate or heavy PAE independently on whether only exposed cases are analyzed or controls are also included (panels C and D, respectively).

DISCUSSION AND CONCLUSIONS

In the present study, we have evaluated the relationship between three indicators of light-to-moderate PAE in the second or third trimester of pregnancy (i.e. drinks ingested/week, drinks ingested/occasion, and days drinking/week), and the concentration of nine different FAEEs in meconium. In most cases, the concentrations were at negligible levels. Human placenta extensive hydrolyzes FAEEs, and therefore their presence in meconium is considered to be exclusively produced by non-oxidative metabolism of alcohol in the fetus (Chan *et al.* 2004). Furthermore, once FAEEs are formed and deposited in the meconium, their concentration is expected to be stable since this biological matrix lacks of the enzymatic machinery to produce or metabolize them. Therefore, our results suggest that ingestion of low-to-moderate amounts of alcohol during pregnancy is not enough to trigger the biochemical formation of FAEEs at quantifiable levels in the fetus. This would add biochemical support to the epidemiological studies showing no neurodevelopmental consequences in the

offspring of mothers with light-to-moderate PAE (Kelly *et al.* 2013, Han *et al.* 2012, O'Leary *et al.* 2013, Robinson *et al.* 2010).

There is an additional quandary on the use of individual FAEEs in meconium as biomarkers of prenatal alcohol exposure: their concentrations may reach considerable levels and exhibit large variability across different populations of abstainer women. For example, although in our study the median concentration of ethyl linoleate in babies born to alcohol-abstainer Korean women was 0.03 (range: <LOQ to 6.20) nmol/g, this was approximately 0.31 (range: <LOQ to 4.8) nmol/g in Jordan babies born to strict alcohol-abstainer women, and approximately 0.38 (range: <LOQ-250) nmol/g in a group of babies born to African-American women living in Cleveland, Ohio (Bearer *et al.* 2005).

Under anaerobic conditions, metabolism of carbohydrates by intestinal microflora produces acetaldehyde, which can be further reduced to ethanol. As reviewed elsewhere by Spruss and Bergheim (2009), this metabolic fate of carbohydrates is favored when there is intestinal overgrowth of bacteria or yeast, or if carbohydrates are consumed excessively. This may result into measurable blood alcohol levels which, in a few rare circumstances, may reach abnormally high concentrations (Ostrovsky *et al.* 1989, Logan and Jones 2000, Spinucci *et al.* 2006). A mild increment of endogenous blood alcohol concentrations, which did not correlate with blood glucose values, has also been reported in diabetic subjects (Simic *et al.* 2012).

It is very difficult to know whether any of these factors played a relevant role in the marginally elevated individual FAEE concentrations in meconium observed in some unexposed babies in the present study, or in the much higher FAEE concentrations reported by Bearer *et al.* (2005) in Jordan and Cleveland control babies. However, the possibilities that the individual FAEEs are found at elevated concentrations jeopardizes their use at a population level, especially if history of PAE is absent or unreliable.

Another interesting result in the present study is that only two cases had relevant concentrations of individual FAEEs, which corresponded to two babies born from mothers exposed to >3 drinks/week (Figure 3). The data points were distant enough from the cluster of negative data that produced statistically significant linear relationships between individual FAEE concentrations and drinks/week, drinking days/week and, in a lesser extent, drinks/occasion. The stronger correlations were observed between drinks/week and the meconium concentrations of ethyl laurate, ethyl myristate, ethyl oleate, ethyl linoleate, and ethyl linolenate, individually. If our conclusions were based on the results generated by the linear regression analyses, we would have considered that our results were in agreement with other studies. For example, Bearer *et al.* (1999, 2003, 2005) found that ethyl linolate and ethyl oleate, separately, were linearly

related to PAE, and therefore considered them as reliable biomarkers of this type of exposure.

However, in our study, the two individual FAEEs from babies born from mothers exposed to >3 drinks/week were not enough to affect the receiver operating characteristic analysis, which did not identify any AUC at a significant level. This analysis together with the visual inspection of the plots clearly suggest that the evaluation of any biomarker of PAE should be conducted under different perspectives, especially if a false positive test might represent an enormous social and legal burden, such as those faced by pregnant women who are mislabeled as alcohol drinkers.

In summary, the present study provides evidence that the correlation between prenatal alcohol exposure and individual FAEE concentrations in meconium is non-linear, with a threshold probably at 3 drinks/week. Our results also emphasize the need for a thorough characterization of any biomarker that is intended to be used as a diagnostic test of women with PAE in order to avoid misrepresenting their exposure.

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