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Date

Ethyl Linolenate is Elevated in Meconium of Very Low Birthweight Neonates Exposed to Alcohol *In Utero*

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An abstract of
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James T. Laney School of Graduate Studies of Emory University
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ABSTRACT

Objectives: In an effort to improve research on the specific health complications of alcohol exposure during development, this study is to evaluates fatty acid ethyl esters (FAEEs) in meconium as potential biomarkers of *in utero* alcohol exposure in very low birth weight (VLBW) neonates. Meconium FAEEs have been studied in full term neonates with alcohol exposure in high quantities late in pregnancy. To study meconium FAEEs in VLBW neonates, first it needs to be determined if FAEEs are present in quantifiable amounts in VLBW neonates. Then an association between alcohol exposure *in utero* and meconium FAEE concentration needs to be established. Finally, a model will be developed to identify which VLBW neonates were exposed to alcohol *in utero.*

Methods: This retrospective cohort study included 70 neonates weighing less than 1500 grams who were admitted to the NICUs at Grady Memorial Hospital and Emory University Hospital Midtown. Meconium samples from each neonate were processed with gas chromatography mass spectrometry to quantify the concentration of FAEEs. Mothers underwent an in depth structure interview to determine the timing and quantity of alcohol consumption during pregnancy. Alcohol consumers were classified as those drinking at least one alcoholic beverage during the first trimester, and alcohol abstainers as those who drank zero. FAEE concentrations were compared between alcohol consumers and alcohol abstainers.

Results: Thirty percent of women reported drinking alcohol during the first trimester of pregnancy. FAEE concentrations were measurable in the meconium of 69 out of 70 neonates. The FAEE ethyl linolenate was significantly elevated when mothers reported alcohol consumption during trimester one ($p=0.02$). A simple logistic regression model including meconium ethyl linolenate concentration, birth weight, and maternal drinking status before pregnancy differentiated the alcohol abstainers from the alcohol consumers with 89% sensitivity and 87% specificity.

Conclusions: Meconium FAEEs were measurable in over 98% of VLBW neonates, with ethyl linolenate showing a significant positive association with alcohol consumption during pregnancy. A model based on this FAEE could accurately differentiate alcohol abstainers from consumers with 89% sensitivity and 87% specificity. In future, this biomarker may help in risk management of neonates exposed to alcohol in utero.

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INTRODUCTION

Despite common knowledge of the negative effects of alcohol use during pregnancy, 20-45% of women report drinking during their pregnancy [1]. Alcohol has been associated with preterm delivery and learning disabilities in young children [2,4]. In vivo studies of the health implications of alcohol exposure in utero on neonates have been limited due to difficulties in identifying mothers who consumed alcohol during pregnancy. The identification of alcohol exposure during pregnancy often relies on maternal self-report of drinking during pregnancy, which can be unreliable due to both inaccurate recall and the fear of repercussions or the stigma associated with maternal drinking during pregnancy [4-7]. The inability to identify those who have been exposed to alcohol significantly hampers research into health impact of alcohol exposure on neonates.

There have been several efforts to develop a more reliable measure of alcohol exposure in utero. One such promising tool is fatty acid ethyl esters (FAEEs). FAEEs are metabolic byproducts of alcohol. They can be measured in many tissues of the body, including the placenta and the meconium. When a pregnant woman drinks alcohol, it crosses the placenta freely, and fetal tissues metabolize the alcohol, depositing FAEEs in the developing GI tract lumen. Soon after birth, the neonate excretes the FAEEs along with any other contents of the GI tract lumen as meconium. Therefore, meconium has the ability to identify the fetus exposed to alcohol during pregnancy. Meconium FAEEs have been well described as potential biomarkers to identify term babies exposed to alcohol in utero [4,6,8-15], and can be predictive of adverse neurological outcome in these children [16,17].

However, meconium FAEEs have never been studied in very low birth weight (VLBW) babies. It is desirable to study the VLBW population since this population in the newborn intensive care unit (NICU) is at high risk for multiple morbidities. Not only could alcohol effects be more severe in this population of tenuous health, but alcohol exposure is also likely more common, as alcohol is linked to preterm birth [2,3]. Understanding the health complications of *in utero* alcohol exposure in the VLBW NICU population is of paramount importance.

Broad Research Question

Can meconium FAEE concentration be used to identify which VLBW neonates in the NICU have been exposed to alcohol in utero?

BACKGROUND

As many as 45% of women report alcohol consumption during pregnancy [13]. In inner city Washington, DC, 42% of women reported drinking during pregnancy [18]. Particularly since many pregnancies are unplanned (about half in one study), alcohol use early in pregnancy is common [14]. Alcohol freely crosses the placenta, and within an hour the concentration of alcohol in the fetus is equal to that in the mother [19]. Ethanol in amniotic fluid is about half to nearly equal to that of the mother's blood ethanol level, but lasts longer, clearing at about half the rate [19].

Alcohol has known negative impacts on the developing fetus. Ethanol causes dose-dependent placental vasoconstriction, which likely impairs oxygen transport to the fetal tissues and cause acidosis [19]. This placental vasoconstriction may be tied to growth restriction, with small for gestational age (SGA) more common in drinkers (13%) than controls (1%) [11]. Similarly, the risk of preterm birth is significantly increased in alcohol users (OR 12.1), and over 14 drinks per week is linked to still births [19]. Alcohol is associated with a 25 fold increase in risk of extreme premature birth [14]. The impact of alcohol is particularly important in VLBW neonates because alcohol exposure is expected to be more common in this population, as alcohol is linked to preterm delivery.

Preterm fetuses already have underdeveloped lungs and higher health risks, and alcohol has known detrimental effects on lung health. In adults alcohol has been shown to damage lungs and immune function. It increases the risk and severity and mortality of pneumonia, ARDS, and VAP [20]. Alcohol increases lung epithelial cell death, decreases surfactant production, and decreases alveolar macrophage phagocytosis and cytokine

production (part of immune signaling) [20]. Alcohol depletes glutathione, and therefore increases susceptibility to acute lung injury [20]. In rat and lamb fetuses, alcohol inhibits appropriate lung development, resulting in hypoplastic lungs [14]. Specifically, there is a decreased amount of VEGF, a factor required for angiogenesis, and surfactant, a substance required for healthy lungs [14].

The impact of alcohol during fetal development can also negatively impact immune function. In humans, small for gestational age babies born at term had a 2.5 fold higher rate of infection if mother consumed any alcohol versus abstaining, and an over 3 fold rate of infection if mother abused alcohol versus abstaining [21]. Alcohol exposure increases risk of early onset sepsis (15 fold) in very low birth weight neonates, hypothetically because of the negative effects of alcohol on the immune system [22]. Further down the line, alcohol is also linked to developmental problems in young children, known as fetal alcohol syndrome disorder (FASD) [14].

However, our understanding of alcohol's full impact on fetal and neonatal health is incomplete [19]. There is still a great deal to understand about the pathophysiology of alcohol exposure in utero, the effect of timing and quantity of exposure, and the morbidities of alcohol-exposed neonates. This is largely because most recognition of alcohol exposure in neonates relies on the mother's report of alcohol consumption.

Maternal surveys on alcohol consumption are known to be unreliable [23]. This is often due to the stigma associated with drinking alcohol during pregnancy. Women cite denial, embarrassment, and litigious fears [23]. As in any retrospective survey, there is also the problem of recall. Finally, most alcohol surveys were designed for men, and have not been validated for use in women in the context of pregnancy. Survey tools such as

AUDIT, TWEAK, T-ACE, MAST, TLFB can identify problem drinkers but have not been developed or necessarily validated within the pregnant population. When tested in pregnant women, these tools are best at identifying heavy drinkers [23]. For example, the AUDIT survey had high sensitivity of 95% and specificity of 85% for "risk drinking," which is at least 1 oz of alcohol per day or in other studies as 2 drinks/day, or more than 7 drinks/week. Such surveys are optimal at identifying heavy drinking, but not mild drinking which is the more prevalent case during pregnancy [24]. Although there is no gold standard for identifying alcohol consumption in pregnant women, the best approximation is commonly a structured in-depth interview with the mother. These interviews mitigate the issues of reliability in maternal survey, but they are highly impractical due to the time required to perform a structured in depth interview, and the trained personnel required to conduct such interviews.

The inaccuracy of maternal surveys and the clinical impracticality of in-depth structured interviews has led to a search for biomarkers. Ethanol is metabolized by both oxidative and non-oxidative pathways. In the oxidative pathway, acetaldehyde is produced via ADH (antidiuretic hormone) by aldehyde dehydrogenase or in the microsome by oxidases [19]. This primarily happens in the liver in adults, or by class I and class IV alcohol dehydrogenase in the fetal developing lung [14]. Acetaldehyde produced in the fetus or in the adult (if not bound by the placenta), can enter fetal circulation and cause harm to the fetus [19]. Similarly, the oxidases in the microsome produce reactive oxygen species when metabolizing alcohol, leading to a depletion of microsomal stores of the antioxidant glutathione and causing oxidative damage to tissues [20]. It is acetaldehyde and the reactive oxygen species generated in alcohol metabolism

that can damage fetal tissue through protein oxidation, lipid peroxidation, and DNA oxidation [14]. Developing organs do not have very robust antioxidant systems, so oxidant stress is particularly damaging for the fetus [14]. The amount of damage caused by acetaldehyde and oxidase system has high inter-individual variability. The amount of acetaldehyde that reaches the fetus is variable because there is a 2-3 fold range in the placental binding of acetaldehyde [19]. Also, the amount of oxidases present (and the amount of ensuing oxidative damage) can increase with the amount of alcohol consumed [14]. Genetic variations can also impact how much of the alcohol is metabolized by oxidases versus alcohol dehydrogenase [14].

Ethanol and the oxidative metabolites of ethanol, like acetaldehyde, are not practical as potential biomarkers because they have a short half life [13]. Levels of alcohol in vivo cannot address long term fetal exposure because of such rapid elimination [23]. Routine serum biomarkers routinely used to identify alcohol exposure in adults such as gamma-glutamyl transferase, mean corpuscular volume, hemoglobin associated acetylaldehyde, and carbohydrate deficient transferring are not reliable during pregnancy as they demonstrate only a 40-70% association with maternal alcohol consumption [23].

In the non-oxidative pathway, ethanol is conjugated to free fatty acids, forming FAEEs via fatty acid ethyl ester synthase [11,19]. FAEEs persist in blood for more than 24 hrs and concentrate in adipose tissue with a half-life of 16.5 hrs [23]. While alcohol can cross the placenta, FAEEs do not [23,25]. Therefore, neonatal FAEEs represent alcohol which was metabolized in fetal tissues [23]. In term placentas injected with alcohol, there is an 89% increase in production of free fatty acids [19].

FAEEs are stable, and accumulate in body tissues [23]. In particular, FAEEs accumulate in biological matrices such as hair and meconium. Evaluation of meconium is attractive compared to urine or blood sampling since it has been used for in utero drug exposure, is routinely discarded and can be collected noninvasively [23]. Meconium formation begins at approximately week 12 of gestation, and is generally thought to serve only as a chemical reservoir for exposures during the second and third trimester [11]. FAEEs in meconium are accurately measured with gas chromatography mass spectroscopy (GC/MS) [13]. GC/MS has been verified as a sensitive and reproducible method to measure FAEEs in meconium [12,13].

There are many types of FAEEs formed after exposure to alcohol, depending on the carbon length of the fatty acid. They include: Lauric (E12), Myristic (E14), Palmitic (E16:0), Stearic (E18:0), Oleic (E18:1), Linoleic (E18:2), Linolenic (E18:3), and arachidonate (E20). Each study emphasizes a slightly different combination of FAEEs, or single FAEE, as an indicator for alcohol exposure. It is thought that ethnicity may impact the type of FAEE that is most increased in alcohol exposed neonates [23]. One study found that ethyl oleate levels in term meconium above 32ng/g had a sensitivity of 84% and specificity of 83% in identifying women who drank over 1.5oz of alcohol per day during their second and third trimester [26]. Another found that ethyl linoleate had a sensitivity of 88% and specificity of 64% in identifying abstainers (did not drink before or during pregnancy) from non-abstainers (those who drank before or during pregnancy). However, the study found significantly better identification of those who drank at least 7 drinks per day [9]. The types of FAEE most often used to identify alcohol exposure have

been oleate and linoleate, and sometimes stearate, palmitate, and arachidonate, or a combination of the above [10].

There are low levels of FAEE present in meconium in babies whose mothers did not drink any alcohol [12]. This is because ethanol is produced endogenously in small amounts as a by-product of normal gut physiology [8,23]. Low levels of FAEE were present in meconium from nondrinkers in populations studied in both Toronto and Jerusalem [23,27]. Therefore, past studies have suggested the use of 'cut off values,' below which FAEE concentration is considered normal without alcohol consumption, and above which alcohol consumption has occurred. Cut off values are also useful because of the issue of lower limits of detection by GC/MS. Very small quantities of FAEE are not reliably measured even by GC/MS. Limit of detection (LOD) in 9 FAEEs have been cited ranging from 0.16 nmol/g to 0.22 nmol/g (50ng/g) [23,27].

Cut off values used in previous studies depend on the type of FAEE or combination used. A study using ethyl linolenate used a cut off of 100ng/g to detect those who drank > 21 drinks/week, and 50 ng/g for oleate and linoleate [10]. In a study using a combination of FAEEs, the cut off value was 0.5nm/mg for the sum of myristic, palmitic, oleic, stearic, linoleic, and linolenic acid for significant alcohol exposure [12]. Another used a cut off of 2nmol/g (600ng/g) for the combination of ethyl palmitate, stearate, oleate, and linoleate to identify heavy drinkers from non-drinkers with a sensitivity of 100% and a specificity of 98% [23,27]. However, these values are from studies of term neonate meconium, and therefore are likely not applicable to the VLBW population.

Quantity of alcohol is not the only factor that influences concentration of FAEE. There is high inter-individual variation in FAEE from term meconium among women

who reported drinking [13]. Ethyl Myristate has been described to increase with gestational age, while ethyl palmitate decreased with maternal age and with birth weight [27]. Prenatal vitamin use was also associated with decreased FAEE concentration [27]. Even maternal diet has been reported to impact term meconium FAEEs. There was an association between olive oil use and increased total FAEE concentration [27].

With high inter-individual variation and the complexity of the relationship between meconium FAEE concentration and alcohol consumption in pregnancy, most studies of term newborns only cite strong correlations between heavy drinkers and abstainers, rather than identifying moderate drinkers [23,12]. Commonly cited values are at least 7 drinks per day, or at least 21 drinks per week [10].

Furthermore, most studies have also focused on drinking in the second or third trimester. Ethyl oleate in term neonate meconium was tied to alcohol consumption in the second and third trimester [10,13,26], while ethyl linoleate strongly correlated with alcohol consumption in later stages of pregnancy [9].

VLBW babies are often premature, and are often born before the third trimester. This is a high risk population that has not been studied, as past studies have focused on full term babies. Past studies have also tended to emphasize the relationship between drinking later in pregnancy and FAEE in neonatal meconium, but did not concentrate on alcohol consumption early in pregnancy, which is the most common pattern of maternal drinking [9,10,13,26]. Since meconium is thought to begin accumulating at week 12 of gestation [11], it is unclear if meconium FAEE accumulation occurs in the VLBW infant with maternal consumption of alcohol during the first trimester. It is generally thought that a 5g sample of meconium is necessary to accurately detect FAEE. Since meconium

accumulation increases exponentially across gestation it is estimated that only approximately 1 g of meconium is formed at 23-26 weeks of gestation [10,13].

Meconium FAEE concentration has not been studied in VLBW babies, nor has meconium FAEE been investigated as a biomarker of intrauterine alcohol exposure in this population. These babies are born often before the third trimester, so it is unknown whether there will be adequate meconium accumulation to measure FAEE concentration and if FAEEs accumulate in meconium with maternal alcohol consumption early in pregnancy. Since the VLBW population is already at a high risk for morbidities, the superimposed health impacts of alcohol exposure could be severe. Therefore, it would be very beneficial if FAEEs in VLBW meconium could be used to identify alcohol exposure in this population. Identification of the alcohol-exposed VLBW infant would advance our understanding of the immediate health impacts of such exposure in the neonatal period.

METHODS

Specific Aims

1. To determine if FAEE is detectable by gas chromatography mass spectroscopy (GC/MS) in meconium of VLBW neonates.

2. To estimate the association between meconium FAEE concentration and alcohol consumption during pregnancy in VLBW neonates.

3. To form a model to identify the VLBW neonates that were exposed to alcohol in utero.

Objectives

The *objectives* of this study were to measure meconium FAEEs in very low birth weight (VLBW) newborns (≤ 1500 grams at birth) and to evaluate meconium FAEEs as potential biomarkers for in utero alcohol exposure in premature neonates. We *hypothesized* that FAEEs are detectable in the meconium of VLBW newborns and that meconium FAEEs would be elevated in the alcohol-exposed VLBW newborn. We hypothesize that ethyl oleate, linoleate, linolenate, or a combination of them will be elevated in the alcohol consumer group as compared to the alcohol abstainer group. A model based on meconium ethyl FAEEs will be constructed to quantify alcohol consumed, which should match closely to the alcohol consumption reported by mothers in the in-depth maternal interview.

Study Population

Mothers who delivered premature newborns weighing ≤1,500 grams who were admitted to the Newborn Intensive Care Units of Grady Memorial Hospital or Emory

University Hospital Midtown, two newborn intensive care units in the Emory University system were eligible for enrollment into the study. The population at Grady Memorial Hospital is primarily urban, while that at Emory University Hospital Midtown is a mix of suburban and urban. Only adult women and emancipated minors were approached for enrollment and written informed consent was required. Exclusion criteria included maternal refusal to participate, multiple congenital anomalies on physical exam, and clinically suspected or confirmed chromosomal abnormality. Mothers with a history of HIV were excluded for the safety of our laboratory workers. This study was approved by the Emory University IRB (IRB00000976, Gauthier, PI). Newborns were all under the care of attending neonatologists unrelated to this research study to ensure that participation did not alter patient care. A total of 70 patients were enrolled.

Study Design

This was a retrospective cohort design, with the cohort being all women enrolled at the two sites from May 2009 to November 2013 (**Figure 1**). Meconium was collected from neonates whose mothers had enrolled in the study at the two hospital sites. Each hospital had a dedicated research nurse to enroll patients and conduct structured maternal interviews. Mothers underwent an in depth structured interview while at the hospital, within two weeks of delivery, to determine how much alcohol was consumed during pregnancy, and when it was consumed. This interview information defined the comparison groups. The comparison groups used were alcohol consumers and alcohol abstainers.

Comparison Groups

Alcohol consumers were defined as those reporting consumption of at least one alcoholic beverage in their first trimester. Alcohol abstainers were defined as those reporting consumption of zero alcoholic beverage consumption. Sensitivity analyses were performed using alternative definitions of consumers and non-consumers.

Measurements

Maternal Survey Data: Mothers were interviewed with by one of two trained research nurses using a structured in-person interview which incorporated the AUDIT and Time-Line Follow Back (TLFB) questionnaires within 14 days of delivery [21,28]. During this in-depth structured interview, questions about alcohol were embedded within more than 50 questions. These questions ranged from "how many drinks of wine did you have during trimester one" to, "did your partner drink alcohol," and generally took over 30 minutes to conduct. The AUDIT questionnaire is an indirect line of questioning based around the negative effects of excessive drinking [1]. It was validated in pregnant African American women in Washington D.C. [18], and has a specificity of 97% [24]. The TLFB is a direct line of questioning providing more detail on quantity and frequency of alcohol consumption [1]. These two validated surveys, the AUDIT questionnaire and TLFB, were combined to create one comprehensive set of questions for a structure interview. For these reason, this in-depth interview is believed to be significantly better than standard maternal surveys about alcohol use during pregnancy. Additionally, the dichotomization of consumers versus non-consumers reduces the misclassification of alcohol consumption, compared to looking at alcohol as a continuous variable.

Meconium Data: First passed meconium samples from neonates were saved in the diaper, labeled with a de-identified study number, placed on ice, and transported

immediately to the Gauthier laboratory. Each meconium sample was scraped from the diaper, with consecutive samples separately labeled and stored at -80° C until batch analysis by GC/MS using methods we have previously described [28,29]. Briefly, thawed meconium samples (1 gram of wet weight meconium/ sample) were homogenized and FAEEs were extracted using methanol/chloroform. FAEEs of measured included ethyl oleate (18 carbons; cis-Δ9); ethyl linoleate (18 carbons; cis,cis-Δ9,Δ12); ethyl linolenate (18 carbons; cis,cis,cis-Δ9,Δ12,Δ15). A single column GC/MS using a Hewlett-Packard 5890 Series II GC and a Hewlett-Packard 5972A Mass Selective Detector with analysis via Chemstation Productivity Software G1701BA (Version B.01/.01) was used. The concentrations (μg/g) of the individual FAEEs of interest were normalized to the dry weight of a corresponding thawed meconium sample (grams) obtained after drying (48 h at 50°C).

The calibration curves of the FAEEs standards demonstrated a linear fit with a mean coefficient of determination (r^2) ranging from 0.954- 0.979, the lower limit of detection (LOD) ranging from 5.99 – 8.73 μg/ml and the limit of quantification (LOQ) ranged from 8.16 – 37.1 μg/ml as we have previously described [29]. Undetectable concentrations were assigned the lower limit of detection. The LOD for each FAEE type was 315 ng/g for ethyl oleate, 305 ng/g for ethyl linoleate, 448 ng/g for ethyl linolenate.

Sample-size and Power Considerations

We anticipated requiring 80 individual samples to be sufficient for comparison of two groups (alcohol abstainers versus alcohol consumers) to a power of 90%. Sample sizes of at least 25 per group are sufficient to compare FAEEs as a continuous variable

between the two groups, however 80 per group is required to demonstrate its use as a diagnositic marker. In this study, 70 were recruited before funding limitations were met, and full data was acquired for 64.

Analytic Plan

First, presence of FAEE in meconium at detectable levels was determined. Levels of ethyl oleate, ethyl linoleate, and ethyl linolenate were recorded. If no FAEE could be detected above the LOD, the sample was said to have unquantifiable amounts of FAEE.

Then, meconium FAEE concentrations were compared between the alcohol consumer group and the alcohol abstainer group. The FAEE concentrations between these two groups were compared both by each type and in combination. Continuous FAEE concentrations in the alcohol consumers versus in the alcohol abstainers were compared using the non-parametric Mann Whitney-U test. This would determine which FAEE type or concentration was best correlated with alcohol consumption. The log transformation of the FAEE concentration was used in order to minimize the left skew of the data.

A scatter plot of quantity of alcohol consumed against FAEE concentration was examined to assess for outliers and to determine if the nature of the relationship between FAEE and alcohol was linear or based on a threshold model. Though a linear regression model was considered, a logistic regression model was more appropriate given the high inter-individual variation in FAEE concentration expected. The association between alcohol consumption and meconium fatty ethyl esters was not expected to be exact, as the alcohol consumption recorded from maternal interview is not expected to be exact. The

in-depth maternal interview, though more accurate than a maternal survey, is the best approximation of actual alcohol consumption during pregnancy. It is actual alcohol consumption that causes meconium FAEE elevation (see **Figure 2**).

A multivariate logistic regression model was developed to most accurately identify those who consumed alcohol during trimester one. The model was developed using backward regression and clinical reasoning and practicality. Univariate analysis (chi square odds ratios) of demographic and survey data was used to determine variables that should be included in the initial model. Variables were then trimmed from the model to avoid overfitting the data and to simplify the model for practical clinical use. Only information that would be easily obtainable without an in-depth in person interview was (Sincluded.

The accuracy of the model in categorizing a neonate as alcohol exposed or unexposed was determined using receiver operating characteristic (ROC) curves and area under the curve (AUC). A $p \leq 0.05$ was considered statistically significant. Two sensitivity analyses were performed. A sensitivity analysis comparing those who drank over 5 drinks in trimester one compared to abstainers was also performed. A sensitivity analysis considering alcohol consumed throughout pregnancy rather than just during trimester one was also performed. All statistical analyses were performed using SAS Statistics Software version 9.4 (SAS Institute, Cary, NC).

RESULTS

A total of 70 participants were recruited for this study, with 64 that completed the interview (**Figure 1**). The demographics, socioeconomic factors, and substance use characteristics of the study population are described in **Table 1A** for maternal traits and **Table 1B** for newborn traits. The study population of 64 mothers had a median age of 28 with 2.5 pregnancies, and was primarily an African American population. About 22% reported that they were trying to get pregnant, 13% smoked during pregnancy and 14% used illicit drugs during pregnancy. The neonates had a median gestational age of 28 weeks, 1103 grams at birth, with the majority male and a majority African American.

The majority of women reported no alcohol consumption during pregnancy. Only 30% reported one or more drink during pregnancy, while 65% reported drinking during the 3 months prior to pregnancy. Of those who did report alcohol consumption, quantities were very low, and almost exclusively in trimester 1. Of the 18 who drank in trimester 1, only 9 people reported drinking 5+ drinks in the entire trimester. Likewise, though 18 drank in trimester one, only 3 participants reported alcohol consumption in trimester two, and only one participant reported alcohol consumption in trimester three.

FAEEs were measurable in 69 out of 70 of the meconium samples collected. The median and interquartile range was 9.2 (8.0-9.8) ln(ng/g) for ethyl oleate, 8.6 (5.8-9.4) ln(ng/g) for ethyl linoleate, and 6.1 (6.1-7.9) ln(ng/g) for ethyl linolenate. The distributions of the log transformation of each FAEE concentration are depicted in **Figure 3**.

The population of 64 participants that completed the study were divided into a group of 46 alcohol abstainers, who reported zero alcohol consumption during trimester one, and a group of alcohol consumers, who reported at least one alcoholic beverage during trimester one. The characteristics of these two groups are presented in **Table 2A** for maternal traits and **Table 2B** for newborn traits.

The median FAEE concentration between these two groups was compared with a non-parametric Mann-Whitney U test. Although the median FAEE concentrations of ethyl oleate, and linoleate were higher in alcohol consumers compared to abstainers, these elevations did not reach statistical significance. The concentration of ethyl linolenate was significantly elevated in the alcohol consumers (7.4 ln(ng/g) in alcohol consumers versus 6.1 ln(ng/g) in alcohol abstainers) (**Table 3** and **Figure 4**). The combination of ethyl linolenate plus ethyl oleate was also significantly elevated (p= 0.04). Ethyl linolenate alone was able to distinguish alcohol consumers from non-consumers with an AUC of 0.68 in logistic regression modelling, with a p-value 0.02. That is, ethyl linolenate concentration alone could identify an alcohol consumer from an alcohol abstainer with 61% sensitivity and 80% specificity (**Figure 5**).

Next, a multivariate regression model was developed. The univariate analysis of variables that could help identify alcohol consumers are listed in Table 2A and Table 2B. The alcohol consumer group tended to be older mothers, lower birth weight neonates, and be born at a younger gestational age. The alcohol consumer group also tended to smoke more cigarettes, have greater odds of education beyond high school and of drinking before pregnancy, but had lesser odds of trying to get pregnant, living with a partner, and of having a male child. This initial, over-fitted model, had an AUC of 0.95 (95%CI 0.9-

1.0) and a p-value < 0.01. However, only information that was easy to obtain without the same stigma issues that surround asking about alcohol consumption during pregnancy were included. Therefore the number of cigarettes per day, education status, presence of a live in partner, and whether or not the pregnancy was intentional were excluded. To simplify the model and because ideally there should be ten times more 'events' (in this case alcohol consumers) than variables in the model, the model was further trimmed of variables that were not statistically significant including gestational age and maternal age. This model had an AUC of 0.93 (0.86-0.99), and a p-value < 0.01. Finally, child gender was excluded from the model as it was felt that this was most likely a chance occurrence that did not influence the association between alcohol and FAEE concentration.

This left three variables in the model: birth weight, meconium ethyl linoleate concentration, and whether or not the mother drank alcohol before pregnancy. This final variable, whether or not the mother drank alcohol before pregnancy, has a very high odds ratio and insignificant p-value because of statistical convergence failure. This is because everyone who drank during pregnancy had also been drinking before pregnancy, which causes quasi-complete separation, and makes the p value inestimable [30]. However, when the logistic regression of alcohol consumption before pregnancy is run as the only variable to predict alcohol consumption after pregnancy with an odds ratio of -3.0 and a p-value of less than 0.01. This variable was important in the model, and was included in the model for its predictive value.

The final model had an AUC of 0.89 (95%CI 0.80-0.98) and a p-value of 0.01 (**Figure 6**). It included birth weight, ethyl linolenate concentration in meconium, and the single question of did the mother drink before pregnancy or not. The significance and

odds ratio of each of these three variables in the model is described in **Table 4**. This model demonstrated a sensitivity of 88.9% and a specificity of 87.0%.

DISCUSSION

Research into the neonatal health implications of alcohol exposure in utero has been hampered by the difficulties in identifying babies that have been exposed to alcohol in utero due to the stigma attached to drinking during pregnancy, and therefore mothers' hesitance in admitting to alcohol use during pregnancy. This is of especially high importance for the NICU population of VLBW neonates, since these neonates already have tenuous health with underdeveloped immune and pulmonary systems, and are more likely to have been exposed to alcohol in utero due to alcohol's association with premature delivery. Though meconium FAEE has been proposed as a biomarker for alcohol exposure in full term babies, past studies have not looked at the VLBW NICU population where this research is most relevant. Past studies have also not evaluated the accumulation of meconium FAEE after alcohol consumption in the first trimester, as meconium is thought to only start accumulating at week 12 of gestation. Yet, alcohol consumption most commonly occurs in trimester one. Past studies have also focused on identifying heavy drinkers who consume several alcoholic beverages per day, rather than on moderate drinkers who are more common.

This study found that FAEEs were quantifiable in the meconium samples of 98% of the 70 very low birth weight neonates enrolled, despite the mean gestational age of 28 weeks. This study found a significant relationship between consumption of one or more alcoholic beverages during trimester one of pregnancy and ethyl linolenate in meconium, despite the low quantity of meconium present in these VLBW neonates. With the addition of easily attainable data, namely birth weight in grams and whether or not the mother drank before pregnancy, ethyl linolenate in meconium can distinguish those who

drank at least one alcoholic beverage in trimester one from those who abstained from alcohol with a sensitivity of 89% and a specificity of 87%.

Several studies in full term neonates have found an association between meconium FAEEs and alcohol consumption [8]. However, ethyl linolenate is rarely cited as the FAEE with the best correlation. This is in part because ethyl linolenate is rarely measured in these studies. However, it could also be due to the predominantly African American study population, as it has been suggested that ethnicity impacts the FAEE responsive to alcohol [23]. It is also possible that different types of FAEEs predominate at different stages of pregnancy. It is also possible that our predominantly premature, VLBW population, did not accumulate enough of these other FAEEs by the time of delivery.

There are however, limitations in this study. First of all, there is no gold standard to identify alcohol consumption against which to compare our proposed biomarker [10]. The surrogate biomarker used in this study, as in many others investigated alcohol biomarkers, is an in-depth maternal interview. The improper determination of who drank alcohol in trimester one can lead to a misclassification bias, which is a bias towards the null. Though maternal interviews are superior to maternal surveys, they still have issues of the stigma associated with alcohol consumption and problems with recall. Recall bias may be more pronounced in our study because all of these babies are in the NICU, and mothers may be looking for a cause of their tentative health status. Ideally, recall bias could be overcome by a prospective study. However, a prospective study would be much more costly, time consuming, and require a larger commitment from mothers. There would also be a drop out bias in a prospective study, as mothers drinking large quantities would be more likely to decide to drop out of the study due to associated stigma.

Next, there are problems with our small sample size, which was limiting in our analysis. The small sample size did not allow us enough power to construct a model and verify it with a different subset of the sample population. Therefore, the model proposed in this paper is not validated. A new sample would be required to do so. The small sample size was also limiting in that there was too much noise in the FAEE concentration to assess a linear relationship between FAEE in meconium and quantity of alcohol consumed. Therefore, we did not have the power to assess a dose-response relationship.

Finally, our population is not representative of the general population in the US NICUs. Our study had a primarily African-American population. This may be important as it is thought that ethnicity may impact the type of FAEE that is most increased in alcohol exposed neonates [23]. There was an unequal distribution of male babies between the alcohol consumers group and the alcohol abstainers group. This variable was excluded from the model as a sampling error, but it is therefore unclear if male gender influences the relationship between alcohol and FAEE concentration. This study also had a high proportion of twins, with two twin pairs present in the study. There were not enough twins to study the potential relationship of meconium FAEE present in twins which had theoretically been exposed to the same quantity of alcohol in utero. If twins were fraternal and of opposite gender, we could gain insight into the variable impact of genetics and gender on FAEE concentration while holding all other environmental factors constant. The presence of twins in the study also increases the random error in the maternal data, as the same mother was effectively counted twice. This means that the standard error around maternal data, such as maternal age, is slightly underreported. This, however, does not greatly impact our results.

Despite the limitations of this study, meconium ethyl linolenate is proposed as a potential biomarker for trimester one alcohol exposure in the VLBW NICU population. Further studies are necessary to validate this biomarker and this model. The ability of this biomarker to show a dose-response relationship needs to be investigated in a larger study with greater numbers of alcohol consumers. The model to identify alcohol consumers could also be improved by combining meconium ethyl linolenate with other biomarkers, such as placental FAEEs to improve sensitivity and specificity [8]. It would be ideal to increase the sensitivity of the model to avoid false negatives, which would clinically be problematic, as we would not want to miss those neonates that were exposed to alcohol [10].

The ability to identify the VLBW baby that has been exposed to alcohol in utero is the crucial first step to understanding the health complications in the NICU caused by alcohol [14]. Once we can more accurately identify which babies are exposed to alcohol in utero, we can better characterize their early health concerns. The research community will be able to better investigate the pathophysiology and mechanism of alcohol induced fetal injury, leading to better management of these conditions in the future.

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TABLES AND FIGURES

Figure 1. Retrospective cohort study design of 70 women recruited at Emory Midtown Hospital NICU and Grady Memorial Hospital NICU who were divided into alcohol abstainer and alcohol consumer groups.

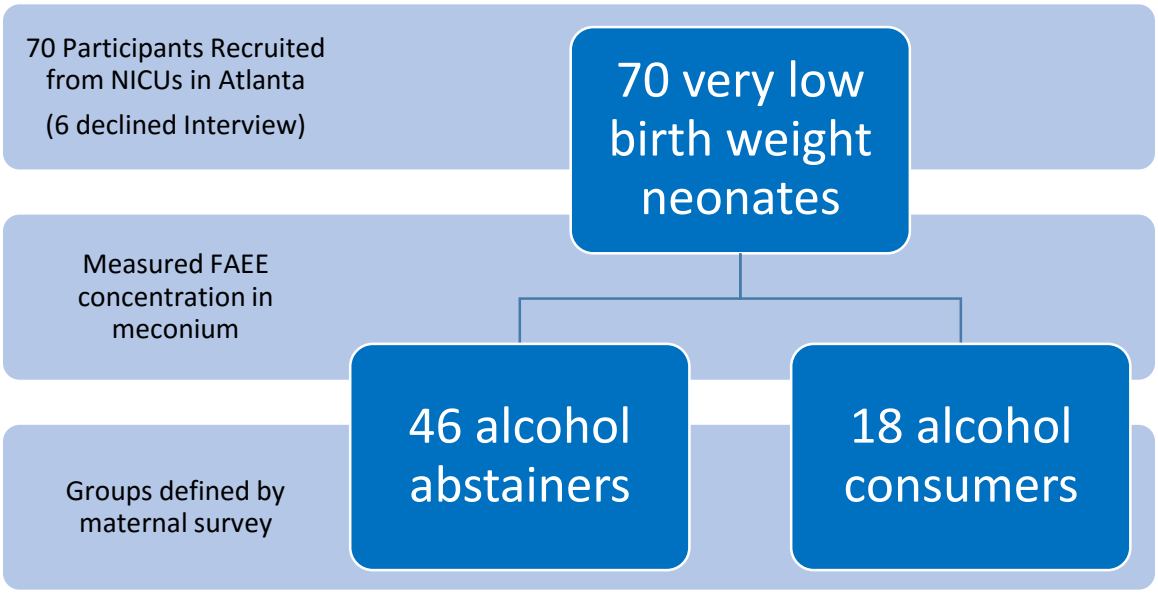
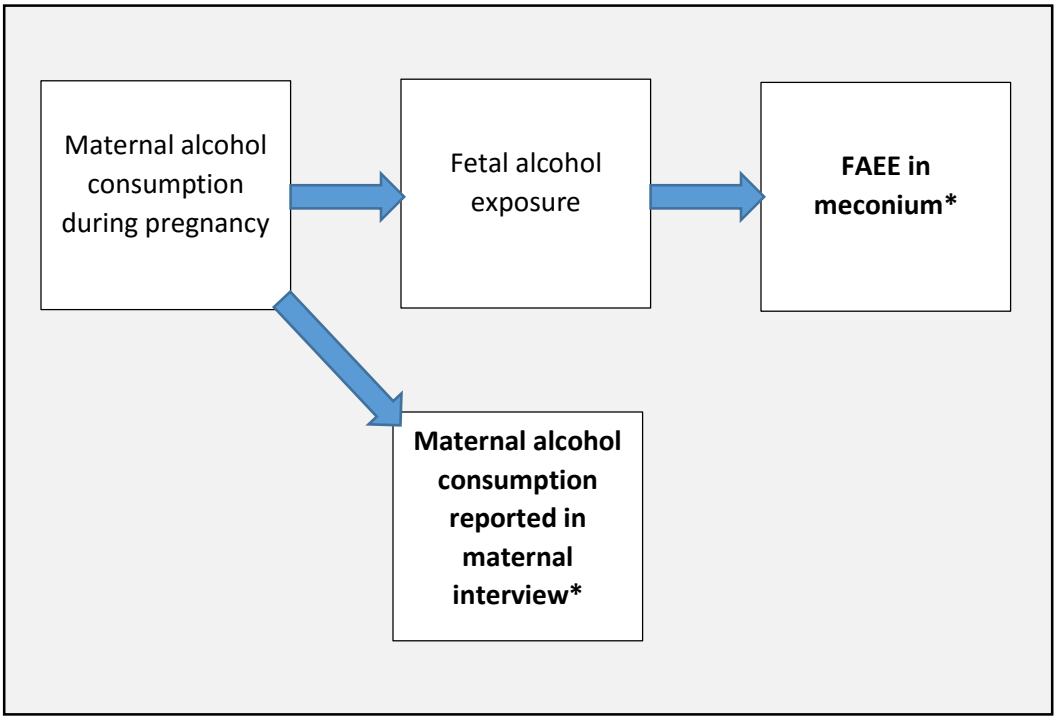


Figure 2. Causal diagram depicting relationship of alcohol consumption, maternal alcohol consumption reported in interviews, and meconium FAEEs.



*Measured in this study.

Table 1A. Maternal Characteristics for 64 very low birth weight neonates from the NICUs of Grady Memorial Hospital and Emory Midtown Hospital

	N (%)
Maternal Age	
Years	28 (23 – 32)*
Race	
White	5 (8%)
Black	57 (89%)
Other	2 (3%)
Gravidity	
Total	3.4 (0-11)*
Livebirth	2.4 (0-9)*
Trying to get Pregnant	13 (20%)
Total Family Income in Last Year	10k to 25k
Dollars	(<10k – 25k to 40k)*
Prenatal Care	
Total Visit	0.9 (1-24)
Visits Missed	0.9 (0-15)
Highest level of Education	
Did not complete high school	9 (14%)
High School	26 (41%)
Some college	21 (33%)
College	5 (8%)
Graduate School	3 (5%)
Marital Status	
Married or Living with partner	13 (20%)
Separated or Divorced	3 (5%)
Single or Widowed	48 (75%)
Illicit Drug Use	
Before pregnancy	12 (19%)
During pregnancy	9 (14%)
Tobacco Smoking,	
During pregnancy	14 (22%)
Cigs/Day	0.7 (0-10)*
Alcohol consumption	
During 3 months before pregnancy	43 (67%)
Binge before Pregnancy	10 (16%)
During Pregnancy	19 (30%)
During Trimester 1	18 (28%)

*values are median (IQR)

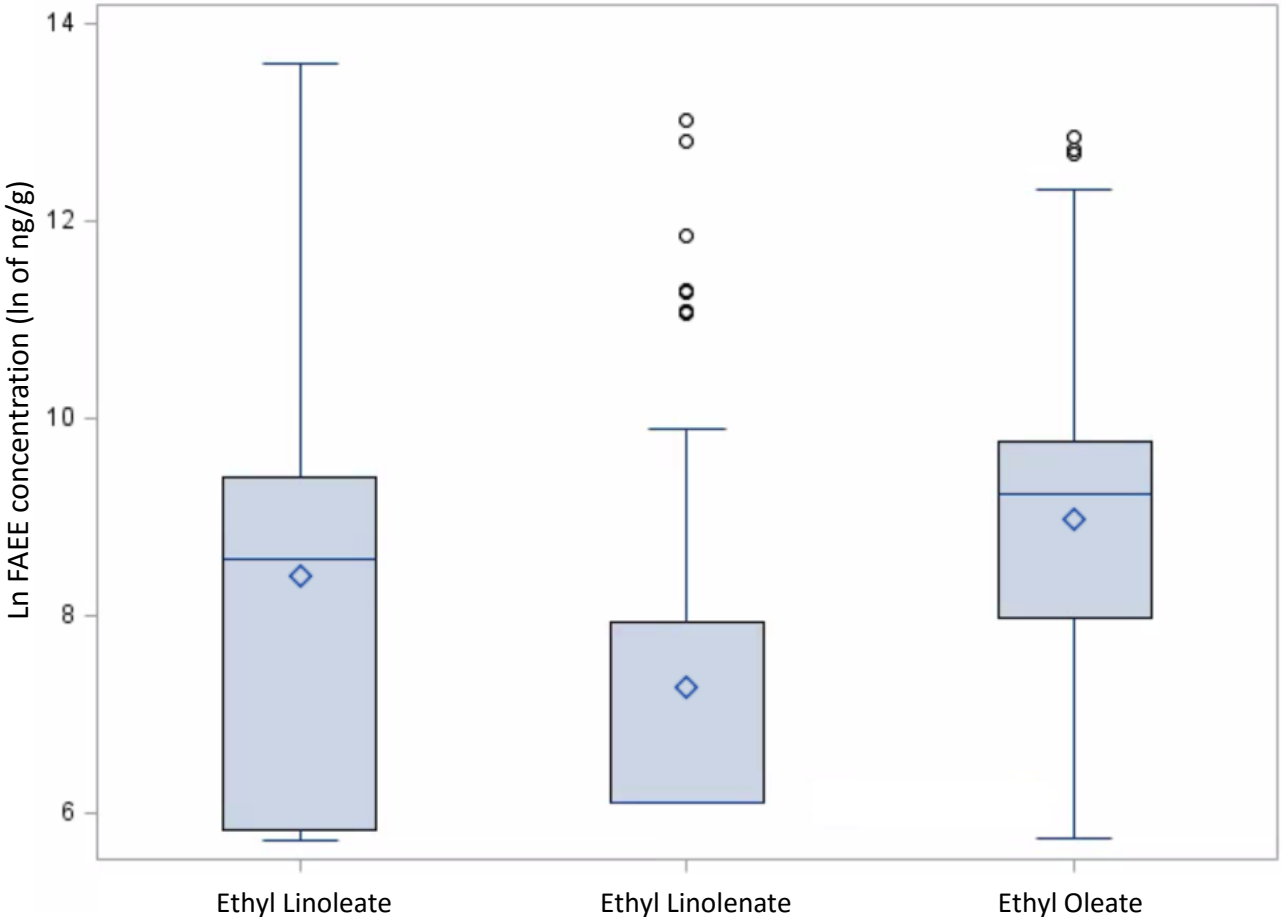
Note: There were two pairs of twins, so two mothers have effectively been duplicated

Table 1B. Characteristics of 64 very low birth weight neonates from the NICUs of Grady Memorial Hospital and Emory Midtown Hospital

	N (%)
Gestational Age in weeks	28.0 (23-35)*
Child Weight in grams	1039 (510 – 1465)*
Male Child	34 (53%)
Child Race White Black Other	 5 (8%) 56 (88%) 3 (5%)
Twin	4 (6%)

*values are median (IQR)

Figure 3. Fatty acid ethyl ester (FAEE) concentration, by type, in meconium of 69 very low birth weight neonates from the NICUs of Grady Memorial Hospital and Emory Midtown Hospital, as determined by Gas Chromatography Mass Spectrometry.



The box plots depict the median line and the first and third quartiles are represented by the lower and upper box edge, respectively. The whiskers indicate the smallest and largest values measured with outliers depicted by a small circle.

Note: 5 of these women declined interview, and were therefore not included in the subsequent dichotomization into alcohol abstainers and consumers.

Table 2A. Characteristics of 64 mothers who had just given birth to very low birth weight neonates in the NICUs at Grady Memorial Hospital and Emory Midtown Hospital

	Alcohol Abstainers (N=46) N(%)	Alcohol Consumers (N=18) N(%)	OR (95%CI)	P value
Maternal Age, years	28 (22-32)*	31 (27-34)*	1.1 (1.0-1.2)	0.08
Race = Black	41 (89%)	16 (89%)	1.0 (0.2-5.6)	0.98
Gravidity				
Total	2 (2-3)*	3 (1-6)*	1.1 (0.9-1.3)	0.37
Livebirths	2 (1-3)*	1 (1-3)*	1.0 (0.7-1.3)	0.81
Trying to get Pregnant	12 (26%)	1 (6%)	0.2 (0.0-1.4)	0.10
Prenatal Care				
Total Visit	8.9 (7.0-8.9)*	9.0 (7.0-14.0)*	1.1 (0.9-1.2)	0.32
Visits Missed	0.9 (0-0.9)*	0 (0-0)*	1.0 (0.8-1.3)	0.98
Education Beyond High School	16 (35%)	13 (72%)	4.9 (1.5-16.1)	0.01
Married or Living with partner	11 (24%)	2 (11%)	0.4 (0.8-2.0)	0.26
Illicit Drug Use before pregnancy	9 (20%)	3 (17%)	0.8 (0.2-3.5)	0.79
Tobacco Smoking				
During pregnancy	9 (20%)	5 (28%)	1.6 (0.4-5.6)	0.48
Cigs/day	0 (0-0)*	0 (0-2)*	1.3 (1.0-1.6)	0.07
Alcohol during 3 months before pregnancy	25 (54%)	18 (100%)	---	---

*values are median (IQR)

Table 2B. Characteristics of 64 very low birth weight neonates in the NICUs at Grady Memorial Hospital and Emory Midtown Hospital

	Alcohol Abstainers (N=46) N(%)	Alcohol Consumers (N=18) N(%)	OR (95%CI)	P value
Gestational Age Weeks	28 (27-30)*	27 (25-29)*	0.8 (0.6-1.0)	0.05
Child Weight Grams	1123 (950-1270)*	802 (650-1230)*	1.0 (1.0-1.0)	0.04
Male Child	21 (46%)	13 (73%)	0.3 (0.1-1.1)	0.06

*values are median (IQR)

Table 3. Fatty acid ethyl ester (FAEE) concentration, by type, in meconium of 64 very low birth weight neonates comparing those had not been exposed to alcohol in utero compared to those who had been exposed to alcohol in utero.

FAEE type	Alcohol Abstainers (N=46) *Median (IQR) of ln ng/g*	Alcohol Consumers (N=18) *Median (IQR) of ln ng/g*	P-Value
Ln Ethyl Linoleate	8.4 (6.7-9.2)	9.1 (5.5-10.7)	0.67
Ln Ethyl Linolenate	**6.1 (6.1-6.9)**	**7.4 (6.1-9.9)**	**0.01**
Ln Ethyl Oleate	9.0 (8.0-9.7)	9.5 (7.9-10.0)	0.31
All 3	23.4 (21.0-25.2)	25.6 21.4-30.3)	0.25
Ethyl Linolenate + Ethyl Linoleate	14.7 (13.2 – 16.3)	16.1 (11.8-20.6)	0.26
Ethyl Linolenate + Ethyl Oleate	15.3 (14.1-16.5)	16.6 (15.7 – 19.6)	0.04
Ethyl Oleate + Ethyl Linoleate	17.0 (14.9-18.7)	18.4 (13.6-20.4)	0.47

Figure 4. Ethyl linolenate concentration in meconium of 64 very low birth weight neonates comparing those had not been exposed to alcohol in utero compared to those who had been exposed to alcohol in utero.

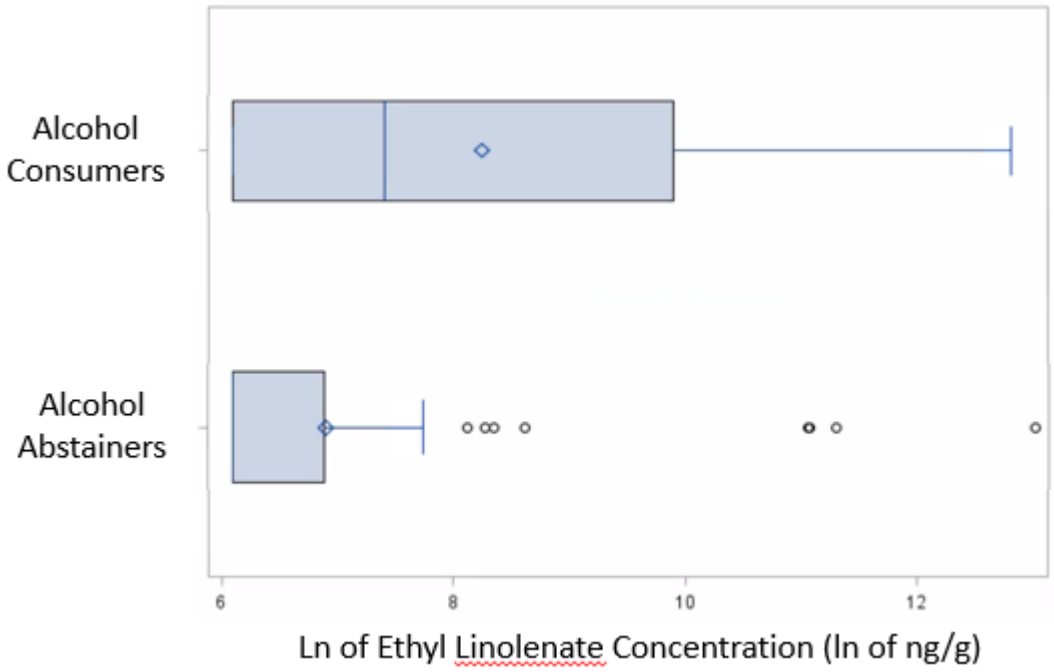


Figure 5. Receiver Operating Curve of logistic regression model characterizing 64 very low birth weight neonates as alcohol exposed or unexposed based on Ethyl Linolenate only.

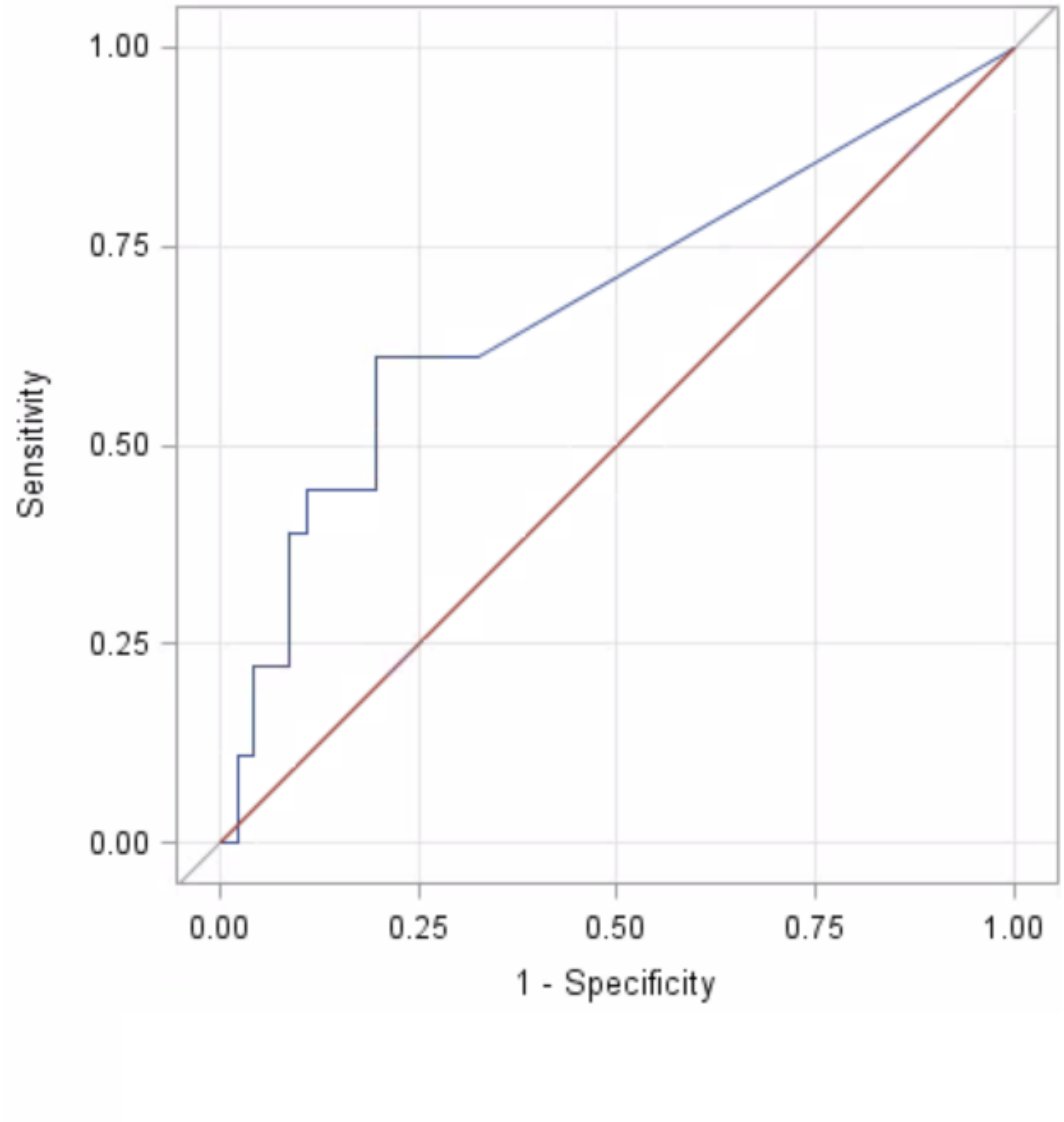


Figure 6. Receiver Operating Curve of multivariate logistic regression model characterizing 64 very low birth weight neonates as alcohol exposed or unexposed based on Ethyl Linolenate with adjustments for demographic variables.

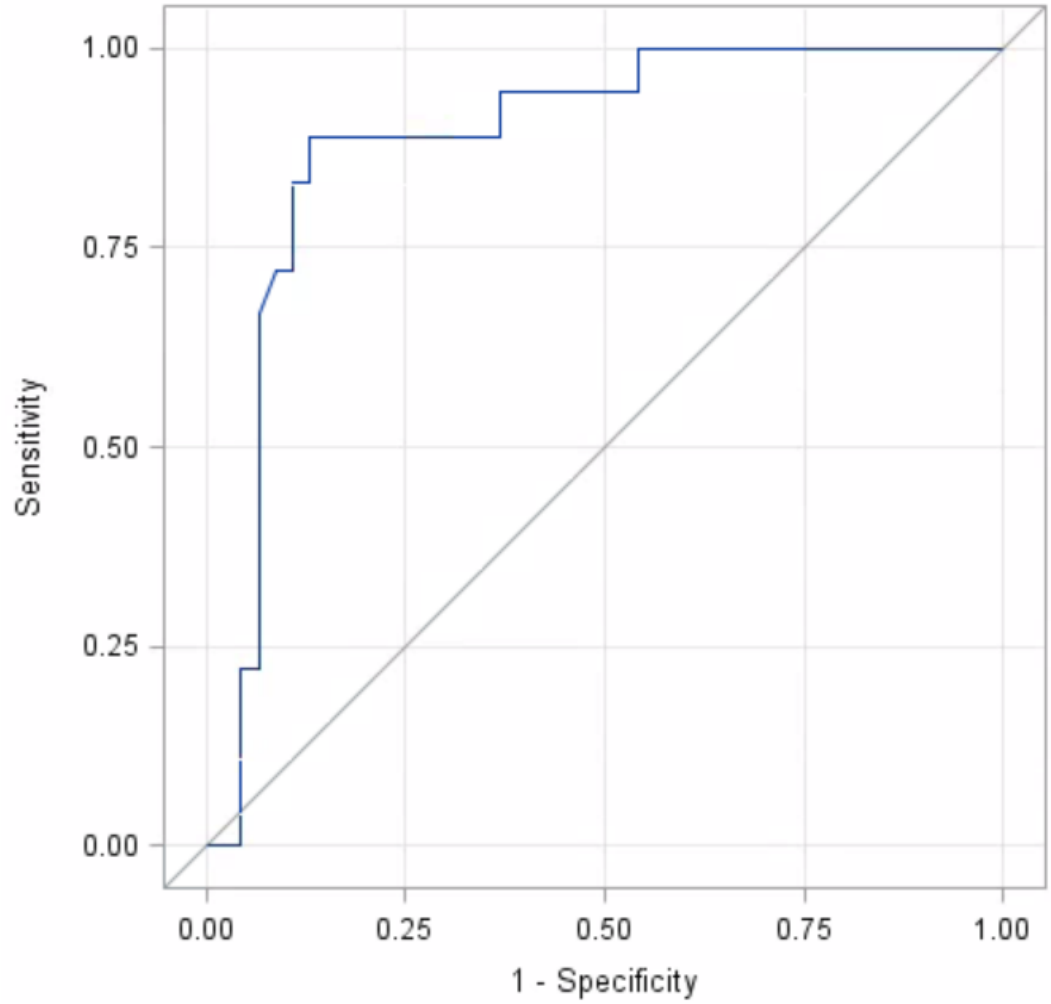


Table 4. Odds Ratios of variables in final fully adjusted logistic regression model used to characterize 64 very low birth weight neonates as part of the alcohol abstainer group or the alcohol consumer group.

	OR (95% CI)	P-value
Ln Ethyl Linolenate Concentration	1.5 (1.1-2.2)	0.02
Birth Weight	1.0 (1.0-1.0)	0.12
Drank before Pregnancy	>999.9 (<0.01 - >999.9)	0.94

Variables chosen through clinical reasoning and statistical significance with forward stepwise selection. Number of variables in the model was kept to a minimum for simplicity and to avoid over-fitting the model.