Published in final edited form as:

J Pediatr. 2005 June ; 146(6): 824–830. doi:10.1016/j.jpeds.2005.01.048.

FATTY ACID ETHYL ESTERS: QUANTITATIVE BIOMARKERS FOR MATERNAL ALCOHOL CONSUMPTION

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Abstract

Objective—To develop a laboratory marker to identify newborns exposed to alcohol.

Study design—Meconium was collected from 30 infants from Jordan who were unexposed and from 248 Cleveland study infants of varying exposure status. Retrospective maternal alcohol histories were obtained. Fatty acid ethyl esters (FAEEs) were quantified with gas chromatography/flame ionization and compared between abstainers and non-abstainers to identify FAEEs of interest. The area under the receiver operating characteristic curve, sensitivity, specificity, and positive and negative predictive values were calculated by using definitions of drinking obtained from a graphical representation.

Results—Six of 7 FAEEs were significantly different between the non-abstainers and at least 1 of 2 of the abstaining groups. FAEEs best predicted drinks per drinking day, and ethyl linoleate had the greatest area under the curve (76%), with a sensitivity rate of 88%, a specificity rate of 64%, a positive predictive value of 9%, and a negative predictive value of 99%. No combination of FAEEs was better than a single ester for identifying drinkers.

Conclusion—Ethyl linoleate in meconium is a useful biological marker for identifying infants not exposed in utero to high levels of alcohol in a high-risk, substance-abusing, clinic-based sample.

Heavy drinking during pregnancy is the cause of fetal alcohol syndrome (FAS), the leading known cause of mental retardation.¹ Conservative estimates place the incidence of FAS at 0.33 of 1000 live births.¹ More prevalent are infants with a spectrum of outcomes, including alcohol-related birth defects, alcohol-related neurodevelopmental defects, and subtle effects on a variety of behavioral, educational, and psychological tests, resulting from low to moderate levels of drinking during pregnancy. Together, these effects are estimated to be present in 1% of all newborns² and to cost from \$75 million³ to \$9.7 billion per year.⁴

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Cynthia F. Bearer, MD, PhD, currently holds a patent for the measurement of fatty acid ethyl ester in meconium. Currently, there are no business relationships based on this patent.

There is a lack of clinical tools for assessing levels of drinking in pregnant women and identifying newborns who were exposed to alcohol. In one study, the diagnosis of FAS was missed in 100% of newborns in whom FAS was subsequently diagnosed in childhood.⁵ Identification of affected newborns is desirable to facilitate early intervention, minimize secondary disabilities,⁶ and identify mothers at high risk for drinking during gestation. Recent research has focused on developing biomarkers to identify maternal drinking during pregnancy. Maternal biomarkers showing promise include a combination of 4 maternal blood measurements: hemoglobin acetaldehyde adducts, gamma glutaryl transferase, mean corpuscular volume, and carbohydrate deficient transferrin.⁷

For identification of exposed neonates, fatty acid ethyl esters (FAEEs), non-oxidative metabolites of ethanol, in meconium are being investigated. In adults, FAEEs have been tested as a biological marker for alcohol-related fatalities.⁸ Initially described in the cord blood of an infant born to an alcoholic mother,⁹ FAEEs were subsequently identified in meconium.¹⁰ In our initial study of a Cleveland population, mothers of infants whose meconium contained ethyl linoleate reported drinking an average of 10 drinks per week in the month before pregnancy.¹¹ The amount of reported drinking (3 drinks/week) by mothers whose infants did not have ethyl linoleate in their meconium was significantly different.¹¹ Four-fold higher levels of FAEEs were found in the meconium of an infant born to an alcoholic compared with that of a control infant.¹² FAEEs are also found in the meconium of infants born to mothers from abstaining populations.¹³ Thus, there is a need to quantify FAEEs to determine the level of alcohol exposure. With a highly alcohol exposed mixed race/Cape Colored population from South Africa, the sensitivity and specificity rates of ethyl oleate for detecting women who drank an average of 3 or more drinks per drinking day was 84.2% and 83.3%, respectively.¹⁴ These results prompted a quantitative reanalysis of our previous work in a Cleveland population.¹² With gas chromatography/flame ionization detection (GC/FID), we quantified the FAEEs from the meconium of the Cleveland population¹¹ and a comparison Jordan population to further investigate the relationship between FAEE quantity and maternal self-reported drinking. In addition, we investigated the clinical usefulness of this biomarker.

METHODS

Cleveland Study Subjects

Postpartum women were recruited from a large urban, teaching hospital in Cleveland, Ohio, to participate in a 2-year longitudinal study on the neurobehavioral effects of prenatal cocaine exposure.^{15–18} Women were predominantly African-American (83%), of low socioeconomic status (99%), and identified from a population screened for risk of substance abuse during pregnancy. Informed consent was obtained as approved by the institutional review board of MetroHealth Medical Center.

Jordan Comparison Subjects

Postpartum Muslim women and their infants were recruited from a large urban hospital in Amman, Jordan. The meconium from Jordan was expected to have fewer false negative

results than abstainers from the Cleveland study group because there is a strong religious, cultural, and societal prohibitive influence against drinking by Jordanian Muslim women.

Meconium Collection

A total of 275 samples of meconium were obtained from 248 study infants in Cleveland and 30 samples from 30 comparison infants in Jordan. Meconium stool from each infant was scraped from the diaper, collected into falcon tubes (15 mL, polypropylene, Becton-Dickinson), and frozen at -80°C until analysis.

FAEE Analysis

The analyses were performed by investigators who were blinded to the questionnaire results. A total of 1 g wet weight of meconium was used for analysis. An a priori exclusion criteria was constructed to ensure the best representation of each meconium sample (Figure 1). For samples <1 g (83 samples), the whole sample was used. Samples <0.5 g (38 samples) were excluded. The analysis of FAEEs has been previously described.^{11,19} In brief, 100 μL of 1 mM/L ethyl heptadecanoate was added to each meconium sample as an internal standard. Only samples with ethyl heptadecanoate percent recovery $\geq 50\%$ were analyzed (230 samples). FAEEs were extracted with acetone/hexane and isolated with silica column chromatography. The isolated FAEEs were identified and quantified with gas chromatography using a flame ionization detector (HP5890 Series II). Peak areas were obtained by integration with HP Chemstation software and valley to valley baseline. Peaks with an area ≥ 500 and with retention times ± 0.1 minutes of authentic standards were used in the analysis. FAEEs from 10 samples were confirmed with gas chromatography with mass spectroscopy detection (GC/MS). The limit of detection was 2 pmole of ethyl heptadecanoate on column. For individuals with >1 sample (14 individuals), the sample with greater weight and higher recovery was selected. Of these 216 samples, 22 had no maternal interview data. All the Jordanian samples were 1 g and had recovery $\geq 50\%$. The remaining 194 samples from Cleveland and all 30 samples from Jordan were used for further analyses (Figure 1).

Maternal Prenatal Substance Abuse Assessment

The maternal postpartum interview^{20–22} was used to estimate maternal alcohol use during the month before and for each trimester of pregnancy. For each period, mothers were requested to recall both the amount and frequency of alcohol use, and 3 drinking measures of alcohol intake were calculated: (1) number of drinks per drinking day (DRDD), (2) number of drinking days per week (DYWK), and (3) number of drinks per week (DRWK). To estimate the amount of alcohol used, the DRDD was computed on the basis of the amount of beer, wine, or hard liquor consumed, with 12 oz of beer, 4 oz of wine, or 1 oz of hard liquor assumed to be equivalent to 1 drink (0.5 ounces of absolute alcohol). DYWK was estimated on a Likert-type scale, ranging from 0 (less than once per month) to 7 (daily use). The DRWK was calculated by multiplying DRDD by DYWK. The postpartum interview was conducted as soon as possible after the birth of the child, generally within 1 month.

Statistical Analysis

The purpose of the analyses was to develop a clinically useful tool to aid in screening infants for prenatal alcohol exposure. Analyses were carried out first to eliminate any FAEEs that lacked the potential to be predictive of alcohol intake, second to determine by a principal component analysis whether some combination of FAEEs reduced noise and redundancy, third to define subjects as “drinkers” or “non-drinkers” on the basis of each of the 3 measures of alcohol intake, and fourth to use these definitions to estimate area under the receiver operating characteristics curve (AUC), sensitivity rate, specificity rate, and positive (PPV) and negative (NPV) predictive values.

Abstainers were strictly classified as Cleveland mothers who denied using alcohol, cocaine, tobacco or marijuana ($n = 25$) during the month before and during pregnancy. The remainder were classified as non-abstainers ($n = 169$). Subjects in the non-abstainer group reported the use of any drug, but not necessarily alcohol. This effectively created an internal comparison group, in addition to the external (Jordan) comparison group (Figure 1). This conservative approach to classification of abstainers and non-abstainers makes it less likely that we will be able to find significant associations, because undoubtedly our non-abstaining group includes some abstainers. There was no significant difference in the proportion of African American mothers in the 2 groups (79% non-abstainers vs 92% abstainers, $P = .18$ with Fisher exact test). The alcohol use of the non-abstainers is summarized in Table I.

To screen for the most predictive FAEE(s), the means of the $\log_{10}$ transformed FAEE concentrations were compared with 2 sample t tests to compare Jordan control subjects, Cleveland abstainers, and Cleveland non-abstainers. The FAEE(s) with significant differences at a level of $P < .05$ between the Cleveland non-abstainers and at least 1 of the comparison groups were selected for further analysis (Table II).

Second, a principal component analysis with only the FAEEs with a significant difference between groups showed that only 1 linear combination of the FAEEs explained 91% of the variance of these highly correlated measures. A score for each subject was calculated by using this linear combination.

Third, each measure of alcohol intake (X-axis) was plotted against $\log(\text{ng/g}+100)$ FAEE (Y-axis) for each period. On the basis of the graphical representation of the data, subjects were defined as “drinkers” or “non-drinkers” for the respective measure. Figure 2 is an example of such a graphical representation with DRDD as the drinking measure. As can be seen, defining drinking as ≥ 7 drinks per drinking day (solid line) allows a cutoff value of ethyl linoleate (dotted line) in which only 1 subject is misclassified. With these definitions, receiver operating characteristics (ROC) curves were generated to select the FAEE concentrations that yielded the best sensitivity and specificity and at which the values would be dichotomized into “positive test” and “negative test”. Figure 3 shows a representative ROC curve for the 2 most useful FAEEs and the score from the principal component analysis using the value of ≥ 7 DRDD in the month before pregnancy to define drinkers. The results of the ROC analysis for select FAEEs are presented as AUC, sensitivity rate, specificity rate, PPV, NPV, and the cutoff point in both ng/g and $\log(\text{ng/g}+100)$ (Table III).

All analyses were done with $\log_{10}$ transformations of the FAEE levels. Log transformations are routinely used to convert values that increase exponentially to a scale in which the increase is linear, thus allowing for standard statistical methods to be used. A constant value of 100 was added, which effectively set the value of the samples with values below the limit of detection at 100 to allow for log transformation. The alpha level was set at 0.05. All analyses were done with SAS software version 8.1 (SAS Institute, Carey, NC).

RESULTS

Study Group Differences in FAEE

Table II represents the medians and ranges of each FAEE by study group. Four of the 7 FAEEs being studied (ethyl myristate, ethyl palmitate, ethyl oleate, ethyl linoleate) have statistically significant differences in the mean ($\log_{10}$) concentration between the Cleveland non-abstainers and both the Jordan control subjects and the Cleveland abstainers. Ethyl linolenate is significantly different only between meconium samples of Cleveland non-abstainers and the Jordanians, and ethyl arachidonate is different only between the Cleveland non-abstainers and the Cleveland abstainers. Ethyl palmitoleate is the only FAEE with no significant differences among the study groups and is excluded from subsequent analysis. Although both control groups have limitations (Jordanian group may have different diets, genetic background, and environmental exposures; the Cleveland abstaining group may have more underreporting), the finding that both groups have FAEE levels that are significantly less than those of the Cleveland non-abstainers strengthens this finding.

Principal Component Analysis

Only 1 linear combination of FAEEs is required to explain the variance between the FAEEs. Scores for each subject using the 4 or 5 most heavily weighted FAEEs or all 6 FAEEs were calculated. Further evaluation showed no difference in predictive value of these 3 scores. Thus, only scores calculated with the 4 FAEEs are shown. Comparison of these scores between study groups is shown in Table II. The scores are significantly different between the Cleveland non-abstainers and both the Cleveland abstainers and the Jordanian groups for all FAEEs except ethyl palmitoleate.

Definition of “drinking” for Each Measure of Exposure

The graphical analyses of the data indicate that not only are the Cleveland abstainers different from the non-abstainers in overall FAEE concentrations, but that within the non-abstaining group, the concentrations are dependent on the level of drinking. An example of a graphical analysis for DRDD versus ethyl linoleate is shown in Figure 2. As can be seen in the figure, defining drinking as ≥ 7 DRDD (solid line), and using a cutoff value of 2.8 (631 ng/g; dashed line) yields only 1 false negative result. This pattern was the same for all periods studied. Therefore, to analyze the predictive values of the FAEE to detect “drinking,” we chose the definition of “drinking” as ≥ 7 DRDD. Using the same approach, we defined “drinking” for each of the other measures. For DRWK, “drinking” was defined as ≥ 21 DRWK, and for DYWK, “drinking” was defined as ≥ 5 DYWK for all prenatal periods.

ROC Analysis

The first 2 analyses aforementioned established a relationship between reported drinking and FAEE concentrations in meconium. The ROC analyses were used to determine which of the measures of alcohol exposure was best associated with FAEE concentration, for which period(s) the concentrations were the most predictive, and which of the individual FAEEs or the score from the linear combination of FAEEs was the most useful in the clinical setting. In general, values of AUC, sensitivity rate, specificity rate, PPV, and NPV for each of the 3 drinking measures were consistent across periods. The FAEEs and the score were most predictive of DRDD (Table III). For DRDD, all the FAEEs had AUCs between 63% and 76% during all prenatal periods. The FAEEs with the best performances for DRDD were ethyl oleate, ethyl linoleate, and ethyl arachidonate (Table III).

The ROC analysis for DRWK is reported in Table III. Using 21 DRWK as the definition of “drinking,” the AUC ranged from 54% to 71% for all periods, FAEEs, and score values. Ethyl arachidonate showed the most consistent result with AUCs of 63% to 70%, sensitivity rates of 72% to 88%, specificity rates of 58% to 63%, PPV of 9% to 18%, and NPV of 96% to 99%.

For DYWK, all the FAEEs had AUCs ranging from 52% to 67% (data not shown). Because of the low values of the AUCs, no further analysis with this measure of drinking was done.

We conclude that FAEE concentrations in general are related to prenatal alcohol exposure, particularly at high levels of DRDD and DRWK. With NPVs 98% and 94%, respectively, FAEE levels in meconium are particularly well suited for identifying those babies who have not been exposed to these high levels of drinking in utero. In addition, the principal component score had no better predictive value than the single most predictive FAEE for each alcohol measure.

Discussion

Screening of newborns for prenatal ethanol exposure has been done routinely by history-taking alone. The identification of a biological marker that better represents the amount of prenatal drinking to which an infant has been exposed is a challenging task. This study showed that, in a high-risk, predominately African American Cleveland population using a GC/FID assay, FAEE in meconium was most related to the self-reported DRDD. These findings are consistent with our published findings from a cohort of women in South Africa.²³ In that study, we found that ethyl oleate correlated best to DRDD and that the correlation of ethyl oleate to maternal history increased with stage of pregnancy. However, there are important differences between these 2 studies. First, women in the South African population were heavier drinkers. The DRDD, DYWK, and DRWK medians were 5.2 ± 4.0 (range, 0–15), 1.3 ± 1.5 (range, 0–6.7), and 12.6 ± 21.0 (range, 0–102.8), respectively. In the Cleveland non-abstaining population, these values were 2.54 ± 4.64 (range, 0–39.6), 2.56 ± 2.03 (range, 0.25–7.0), and 8.19 ± 19.36 (range, 0–138.6). Having a broader range of drinking in the South African population would result in better correlation.

Second, the degree of error or noise in the South African reports of drinking may be less than that of the Cleveland population. The South African population was followed prospectively, whereas the Cleveland group had retrospective alcohol histories. Previous studies have shown that antenatal self-reported drinking more accurately predicts outcome than retrospective reports.²⁴ The South African population is expected to more accurately report drinking because of a greater acceptance of drinking during pregnancy among this population.²³ In addition, there may be cultural differences in the amount of alcohol reported in a drink. One report has shown that women markedly underestimate the amount of alcohol they consume because of the volume of their drinks.²⁵ This study, although converting type of drink to standard drink, did not try to estimate the size of each reported drink.

Third, the sensitivity and specificity rates of the measurement of the FAEE were better in the South African study. The South African meconium was analyzed with a GC tandem mass spectrometer, an instrument with greater sensitivity and specificity than the GC/FID used to analyze the samples from the Cleveland and Jordan populations. Thus, higher predictive values in the South African study may be caused by lower noise in both the questionnaire data and the meconium data.

Fourth, the meconium analyzed in this study was from 1 meconium stool selected arbitrarily from all the meconium excreted by the infant. Preliminary data from other laboratories suggest that meconium is formed sequentially, with earlier gestational ages represented in the first meconium passed, and subsequent ages represented in subsequent meconium stools.²⁶ Alternatively, FAEEs could accumulate uniformly throughout meconium and not in concurrently forming meconium at the time of exposure. The samples used in our study, which were unsystematically scraped from the neonate's diaper, may not represent accurate levels of FAEEs corresponding to a particular gestational age. Future studies will need to serially collect all meconium to determine whether FAEE amounts vary within samples from the same infant.

Fifth, the 2 populations studied were culturally and ethnically distinct. Thus both genetic and dietary differences may account for the different findings in these 2 studies.

Several important questions need to be addressed in future studies. Do FAEEs distribute evenly throughout meconium? Animal studies would permit both control over the timing and quantity of alcohol consumption and the sequential collection of meconium. Do FAEEs identify a high-risk population of children? Studies on FAEE and outcome should be pursued to determine how well the biomarker can predict future adverse events in infants exposed to prenatal alcohol, establish a gold standard test, and implement early intervention programs for exposed children. FAEE in meconium show promise as a useful biological marker for determining alcohol exposure in utero.

Acknowledgments

Supported by grants from the National Institute on Environmental Health Science/National Institutes of Health/Environmental Protection Agency (P01 ES11261), Association of American Medical Colleges/Centers for Disease Control and Prevention/Agency for Toxic Substance and Disease Registry Cooperative Agreement

(MM-0122-02/02), Association of Retarded Citizens of the United States (C.F.B.), and National Institute on Drug Abuse (R01-07259; L.T.S.).

Glossary

AUC	Area under the receiver operating characteristics curve
DRDD	Drinks per drinking day
DRWK	Drinks per week
DYWK	Drinking days per week
FAS	Fetal alcohol syndrome
FAEE	Fatty acid ethyl ester
GC/FID	Gas chromatography with flame ionization detection
GC/MS	Gas chromatography with mass spectroscopy detection
NPV	Negative predictive value
PPV	Positive predictive value
ROC	Receiver operating characteristics

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Figure 1.
Flow chart showing selection scheme for meconium analysis.

Figure 2.

Scattergram to define drinking. Example shows the $\log_{10}(\text{ng/g} + 100)$ of ethyl linoleate versus DRDD in the month before pregnancy (MP). The solid line dichotomizes the population at <7 or ≥ 7 DRDD. The solid line indicates a cutoff value of ethyl linoleate that gives 1 false negative result.

Figure 3. ROC curves for definition of drinking as ≥ 7 DRDD in the month before pregnancy with ethyl oleate, ethyl linoleate, or the score from the principal component analysis.

Table I

Characteristics of drinking of Cleveland non-abstainers (n = 169)

Measure	Period	Mean $\pm$ SD	Median	Range
DRDD	Month before	2.54 $\pm$ 4.64	1.0	0–39.6
	1st trimester	2.00 $\pm$ 4.31	0	0–39.6
	2nd trimester	1.09 $\pm$ 2.72	0	0–22.0
	3rd trimester	1.02 $\pm$ 2.83	0	0–22.0
DYWK	Month before	2.56 $\pm$ 2.03	1.5	0.25–7.0
	1st trimester	2.75 $\pm$ 2.18	1.5	0.25–7.0
	2nd trimester	2.80 $\pm$ 2.28	1.5	0.25–7.0
	3rd trimester	2.07 $\pm$ 1.94	1.5	0.25–7.0
DRWK	Month before	8.19 $\pm$ 19.36	0	0–138.6
	1st trimester	6.71 $\pm$ 17.41	0	0–138.6
	2nd trimester	3.75 $\pm$.2312	0	0–110.0
	3rd trimester	3.41 $\pm$ 13.52	0	0–110.0

Table II

Comparison between study groups

FAEEs	Cleveland non-abstainers (median [range])	Jordan (median [range])	Cleveland abstainers (median [range])
N	169	30	25
Ethyl myristate *	52 ^{‡§} (0–21461)	32 (0–233)	35 (0–2697)
Ethyl palmitate *	166 ^{‡§} (0–27245)	72 (27.4–336)	65 (0–3524)
Ethyl palmitoleate *	695 (124–50241)	805 (290.5–2109)	574 (65–2172)
Ethyl oleate *	317 ^{‡§} (0–344047)	108 (0–569)	142 (0–55213)
Ethyl linoleate *	282 ^{‡§} (0–627705)	97 (0–1490)	118 (0–77056)
Ethyl linolenate *	143 [§] (0–173558)	79 (0–421)	80 (0–31389)
Ethyl arachidonate *	208 [§] (0–23560)	144 (0–2586)	0 (0–2349)
Score [‡]	1.73 ^{‡§} (1.42–3.55)	1.58 (1.45–1.98)	1.60 (1.42–2.95)

*
ng/g wet weight.

[‡] Unitless because it is the result of the principal component analysis.

[‡] Cleveland non-abstainers mean log (FAEE + 100) of meconium statistically significantly different than Jordan, with $P < .0001$.

[§] Cleveland non-abstainers mean log (FAEE + 100) of meconium statistically significantly different than Cleveland abstainers, with $P < .05$.

Table III

Receiver operating characteristics curve analysis

FAEE prenatal period	AUC (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	FAEE cutoff point (ng/g)	FAEE cutoff point Log(ng/g+100)
Drinker 7 drinks/drinking day							
Ethyl oleate							
Month before	74	92	61	14	99	676	2.83
1st trimester	73	88	61	9	99	837	2.92
2nd trimester	70	78	59	8	98	445	2.65
3rd trimester	68	80	58	5	99	445	2.65
Ethyl linoleate							
Month before	73	85	65	14	98	680	2.83
1st trimester	76	88	64	9	99	761	2.88
2nd trimester	72	89	58	9	99	383	2.58
3rd trimester	70	80	57	5	99	390	2.59
Ethyl arachidonate							
Month before	71	92	59	13	99	214	2.33
1st trimester	74	88	58	8	99	213	2.33
2nd trimester	71	78	54	7	98	202	2.31
3rd trimester	71	80	62	5	99	306	2.49
Score							
Month before	72	85	63	14	98	74*	1.87
1st trimester	75	88	62	9	99	74*	1.87
2nd trimester	71	78	60	8	98	68*	1.83
3rd trimester	69	80	59	5	99	68*	1.83
Drinker 21 drinks/week							
Ethyl oleate							
Month before	69	79	63	18	97	802	2.90
1st trimester	59	61	61	13	94	836	2.92
2nd trimester	60	64	58	8	97	443	2.65
3rd trimester	62	75	58	7	98	445	2.65
Ethyl linoleate							

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FAEE prenatal period	AUC (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	FAEE cutoff point (ng/g)	FAEE cutoff point Log(ng/g+100)
Month before	71	84	58	17	97	361	2.56
1st trimester	59	67	57	13	95	361	2.56
2nd trimester	59	64	58	8	96	384	2.58
3rd trimester	68	75	58	7	98	390	2.59
Ethyl arachidonate							
Month before	70	84	60	18	97	213	2.33
1st trimester	63	72	58	14	96	213	2.33
2nd trimester	64	73	63	10	98	306	2.49
3rd trimester	70	88	63	9	99	306	2.49
Score							
Month before	71	84	51	15	97	49*	1.69
1st trimester	60	72	50	12	95	49*	1.69
2nd trimester	64	73	54	8	97	54*	1.73
3rd trimester	66	75	60	7	98	68*	1.83

* Unitless value.