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# Effects of Maternal Folate Levels and Prenatal Alcohol Exposure on Fetal Growth, Infant Outcomes and Later Development

By

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A Thesis Submitted to the Graduate Faculty of Georgia State University in Partial Fulfillment of the Requirements for the Degree

MASTER OF PUBLIC HEALTH

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30303

Effects of Maternal Folate Levels and Prenatal Alcohol Exposure on Fetal Growth, Infant Outcomes and Later Development

By

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July 27, 2015

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## Abstract

### Objectives

Prenatal alcohol exposure can lead to fetal alcohol spectrum disorders (FASD), which include a broad range of cognitive, growth, behavior, and physical abnormalities. Early detection of the teratogenic effects of prenatal alcohol exposure is necessary to identify early interventions. The aim of this study is to identify the effects of prenatal alcohol exposure on growth and infant development, to determine if ultrasound imaging can be used as an early identification tool, and to determine if maternal folate supplementation can mitigate the detrimental effects on growth and infant development.

### Methods

A prospective cohort study and randomized trial from 2008 to 2014 conducted in two sites of Western Ukraine was analyzed. A sample of pregnant women who reported moderate-to-heavy alcohol consumption during pregnancy, and a sample reporting little-to-no alcohol use during pregnancy participated in a comprehensive maternal interview and screening process. Women were further randomized into micronutrient supplementation groups. Standard ultrasound examinations during pregnancy including study specific brain growth measurements, along with blood and urine samples were obtained during follow-up visits. A biometric screening was conducted at birth, along with Bayley Scales of Infant Development-II Mental Development Index (MDI) and Psychomotor Development Index (PDI) assessments at 6 and 12 months of age.

### Results

Estimated fetal weight (EFW), abdominal circumference (AC), biparietal diameter (BPD), transverse cerebellar diameter (TCD), occipitofrontal diameter (OFD), caval-calvarial distance (CCD), and orbital diameter (OD) were significantly reduced by alcohol exposure at third trimester ultrasound ($p<0.05$). Birth weight, length and head circumference as well as Bayley MDI and PDI scores at 6 and 12 months of age were significantly reduced by alcohol exposure ($p<0.05$). Generalized linear modeling was used to examine the relationship between outcomes significantly associated with alcohol exposure and multivitamin supplementation (MVM), absolute ounces of alcohol per day at conception (AA/day), and serum folate concentration. MVM was significantly associated with increased EFW at third trimester ($X^2_{(7)}=18.044$, $p=0.012$), AC at third trimester ($X^2_{(5)}=17.955$, $p=0.003$), and birth weight ($X^2_{(6)}=75.058$, $p<0.001$). Serum folate concentration during third trimester was significantly associated with increased Bayley MDI scores at 6 months of age ($X^2_{(11)}=63.051$, $p<0.001$).

### Conclusions

Significant reductions in fetal growth measurements during third trimester ultrasound suggest that the effects of prenatal alcohol exposure may be detectable in late pregnancy. Significant associations between multivitamin supplementation and specific growth measurements suggest that micronutrient supplementation during pregnancy, including high doses of folate, may be an early intervention to reduce the harmful effects of prenatal alcohol exposure. Further studies are needed to identify the specific micronutrients producing these effects, and to assess the appropriate level of these micronutrients necessary to provide the greatest benefit without exceeding the safe limit.

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# INTRODUCTION

## 1.1 Background

Fetal Alcohol Syndrome (FAS) is the most severe form of a group of disorders known as Fetal Alcohol Spectrum Disorders (FASDs). The disorder was first recognized in 1973 by Jones and colleagues and remains one of the leading causes of developmental disabilities worldwide.[1,2] Alcohol passes to the fetus through the blood stream, and currently there is no known safe level of alcohol use during pregnancy.[3] The estimated prevalence of FAS in the United States ranges from 0.2 to 1.5 infants for every 1,000 live births. The full range of FASD's are estimated to affect as many as 2% to 5% of the population in the United States and Western European Countries.[4] FAS is characterized by four broad areas of clinical features including abnormal facial features (smooth philtrum, short palpebral fissures, and thin upper lip), growth retardation, central nervous system dysfunction, and alcohol use during pregnancy.[5] The developmental outcomes can range from mild cognitive deficits to severe impairment, and can result in severe problems in memory, executive function, and adaptive behavior.[5,6,7,8,9]

## 1.2 Nutrition and FASD's

Proper maternal nutrition is necessary for optimal fetal growth. Women with poor nutritional status during pregnancy can give birth to children with low birth weight, physical abnormalities, delayed cognitive development, and poor overall health. Alcohol use during pregnancy can displace essential nutrients and deprive the developing fetus of the nutrition necessary for proper growth and development.[10] Micronutrients such as vitamin A, DHA, zinc, calcium, folate, and choline serve a variety of roles in fetal development. Animal models using micronutrient supplementation have shown beneficial results in prevention of fetal alcohol effects. Human studies are needed to examine the association between micronutrient

supplementation and FASD outcomes. Maternal supplementation with these micronutrients has been the focus of recent research as part of the Collaborative Initiative on Fetal Alcohol Spectrum Disorders (CIFASD) in Ukraine.[11] The current project aims to examine the role of maternal nutrition on FASD outcomes.

### 1.3 Alcohol Exposure and Growth

Alcohol exposure can affect prenatal and postnatal growth including height, weight, head circumference, and brain growth. The severity of growth restriction can vary with timing and quantity of exposure. Heavy alcohol exposure and binge drinking have the greatest effect on preterm birth and growth restriction, with several studies showing a dose-response relationship between alcohol consumption and preterm birth, small birth size, and low birth weight.[12,13-15] The timing of alcohol exposure necessary to produce effects is widely debated, with studies showing a significant association between heavy alcohol exposure in second trimester and small birth size, and no association between alcohol exposure in first trimester and birth size.[14] Alcohol exposure affects overall head circumference as well as individual structures in a variety of regions of the brain. These regions include the cerebellum, the corpus callosum, the basal ganglia, and the frontal cortex.[16] Imaging techniques such as magnetic resonance imaging (MRI) have shown significant reductions in brain size and shape, reductions in frontal lobe and basal ganglia size, and reduction in cerebellar volume with prenatal alcohol exposure.[17] Ultrasound technology could be used as an early predictor of the effects of prenatal alcohol exposure on the brain. Few studies examine this effect and show conflicting results with one study showing no association between alcohol exposure and ultrasound measures of brain growth, and others showing a significant association between ultrasound measures of frontal lobe size in utero in alcohol exposed groups.[18,19,20]

### 1.4 Alcohol Exposure and Development

The impact of fetal alcohol exposure includes growth restriction, diminished cognitive ability, physical malformations, and behavior abnormalities and extends throughout the lifetime of an individual. Many neurological and behavioral abnormalities can result from prenatal alcohol exposure including intellectual performance, attention, learning and memory, language, executive functioning, fine and gross motor skills, social skills, and adaptive behavior.[17,21] Children with FASD's are at an increased risk for seizure disorders, cerebral palsy, and visual-motor deficits.[5] These abnormalities result in poor educational outcomes, communication deficits, and poor reasoning and judgment. There is no cure for FASD's, however, educational and environmental interventions can improve development. Treatment options include medications for some symptoms, behavior and education therapy, parent training, and special education and social services.[2]

### 1.4 Purpose of Study

The teratogenic effects of alcohol on physical features, growth, cognition, behavior, and infant outcomes have been studied extensively since first discovered in 1973 by Jones and colleagues.[1] The consequences of prenatal alcohol exposure are well known, and extend into adulthood. Early diagnosis of FASD's is necessary to ensure proper educational interventions and prevent adverse outcomes later in life.[22] Imaging techniques such as magnetic resonance imaging (MRI) and prenatal ultrasounds have shown promise in detecting brain structure abnormalities that may result from alcohol exposure. The effect of prenatal alcohol exposure on cognition and postnatal growth restriction can cause lasting effects. Folate has demonstrated the ability to mitigate the effects of prenatal alcohol exposure on development. The purpose of this study is to identify the effects of prenatal alcohol exposure on prenatal and postnatal growth and

infant development using ultrasound imaging. The results have implications for whether ultrasound can be used as an early identification tool for prenatal alcohol effects, and whether if maternal folate supplementation can mitigate the detrimental effects of prenatal alcohol exposure on growth and infant development.

## REVIEW OF THE LITERATURE

### 2.1 Prevalence and Characteristics of FASD's

The prevalence of FASD's is difficult to determine due to the complex nature of the diagnosis. Centers for Disease Control and Prevention (CDC) studies using medical records have estimated the prevalence of FAS to range from 0.2 to 1.5 infants for every 1,000 live births.[23] Another CDC study has estimated approximately 0.3 out of every 1,000 children aged 7-9 suffers from FAS.[24] Additional studies estimate the full range of FASD's to be as high as 20 to 50 out of 1,000 school children in the United States and Western Europe.[25] Studies have shown that approximately 7.6% of pregnant women in the U.S. report using alcohol during pregnancy and 1.4% of pregnant women report binge drinking in the past 30 days.[26,27] However, the true prevalence of drinking during pregnancy is likely underreported due to the stigma associated with it. Fetal alcohol syndrome is estimated to cost an individual approximately $2 million over their lifetime, and is estimated to cost the United States over $4 billion annually.[28]

Globally, the issue is more complex due to limited resources, lack of surveillance and underreporting. In South Africa the prevalence of FAS ranges from 40.5 to 46.4 out of every 1,000 children aged 5-9 years according to one study conducted in a small community in the Western Cape.[29] Additional studies in South Africa estimate the prevalence ranges from 19 to 89.2 children out of every 1,000.[30,31] In Australia one study indicated that approximately 60% of non-indigenous women reported drinking during pregnancy.[32] A separate national survey indicated that approximately 20% of indigenous Australian women reported alcohol use during pregnancy.[33,34] The prevalence of FASD's are estimated to be 0.01 to 1.7 for every 1,000 live births in Australia, however these estimates likely underestimate the true prevalence due to lack of routine assessment and screening, and a lack of national diagnostic criteria.[33] In Ukraine,

heavy episodic drinking is common among women with 92.7% of women reporting being ever-drinkers in one study. The same study indicated that 54.8% of ever drinking women reported drinking in the month around conception, and 46.3% continued to drink during pregnancy.[35]

The characteristics of fetal alcohol syndrome include facial, growth, and central nervous system abnormalities when prenatally exposed to alcohol. Facial dysmorphia includes three sentinel facial features including smooth philtrum, thin vermillion border, and small palpebral fissures.[5,7,8,9] Facial dysmorphia must be present in all three sentinel features with palpebral fissure length at or below the 10$^{th}$ percentile, adjusted for gestation, age, sex and race, and philtrum and upper vermillion ranked at a 4 or 5 on the Lip-Philtrum Guide.[5,9,36] Growth retardation is determined by prenatal or postnatal height or weight at or below the 10$^{th}$ percentile, adjusted for gestation, age, sex and race.[5,36,37,38,39] Central nervous system abnormalities include structural abnormalities observed through imaging or head circumference at or below the 10$^{th}$ percentile adjusted for age and sex, neurological problems including soft neurological signs, and deficits in cognitive ability or performance.[5] Additional diagnoses in the FASD spectrum include partial FAS (pFAS), characterized by at least two of the three criteria above in the presence of confirmed alcohol exposure, and Alcohol Related Neurodevelopmental Disorder (ARND), characterized by cognitive deficits when alcohol exposure is confirmed.[40]

**2.2 Prenatal Alcohol Exposure and Fetal Growth**

Prenatal and postnatal growth deficiency was first recognized in patients with prenatal alcohol exposure in 1973 by Jones and colleagues.[1] The primary growth parameters associated with fetal alcohol syndrome include height, weight, and head circumference.[5] The severity of growth retardation varies by timing and quantity of alcohol exposure. There are conflicting results on the effect of drinking low-to-moderate amounts of alcohol on fetal growth and preterm

birth. A systematic review by Henderson, et al. found inconsistent evidence with some studies showing a protective effect on growth, and others showing a detrimental effect on growth.[41] A cohort study from Western Australia reported little difference in infant outcomes with low-to-moderate drinking compared to those who did not drink during pregnancy. Binge drinking and heavy drinking in this study were significantly associated with preterm birth and small for gestational age, however, this association was eliminated after controlling for smoking.[12] Studies have shown a significant trend toward low birth weight with increasing alcohol consumption.[13,14,15] Binge drinking during second trimester was significantly associated with small birth size; however, no association was found with alcohol exposure during first trimester.[14] Similar results were found in other studies with a dose-response relationship between alcohol consumption and preterm birth, small birth size and low birth weight.[15]

Head circumference is included in central nervous system abnormalities due to the effect on brain development and cognition.[5] Alcohol exposure not only affects overall head circumference but leads to structural abnormalities in a variety of regions of the brain. These regions include the cerebellum, the corpus callosum, the basal ganglia, and the frontal cortex.[16] Research using magnetic resonance imaging (MRI) has shown significant reductions in overall brain size and shape, reduction in frontal lobe size, reduction in cerebellar volume, agenesis of the corpus callosum, and reduction in size of the basal ganglia.[17] The damage to the central nervous system is thought to be a result of the cytotoxicity and mutagenic nature of ethanol on cells.[21]

Ultrasound technology could be used as an early predictor of the effects of prenatal alcohol exposure on the brain. Ultrasonography has been used as a tool to identify conditions such as Down's syndrome and lethal trisomies. Prenatal intracranial measurements, such as

frontothalamic distance and caval-calvarial distance have shown significant associations with fetal frontal lobe size in fetuses with these syndromes that appear normal on other measures.[42] Currently, few studies examine the potential benefit of using this technology to identify the effects of alcohol exposure on the developing fetus. One study showed no association between alcohol exposure and head circumference and transcerebellar diameter in utero, however, the study did not assess the effect on the frontal cortex.[18] Another study using similar intracranial measurements including frontal lobe measurements showed a significant relationship in the variance of frontal lobe size in utero among the alcohol exposed group.[19] A pilot study from the Ukraine further studied this relationship and found significant differences in femur length, caval-calvarial distance, and frontothalamic distance in alcohol exposed fetuses during second trimester ultrasound examinations. Third trimester ultrasound examinations showed similar results for frontothalamic distance and showed a significant reduction in orbital diameter.[20] These results are similar to a mouse model showing brain malformations during critical periods for neural, ocular and craniofacial development.[43]

### 2.3 Prenatal Alcohol Exposure and Infant Development

Prenatal alcohol exposure can result in a variety of structural, neurological and functional deficits. The effect of alcohol exposure on the central nervous system has been studied extensively over the last 30 years, showing short term and long term cognitive outcomes. Neurological damage can include seizures, visual motor difficulties, and motor control issues.[5] Seizures have been observed in 3 to 21% of children with FASD's, with binge drinking during early pregnancy resulting in a 3.15 fold increased risk of neonatal seizures.[44,45,46] Cerebral palsy (CP) is an umbrella term for abnormal motor control, and studies have shown a relationship between heavy prenatal alcohol exposure and CP.[47,48]

Neurological and behavioral abnormalities associated with heavy prenatal alcohol exposure include overall intellectual performance, attention, learning and memory, language, executive functioning, fine and gross motor skills, social skills, and adaptive behavior.[17,21] Neuroimaging studies using magnetic resonance imaging (MRI) have been used to show the structural damage to the brain caused by prenatal alcohol exposure. Numerous studies have used imaging to relate the structural damage to the brain with neurological and behavioral deficits in children with FAS.[49-53] According to one study, children with FAS have a mean IQ score of approximately 74.[54] Additional studies indicate that the average IQ score is between 65 and 72, and scores can range from severe mental retardation to normal ranges.[55,56] Moderate-to-heavy prenatal alcohol exposure can impact learning and memory by reducing the ability to encode information rather than retrieve information.[57]

Individuals with FASD's also have poor receptive and expressive language skills, which may contribute to impaired interpersonal communication.[58] Individuals with FAS have similar attention and activity problems to those with Attention Deficit/Hyperactivity Disorder (ADHD) and are often diagnosed with ADHD and prescribed stimulant medication.[59] Alcohol exposure also contributes to adaptive behavior deficits, including daily living skills, socialization, and communication, which can be exacerbated by ADHD.[60] There are distinct differences between the pattern of adaptive function deficits between individuals with FAS and those with ADHD, and the socialization and communication deficits tend to persist with age for individuals with FAS.[61] Executive function, or higher-order cognitive processes, are significantly impaired in individuals with FAS. Specific executive function deficits include decision making, organization, working memory, and set-shifting.[62] Deficits in non-verbal executive functioning can predict poor adaptive behavior in children with both FAS and ADHD.[63]

## 2.4 Micronutrients and FASD's

Maternal nutrition is required to produce healthy offspring, and pregnant women with poor nutritional status deliver children small for gestational age, with physical malformations, behavioral disorders, and delayed cognitive development. When nutritional status is compromised with alcohol, many essential nutrients are not absorbed, leading to poor development for the fetus.[10] Ethanol alters the uptake of glucose, amino acids and other critical nutrients and alters pathways to regulate biochemical processes.[64] Nutrients such as vitamin A, choline, DHA, zinc, and folic acid influence neuronal development in a variety of ways.[10]

Vitamin A (retinol) is required for cell differentiation and growth, and must be converted to a usable form within the liver in order to be absorbed.[65] Alcohol use during pregnancy can reduce the amount of vitamin A available for the fetus by competing for alcohol dehydrogenase, the rate-limiting step in retinol oxidation.[66] Depletion of vitamin A results in reduced cellular control and cell signaling in fetal development, and impaired neuronal growth and differentiation. Animal models have shown beneficial results with retinol supplementation during pregnancy; however, excess retinol intake can lead to fetal malformations.[65,67] Therefore, vitamin A supplementation programs should be carefully designed.

Choline and metabolites are required for cell structure integrity, cell signaling, and neurotransmission by serving as the precursor to acetylcholine in the developing brain.[68] Alcohol decreases the amount of choline available for these processes by competing for water in choline-related reactions.[10] During pregnancy, large amounts of choline are delivered to the fetus across the placenta and choline biosynthesis is enhanced. Alcohol exposure reduces the availability of methyltetrahydrofolate, the metabolically active form of folate for the human body, increasing the demand for choline.[69] Choline is necessary for neural tube formation in utero, and choline

deficiency can affect stem cell proliferation and increase apoptosis in the brain.[70] Alcohol creates an extra demand for choline during pregnancy which leads to choline deficiency and abnormal fetal development.[69]

Choline supplementation during pregnancy has been studied in both animal and human studies. Animal models using rats have demonstrated conflicting results with some showing mitigated spatial memory deficits and others showing no benefits in spatial learning deficits.[71,72] Additionally, animal models have shown reduced hyperactivity and behavior alterations.[72,73] Choline supplementation also prevented neuronal effects and reduced hypermethylation in the hippocampus and pre-frontal cortex of the brain.[74,75] An observational study examining the effects of choline in pregnancy demonstrated mild increases in child memory and nonverbal communication in 7 year old children.[76] Limited human studies exist relating choline supplementation and alcohol exposure, however; the results of a phase 1 clinical trial indicate tolerable results with minimal adverse effects.[77]

Zinc is involved in DNA and RNA stability, RNA polymerase activity, and serves as a cofactor in enzyme synthesis, such as superoxide dismutase responsible for preventing apoptosis in the brain.[10,78] Zinc deficiency during pregnancy can produce fetal death, intrauterine growth restriction, and teratogenesis, as well as behavioral abnormalities postnatally.[79] Alcohol consumption decreases the amount of zinc available through the placenta, which can result in increased embryonic oxidative stress leading to increased apoptosis, and oxidative damage to DNA, lipids and proteins.[80] Animal models studying the relationship between ethanol exposure and zinc supplementation demonstrate conflicting results in reduction of physical abnormalities, mortality, Purkinje cell loss, and placental uptake.[78,81-83]

Folic Acid (folate) is an essential B vitamin that functions as a coenzyme in nucleic acid synthesis and amino acid metabolism. Folate is also involved in many reactions including DNA methylation required for proper cell division.[84] Folate is responsible for proper fetal growth and development and the demand increases during pregnancy.[85] Animal and cell studies suggest that alcohol impairs folic acid transport across the placenta.[86] Folate deficiency and alcohol consumption during pregnancy may also produce neural tube defects, congenital heart defects, and limb malformations.[85] Studies have shown that folic acid may mitigate the effects of alcohol use during pregnancy by reducing the oxidative stress to the fetus.[87] Chronic and heavy alcohol use is significantly associated with impaired folate transport, reduced folate in cord blood, and reduced cognitive function using Bayley Scales of Infant Development.[85] One animal study of chronic ethanol exposure indicated no significant reduction in teratogenic effects such as growth restriction, brain weight, and hippocampus weight with folate supplementation.[88] However, additional animal studies have shown significant reductions in intrauterine growth restriction, ethanol-induced cardiac defects, and increased placentation and embryogenesis with folate supplementation.[89,90] Few human studies exist to analyze the mitigating effect of maternal folate supplementation on alcohol teratogenesis.

### 2.5 The current study

The current study aims are (1) to examine the association between alcohol exposure and prenatal and postnatal growth and infant development outcomes, and (2) to examine the impact of maternal folate levels on the effects of prenatal alcohol exposure. The impact of alcohol on prenatal growth measurements will be obtained through ultrasonography, and will have implications for whether the effects of prenatal alcohol exposure can be detected in utero. The effect of micronutrient supplementation and maternal blood folate levels will also be assessed to

determine the impact of folate on fetal growth. We hypothesize that increased levels of alcohol exposure will lead to a decrease in head circumference, fetal weight, abdominal circumference, femur length, biparietal diameter, and individual brain measurements during second and third trimester. We also hypothesize that fetal growth measurements will be greater among mothers who take micronutrient supplementation, and that maternal folate levels in the normal and elevated range will relate to greater fetal growth measurements compared to mothers with folate in the deficient range.

The impact of prenatal alcohol exposure on postnatal growth outcomes including birth weight, birth length, birth head circumference, and palpebral fissure length will be assessed and compared among micronutrient supplementation groups. We hypothesize that birth weight, length, and head circumference will be lower in the alcohol exposure group, and that micronutrient supplementation will relate to increases in these growth measurements. The relationship between maternal folate levels during second and third trimester and birth growth outcomes will be also be assessed. We hypothesize that maternal folate levels in the normal and elevated range will lead to greater birth growth measurements compared to those deficient in folate.

The impact of prenatal alcohol exposure on infant development outcomes including Bayley Scales of Infant Development-II Mental Development Index (MDI) and Psychomotor Development Index (PDI) will be assessed and compared among groups randomized to micronutrient supplementation. We hypothesize that Bayley MDI and PDI scores will be lower in the alcohol exposure group compared to controls and micronutrient supplementation will lead to an increase in scores on these measures. The relationship between maternal folate levels during second and third trimester and infant development outcomes will be also be assessed. We

hypothesize that maternal folate levels in the normal and elevated range will lead to greater scores compared to those deficient in folate.

### 3.1 Methods

*Study Design*.

A prospective cohort study with randomization of maternal nutritional supplements was conducted between 2007 and 2012 in two sites of Western Ukraine as part of the Collaborative Initiative on Fetal Alcohol Spectrum Disorders (CIFASD).[91] The study protocol was approved by the institutional review board at Lviv Medical University in Ukraine and the institutional review board at the University of California, San Diego. All study participants provided written informed consent. The study recruited 370 women who drank moderate to heavy amounts of alcohol during pregnancy, and 322 women who did not drink alcohol during pregnancy. Subjects were identified for the alcohol exposure group based on quantity and frequency of alcohol consumption during pregnancy. The women were further separated into micronutrient supplement intervention groups in which half of the women within each alcohol exposure group were assigned a daily multivitamin supplement (Theravit®), and half received standard prenatal care.

At each of the two study sites in Ukraine, standard ultrasound examinations were performed throughout pregnancy. Additional study specific brain growth and facial measurements were incorporated during second and third trimester of pregnancy to identify alcohol specific fetal growth anomalies. Blood and urine samples were also taken from women during second and third trimester of pregnancy. Micronutrient concentration including zinc, copper, calcium, magnesium, iron, vitamin B12, choline, folate, β-carotene, vitamin C, lycopene,

thiamin, lutein, vitamin D and retinol was assessed. The blood samples were also tested for high-sensitivity C-reactive protein, total protein and triglycerides.

After birth, information was collected on growth, physical features, and other developmental factors. Growth information included birth weight, birth length, and birth head circumference. Physical feature information including palpebral fissure length, philtrum, and upper lip measurements were obtained. At six-months and twelve-months of age, children were invited to return to the study site for developmental assessment using Bayley Scales of Infant Development-II.[92]

*Study participants*

Women were recruited during their first prenatal visit at two prenatal care facilities in Western Ukraine. From 2007 to 2014, greater than 11,000 pregnant women were screened for alcohol use. The screening instrument was administered by a nurse using a set of 17 questions on maternal demographics, tobacco and illicit drug use, medication use, and pregnancy history. For those reported as ever using alcohol an additional eight questions were included about quantity and frequency of alcohol use.[93] All women who reported at least weekly binge-drinking episodes in which 5 or more drinks were consumed, at least 5 episodes in which 3-4 drinks were consumed, or at least 10 episodes of 1-2 drinks consumed in either the month of conception or the most recent month of pregnancy were invited to participate. The comparison group was selected from the non-drinking women meeting screening criteria (no binge episodes and minimal or no alcohol use in the month around conception or the most recent month of pregnancy) at a 1:1 ratio.

After enrollment in the study, an in-person interview by the study nurse at each facility was conducted to obtain demographic information including education, occupation, maternal age, parity, gravidity, multivitamin use and tobacco use. Socioeconomic status was calculated from education and occupation information using the Hollingshead Scale.[94]

*Measures*

Independent variables in the analysis include alcohol exposure group identified during initial maternal interview as women reporting binge-drinking episodes or heavy alcohol consumption in either the month of conception or the most recent month of pregnancy, the comparison group was selected from women not meeting screening criteria. Multivitamin supplementation group was randomly assigned at recruitment with half of each alcohol exposure group receiving a daily multivitamin supplement (Theravit®) and half receiving standard prenatal care. Absolute ounces of alcohol per day at conception is determined from reported type and quantity of drinking during each week during the month around conception which is converted into absolute ounces of alcohol per week, then divided by seven days. Serum folate concentration (ng/mL) is obtained by blood sample at two time points during pregnancy, typically during mid-second trimester and mid-third trimester. Serum folate concentration during each trimester is then categorized into clinically significant blood folate levels with elevated blood folate equivalent to >20 ng/mL, normal blood folate equivalent to 6-20 ng/mL, and deficient blood folate equivalent to <6 ng/mL.

Dependent variables in the analysis include fetal growth, birth growth, and infant development outcomes. Fetal growth measurements were obtained by ultrasound examination at initial prenatal visit and follow-up visits during second and third trimester of pregnancy. Standard prenatal growth outcomes obtained by ultrasound include head circumference (mm),

estimated fetal weight (g), femur length (mm), abdominal circumference (mm), and biparietal diameter (mm) measured as the distance between the two parietal eminences. Additional study specific growth measurements were obtained by ultrasound including transverse cerebellar diameter (TCD) measured as the maximum diameter between cerebellar hemispheres, frontothalamic distance (FTD) measured as the distance between the inner surface of the frontal bone to the posterior thalamus, occipitofrontal diameter (OFD) measured as the distance between the occipital bone to the frontal bone, caval-calvarial distance (CCD) measured as the distance between the inner surface of the frontal bone and the posterior margin of the cavum septi pellucidum, and orbital diameter (OD) measurements. Birth growth outcomes include birth weight (g), birth length (cm), birth occipitofrontal head circumference (cm), and palpebral fissure length (cm). Infant development outcomes are measured at 6 and 12 months of age with Bayley Scales of Infant Development-II (BSID-II). The BSID-II Mental Development Index (MDI) and Psychomotor Development Index (PDI) assessment measures mental, physical, emotional, and social development of children less than three years of age.

To address potential confounding, gestational age in weeks at initial maternal interview, at exam, or at birth was included in the analysis depending on the outcome of interest. Child sex identified at birth was factored into the analysis along with socioeconomic status based on Hollingshead category ratings derived from education and occupation information with one being the highest and five being the lowest; Hollingshead categories four and five are considered below average socioeconomic status. Subjects were recruited from two study sites in Ukraine, the *Rivne Regional Medical Diagnostic Center* and the *Khmelnytsky Perinatal Center*. Testing site was factored into the analysis to control for differences in methods or populations between each site. Maternal smoking status during pregnancy categorized as current smoker, past

smoker-quit during pregnancy, past smoker-quit pre-pregnancy, or never smoker was included to control for the effect of tobacco use on fetal growth. Maternal age at interview, gravidity, and multivitamin use in early pregnancy were also factored into the analysis.

*Data Analysis*

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) statistical software for windows version 22.0. Maternal folate levels during second and third trimester among alcohol exposed and unexposed pregnancies by multivitamin supplementation group was assessed using chi-square tests. Maternal demographic, nutritional, reproductive, and lifestyle characteristics were compared between alcohol exposed and unexposed pregnancies by second trimester blood folate levels using ANOVA for continuous variables and Chi-square tests for categorical variables. Maternal demographic, nutritional, reproductive and lifestyle characteristics, were compared between alcohol exposed and unexposed pregnancies by multivitamin supplementation group using ANOVA for continuous variables and Chi-square tests for categorical variables. Fetal growth outcomes were compared using ANCOVA, adjusting for gestational age at exam, among alcohol exposed and unexposed pregnancies by multivitamin supplementation group. The association between absolute ounces of alcohol per day at conception and fetal growth measurements adjusted for gestational age was assessed using simple linear regression. Birth growth outcomes were compared using ANCOVA, adjusting for gestational age at birth, among alcohol exposed and unexposed subjects by multivitamin supplementation group. Gestational age at birth, palpebral fissure length, and Bayley Scales of Infant Development at 6 and 12 months of age were analyzed using ANOVA to compare alcohol exposed and unexposed subjects and multivitamin supplementation group. The association between absolute ounces of alcohol per day at conception and birth growth

measurements adjusted for gestational age, palpebral fissure length, and Bayley Scales of Infant Development-II were assessed using simple linear regression. Variables significantly associated with alcohol exposure or absolute ounces of alcohol per day at conception were analyzed using generalized linear regression models to assess the impact of alcohol use, multivitamin supplementation, and blood folate levels on these outcomes.

### 3.2 Results

A total of 754 women were recruited from 2008 to 2014, of these women a total of 691 had live born children. Among the 691 live infants, a total of 670 mothers had at least one blood folate sample, and at least one ultrasound performed. The alcohol exposure groups consisted of 306 women with confirmed alcohol use, 49 women with highly suspected alcohol use, and 315 women with no alcohol use during pregnancy. The women with highly suspected alcohol use were removed from the analysis due to the lack of reliable reporting of alcohol consumption. Alcohol use was considered highly suspected by the interview nurse if alcohol use was acknowledged but information about timing and quantity of use was not believed to be accurate. The first maternal interview was performed at a mean gestational age of 18.57 weeks. Blood samples and ultrasound measurements were taken at interview date and follow-up visits during second and third trimester.

Blood folate levels in alcohol-exposed and unexposed pregnancies by multivitamin supplementation group are reported in Table 1. There was no significant difference between blood folate levels in alcohol exposed and unexposed pregnancies by multivitamin supplementation group during second trimester; however, blood folate levels were significantly different between alcohol exposure groups and multivitamin supplementation groups during third trimester ($p=0.001$). The alcohol exposure group had a greater percentage of subjects in the

deficient range with 26.6% deficient in the MVM supplement group and 45.3% deficient in the no MVM supplement group. Among the alcohol unexposed group, a greater percentage of subjects were in the elevated range with 38.4% elevated in the MVM group and 18.2% elevated in the no MVM group, and a greater percentage of subjects were in the normal range with 26.5% normal in the MVM group and 60.6% normal in the No MVM group.

Table 1: Blood folate levels in alcohol-exposed and comparison pregnancies, assessed during second and third trimester by multivitamin supplementation group.

| Folate Levels | Alcohol Exposed (n=170)* | | Alcohol Unexposed (n=170)* | | Statistic | p-value |
|---|---|---|---|---|---|---|
| | MVM Supplement (n=95) | No MVM Supplement (n=75) | MVM Supplement (n=97) | No MVM Supplement (n=73) | | |
| **Second Trimester** | | | | | | |
| Elevated (>20 ng/mL) | 20.0% | 20.0% | 35.1% | 26.0% | $X^2_{(2)}<1$ | NS |
| Normal (6-20 ng/mL) | 51.6% | 57.3% | 56.7% | 57.5% | | |
| Deficient (<6 ng/mL) | 28.4% | 22.7% | 8.2% | 16.4% | | |
| **Third Trimester** | | | | | | |
| Elevated (>20 ng/mL) | 25.0% | 14.7% | 38.4% | 18.2% | $X^2_{(2)}=13.28$ | 0.001 |
| Normal (6-20 ng/mL) | 48.4% | 40.0% | 26.5% | 60.6% | | |
| Deficient (<6 ng/mL) | 26.6% | 45.3% | 15.1% | 21.2% | | |

* Sample size varies depending on missing values.
MVM=Multivitamin Supplementation Group

Maternal demographic, nutritional, health and reproductive characteristics are reported in Table 2, by alcohol exposure and blood folate level during second trimester. Women who drank alcohol during pregnancy were more likely to be unmarried, have a greater number of prior pregnancies, and less likely to use multivitamins during the first maternal interview before enrollment in the study. Multivitamin supplement use during early pregnancy was significantly associated with an increase in blood folate level during second trimester ($p<0.001$). Gestational age at first maternal interview was significantly associated with both alcohol exposure group and blood folate levels ($p<0.001$), with later gestational age at maternal interview for the alcohol exposure group and later gestational age for the deficient and normal blood folate levels compared to the elevated group. Gestational age at second trimester ultrasound was significantly

greater in the alcohol exposure group (p=0.011), but was not significantly associated with blood folate levels.

Maternal demographic, nutritional, health and reproductive characteristics are reported in Table 3, according to alcohol exposure and multivitamin supplementation group. Gestational age at maternal interview was significantly higher in the alcohol exposed group compared to unexposed and significantly lower in the multivitamin supplementation group compared to those receiving standard prenatal care ($p<0.001$). Serum folate concentration during 3rd trimester visit was significantly lower in the alcohol exposure group (p=0.008) and higher in the multivitamin supplementation group ($p<0.001$). The number of prenatal visits was higher in the no supplement group (p=0.002) compared to the supplement group, but not associated with alcohol exposure. The alcohol exposure group had higher number of previous pregnancies on average compared to the unexposed group (p=0.024).

Fetal growth outcomes, adjusted for gestational age at second trimester ultrasound, for alcohol exposed and unexposed pregnancies by multivitamin supplementation group are presented in Table 4. Alcohol exposure was significantly associated with an increase in estimated fetal weight ($R^2=0.757$, $F_{(1,279)}=5.67$, p=0.018) at second trimester. Multivitamin supplementation was significantly associated with a decrease in orbital diameter ($R^2=0.273$, $F_{(1,488)}=4.43$, p=0.036) at second trimester. No significant interactions were found between alcohol exposure groups and multivitamin supplementation groups during second trimester.

Fetal growth outcomes, adjusted for gestational age at third trimester ultrasound, for alcohol exposed and unexposed pregnancies by multivitamin supplementation group are presented in Table 5. Alcohol exposure group was significantly associated with a decrease in estimated fetal weight at third trimester ($R^2=0.655$, $F_{(1,491)}=4.02$, p=0.045), abdominal

circumference at third trimester ($R^2$=0.690, $F_{(1,493)}$=7.02, p=0.008), biparietal diameter at third trimester ($R^2$=0.529, $F_{(1,493)}$=7.33, p=0.007), transverse cerebellar diameter at third trimester ($R^2$=0.552, $F_{(1,399)}$=8.86,p=0.003), caval-calvarial distance at third trimester ($R^2$=0.325, $F_{(1,491)}$=5.46,p=0.020), and orbital diameter at third trimester ($R^2$=0.158, $F_{(1,485)}$=5.72,p=0.017). Multivitamin supplementation groups were not significantly associated with any third trimester growth outcomes. No significant interactions were found between alcohol exposure groups and multivitamin supplementation groups during third trimester.

Table 2: Maternal demographic, nutritional, health and reproductive characteristics by alcohol exposure and blood folate level during second trimester.

| Characteristic | Alcohol Exposed (n=170) | | | Alcohol Unexposed (n=170) | | | Statistic | p-value |
|---|---|---|---|---|---|---|---|---|
| | Elevated Folate >20 ng/mL (n=34) | Normal Folate 6-20 ng/mL (n=92) | Deficient Folate <6 ng/mL (n=44) | Elevated Folate >20 ng/mL (n=53) | Normal Folate 6-20 ng/mL (n=97) | Deficient Folate <6 ng/mL (n=20) | | |
| Unmarried | 70.6% | 76.1% | 59.1% | 5.7% | 8.2% | 10.0% | $X^2_{(2)}=8.49$ | 0.014 |
| Low Socioeconomic Status* | 32.4% | 28.3% | 40.9% | 11.3% | 19.6% | 5.0% | $X^2_{(2)}=2.09$ | NS |
| High School Graduate | 94.1% | 85.9% | 95.5% | 100% | 95.9% | 100% | $X^2_{(2)}=5.88$ | NS |
| Multivitamin Use during pregnancy | 97.1% | 63.0% | 43.2% | 98.1% | 75.3% | 40.0% | $X^2_{(2)}=56.70$ | <0.001 |
| Unemployed in last 12 months | 32.4% | 37.0% | 45.5% | 43.4% | 32.0% | 30.0% | $X^2_{(2)}=1.07$ | NS |
| Smoker | | | | | | | | |
| Current | 17.6% | 23.9% | 25.6% | 0% | 2.1% | 0% | $X^2_{(6)}=7.19$ | NS |
| Past, quit during pregnancy | 29.4% | 26.1% | 30.2% | 5.8% | 2.1% | 0% | | |
| Past, quit pre-pregnancy | 17.6% | 12.0% | 9.3% | 9.6% | 6.3% | 0% | | |
| Never | 35.3% | 38.0% | 34.9% | 84.6% | 89.6% | 100% | | |
| Maternal age (years) | 28.06 | 24.97 | 24.52 | 25.17 | 25.63 | 24.05 | EtOH: $F_{(1,619)}<1$ | NS |
| | (5.59) | (4.88) | (6.09) | (3.53) | (4.74) | (4.35) | FOL: $F_{(2,337)}=2.88$ | NS |
| GA at interview (weeks) | 15.06 | 18.32 | 20.23 | 16.01 | 17.64 | 18.09 | EtOH: $F_{(1,619)}=30.48$ | <0.001 |
| | (4.82) | (5.45) | (5.28) | (3.88) | (5.16) | (4.29) | FOL: $F_{(2,337)}=12.04$ | <0.001 |
| GA at 2nd trimester ultrasound | 20.20 | 21.00 | 21.19 | 20.31 | 20.53 | 20.60 | EtOH: $F_{(1,503)}=6.46$ | 0.011 |
| (weeks) | (1.51) | (2.18) | (2.79) | (1.43) | (1.55) | (1.73) | FOL: $F_{(2,304)}=2.80$ | NS |
| Serum folate 2nd trimester (ng/mL) | 38.78 | 11.07 | 4.37 | 32.20 | 12.01 | 4.47 | EtOH: $F_{(1,337)}=3.32$ | NS |
| | (14.54) | (3.98) | (1.13) | (13.24) | (4.13) | (1.04) | FOL: $F_{(2,336)}=357.62$ | <0.001 |
| Number of prenatal visits | 3.79 | 3.98 | 4.18 | 3.60 | 4.15 | 4.30 | EtOH: $F_{(1,612)}<1$ | NS |
| | (2.54) | (2.31) | (2.39) | (1.96) | (2.66) | (3.37) | FOL: $F_{(2,337)}=1.07$ | NS |
| Gravidity | 2.44 | 1.99 | 1.86 | 1.85 | 2.00 | 1.40 | EtOH: $F_{(1,618)}=5.12$ | 0.024 |
| | (1.62) | (1.29) | (1.59) | (0.84) | (1.37) | (0.60) | FOL: $F_{(2,337)}=1.51$ | NS |
| Parity | 0.59 | 0.60 | 0.59 | 0.66 | 0.69 | 0.35 | EtOH: $F_{(1,618)}<1$ | NS |
| | (0.89) | (0.93) | (0.90) | (1.34) | (1.20) | (0.59) | FOL: $F_{(2,337)}<1$ | NS |
| **Alcohol use peri-conception** | | | | | | | | |
| Oz EtOH/per day | 0.715 | 0.596 | 0.554 | 0.005 | 0 | 0 | EtOH: $F_{(1,619)}=354.21$ | <0.001 |
| | (0.637) | (0.498) | (0.408) | (0.028) | | | FOL: $F_{(2,337)}=1.03$ | NS |
| Oz EtOH/per drinking day | 2.809 | 1.698 | 1.263 | 0.038 | 0 | 0 | EtOH: $F_{(1,619)}=276.99$ | <0.001 |
| | (3.626) | (1.521) | (0.725) | (0.195) | | | FOL: $F_{(2,337)}<1$ | NS |
| **Alcohol use first trimester** | | | | | | | | |
| Oz EtOH/per day | 0.080 | 0.060 | 0.088 | 0 | 0 | 0 | EtOH: $F_{(1,619)}=23.73$ | <0.001 |
| | (0.209) | (0.122) | (0.154) | | | | FOL: $F_{(2,337)}=1.92$ | NS |
| Oz EtOH/per drinking day | 0.536 | 0.354 | 0.545 | 0.002 | 0 | 0 | EtOH: $F_{(1,619)}=98.62$ | <0.001 |
| | (1.053) | (0.586) | (0.721) | (0.011) | | | FOL: $F_{(2,337)}=3.11$ | 0.046 |

Values are % or mean (SD).

GA=Gestational Age; Oz=absolute ounces; EtOH=alcohol exposure groups; FOL=Blood Folate (ng/mL)

*Socioeconomic status is based on Hollingshead categories 1-5 derived from education and occupation information with 1 being the highest; Hollingshead categories 4 and 5 are considered below average socioeconomic status.

Table 3: Maternal demographic, nutritional, health and reproductive characteristics by alcohol exposure and multivitamin supplementation group.

| Characteristic | Alcohol Exposed (n=306) | | Alcohol Unexposed (n=315) | | Statistic | p-value |
|---|---|---|---|---|---|---|
| | MVM supplement (n=143) | No supplement (n=163) | MVM supplement (n=159) | No supplement (n=156) | | |
| Unmarried | 37.8% | 35.0% | 7.5% | 12.8% | $X^2(1)<1$ | NS |
| Socioeconomic Status Below average* | 39.2% | 37.4% | 17.6% | 14.7% | $X^2(1)<1$ | NS |
| High School Graduate | 80.4% | 88.3% | 98.1% | 96.8% | $X^2(1)=1.44$ | NS |
| Multivitamin Use during pregnancy | 54.9% | 53.4% | 76.7% | 67.3% | $X^2(1)=2.61$ | NS |
| Unemployed in last 12 months | 47.6% | 52.1% | 38.4% | 36.5% | $X^2(1)<1$ | NS |
| Smoker | | | | | | |
| Current | 26.1% | 24.5% | 0.0% | 2.6% | $X^2(3)<1$ | NS |
| Past, quit during pregnancy | 30.3% | 25.8% | 3.2% | 3.2% | | |
| Past, quit pre-pregnancy | 9.2% | 11.0% | 7.6% | 4.5% | | |
| Never | 34.5% | 38.7% | 89.2% | 89.6% | | |
| Maternal age (years) | 26.11 | 25.98 | 25.87 | 26.47 | EtOH: $F_{(1,619)}<1$ | NS |
| | (5.82) | (5.81) | (4.29) | (5.04) | MVM: $F_{(1,619)}<1$ | NS |
| GA at interview (weeks) | 18.62 | 21.16 | 16.74 | 17.70 | EtOH: $F_{(1,619)}=30.48$ | <0.001 |
| | (7.02) | (6.54) | (5.57) | (5.44) | MVM: $F_{(1,619)}=13.14$ | <0.001 |
| Serum Folate 3rd trimester (ng/mL) | 15.35 | 11.07 | 19.83 | 13.33 | EtOH: $F_{(1,289)}=7.15$ | 0.008 |
| | (12.18) | (10.36) | (15.54) | (9.64) | MVM: $F_{(1,289)}=15.74$ | <0.001 |
| GA at 3rd trimester ultrasound (weeks) | 33.39 | 33.00 | 33.10 | 32.85 | EtOH: $F_{(1,494)}<1$ | NS |
| | (2.86) | (2.59) | (2.76) | (2.27) | MVM: $F_{(1,494)}=1.75$ | NS |
| Number of prenatal visits | 3.46 | 4.24 | 3.71 | 4.19 | EtOH: $F_{(1,612)}<1$ | NS |
| | (2.43) | (2.35) | (2.53) | (2.83) | MVM: $F_{(1,612)}=9.25$ | 0.002 |
| Gravidity | 2.43 | 2.12 | 2.04 | 1.96 | EtOH: $F_{(1,618)}=5.12$ | 0.024 |
| | (1.87) | (1.46) | (1.19) | (1.24) | MVM: $F_{(1,618)}=2.32$ | NS |
| Parity | 0.87 | 0.64 | 0.65 | 0.77 | EtOH: $F_{(1,618)}<1$ | NS |
| | (1.31) | (0.93) | (0.83) | (1.28) | MVM: $F_{(1,618)}<1$ | NS |
| | Alcohol use peri-conception | | | | | |
| Oz EtOH/per day | 0.691 | 0.616 | 0 | 0 | EtOH: $F_{(1,619)}=354.21$ | <0.001 |
| | (0.634) | (0.589) | | | MVM: $F_{(1,619)}<1$ | NS |
| Oz EtOH/per drinking day | 1.861 | 1.861 | 0.021 | 0.021 | EtOH: $F_{(1,619)}=276.99$ | <0.001 |
| | (2.213) | (1.708) | (0.134) | (0.138) | MVM: $F_{(1,619)}<1$ | NS |
| | Alcohol use first trimester | | | | | |
| Oz EtOH/per day | 0.137 | 0.241 | 0 | 0 | EtOH: $F_{(1,619)}=23.73$ | <0.001 |
| | (0.347) | (0.901) | | | MVM: $F_{(1,619)}=2.10$ | NS |
| Oz EtOH/per drinking day | 0.553 | 0.780 | 0 | 0 | EtOH: $F_{(1,619)}=98.62$ | <0.001 |
| | (0.904) | (1.403) | | | MVM: $F_{(1,619)}=3.65$ | NS |

Values are % or mean (SD).

MVM=Multivitamin supplementation group; GA=Gestational Age; Oz=absolute ounces; EtOH=alcohol.

*Socioeconomic status is based on Hollingshead categories 1-5 derived from education and occupation information with 1 being the highest; Hollingshead categories 4 and 5 are considered below average socioeconomic status.

Table 4: Fetal growth outcomes, adjusted for gestational age during second trimester ultrasound, for alcohol exposed and unexposed pregnancies by multivitamin supplementation group.

| Outcomes | Alcohol Exposed (n=217) | | Alcohol Unexposed (n=288) | | Statistic | p-value |
|---|---|---|---|---|---|---|
| | MVM supplement (n=108) | No supplement (n=109) | MVM supplement (n=145) | No supplement (n=143) | | |
| GA at ultrasound (weeks) | 20.87 (2.26) | 20.74 (2.10) | 20.42 (1.37) | 20.37 (1.43) | EtOH: $F_{(1,504)}$=6.44<br>MVM: $F_{(1,504)}$<1<br>E&M: $F_{(1,504)}$<1 | 0.011<br>NS<br>NS |
| Head Circumference (mm) | 181.69 (1.09) | 182.31 (1.09) | 177.11 (0.87) | 177.28 (0.87) | EtOH: $F_{(1,504)}$ <1<br>MVM: $F_{(1,504)}$<1<br>E&M: $F_{(1,504)}$<1 | NS<br>NS<br>NS |
| Estimated Fetal Weight (g) | 393.53 (9.76) | 402.20 (10.15) | 344.26 (6.83) | 351.41 (7.06) | EtOH: $F_{(1,279)}$=5.67<br>MVM: $F_{(1,279)}$<1<br>E&M: $F_{(1,279)}$<1 | 0.018<br>NS<br>NS |
| Femur Length (mm) | 33.56 (0.27) | 33.87 (0.27) | 32.68 (0.23) | 32.92 (0.23) | EtOH: $F_{(1,504)}$<1<br>MVM: $F_{(1,504)}$<1<br>E&M: $F_{(1,504)}$<1 | NS<br>NS<br>NS |
| Abdominal Circumference (mm) | 157.66 (1.06) | 158.80 (1.06) | 153.22 (0.83) | 153.02 (0.83) | EtOH: $F_{(1,504)}$=2.26<br>MVM: $F_{(1,504)}$<1<br>E&M: $F_{(1,504)}$<1 | NS<br>NS<br>NS |
| Biparietal Diameter (mm) | 49.28 (0.33) | 49.66 (0.33) | 48.15 (0.26) | 47.98 (0.26) | EtOH: $F_{(1,504)}$=1.38<br>MVM: $F_{(1,504)}$<1<br>E&M: $F_{(1,504)}$<1 | NS<br>NS<br>NS |
| Transverse Cerebellar Diameter (mm) | 20.95 (0.36) | 21.30 (0.41) | 20.50 (0.28) | 20.58 (0.35) | EtOH: $F_{(1,115)}$=1.87<br>MVM: $F_{(1,115)}$<1<br>E&M: $F_{(1,504)}$<1 | NS<br>NS<br>NS |
| Occipitofrontal Diameter (mm) | 63.55 (0.41) | 63.95 (0.41) | 62.08 (0.33) | 62.55 (0.34) | EtOH: $F_{(1,493)}$<1<br>MVM: $F_{(1,493)}$=1.38<br>E&M: $F_{(1,493)}$<1 | NS<br>NS<br>NS |
| Caval-Calvarial Distance (mm) | 24.11 (0.25) | 24.48 (0.26) | 23.55 (0.20) | 23.50 (0.20) | EtOH: $F_{(1,489)}$=2.03<br>MVM: $F_{(1,489)}$<1<br>E&M: $F_{(1,489)}$<1 | NS<br>NS<br>NS |
| Frontothalamic Distance (mm) | 38.09 (0.47) | 38.08 (0.48) | 37.41 (0.37) | 36.63 (0.38) | EtOH: $F_{(1,489)}$<1<br>MVM: $F_{(1,489)}$<1<br>E&M: $F_{(1,489)}$<1 | NS<br>NS<br>NS |
| Orbital Diameter (mm) | 9.99 (0.13) | 10.13 (0.13) | 9.60 (0.10) | 9.89 (0.10) | EtOH: $F_{(1,488)}$=1.30<br>MVM: $F_{(1,488)}$=4.43<br>E&M: $F_{(1,488)}$<1 | NS<br>0.036<br>NS |

*Sample size may vary due to missing values

Values are mean (SD) for univariate ANOVA, or mean (SE) for univariate ANCOVA.

MVM=Multivitamin supplementation groups; GA=Gestational Age; EtOH=alcohol exposure groups; E&M=Interaction between alcohol exposure groups and multivitamin supplementation groups.

Table 5: Fetal growth outcomes, adjusted for gestational age during third trimester ultrasound, for alcohol exposed and unexposed pregnancies by multivitamin supplementation group.

| Outcomes | Alcohol Exposed (n=229) | | Alcohol Unexposed (n=267) | | Statistic | p-value |
|---|---|---|---|---|---|---|
| | MVM supplement (n=114) | No supplement (n=115) | MVM supplement (n=138) | No supplement (n=129) | | |
| GA at ultrasound (weeks) | 33.39 (2.86) | 33.00 (2.59) | 33.10 (2.76) | 32.85 (2.27) | EtOH: $F_{(1,495)}$ <1<br>MVM: $F_{(1,495)}$=1.85<br>E&M: $F_{(1,495)}$<1 | NS<br>NS<br>NS |
| Head Circumference (mm) | 302.52 (1.86) | 299.77 (1.86) | 303.70 (1.36) | 301.76 (1.41) | EtOH: $F_{(1,493)}$ =3.33<br>MVM: $F_{(1,493)}$=2.03<br>E&M: $F_{(1,493)}$<1 | 0.069<br>NS<br>NS |
| Estimated Fetal Weight (g) | 2113.24 (35.12) | 2099.34 (34.81) | 2141.19 (21.49) | 2108.77 (22.32) | EtOH: $F_{(1,491)}$=4.02<br>MVM: $F_{(1,491)}$<1<br>E&M: $F_{(1,491)}$<1 | 0.045<br>NS<br>NS |
| Femur Length (mm) | 62.46 (0.33) | 62.46 (0.33) | 62.49 (0.29) | 62.34 (0.29) | EtOH: $F_{(1,493)}$=1.16<br>MVM: $F_{(1,493)}$ <1<br>E&M: $F_{(1,493)}$<1 | NS<br>NS<br>NS |
| Abdominal Circumference (mm) | 287.35 (1.57) | 286.26 (1.57) | 289.74 (1.05) | 287.09 (1.09) | EtOH: $F_{(1,493)}$=7.02<br>MVM: $F_{(1,493)}$=2.00<br>E&M: $F_{(1,493)}$<1 | 0.008<br>NS<br>NS |
| Biparietal Diameter (mm) | 82.78 (0.45) | 83.47 (0.45) | 83.81 (0.33) | 83.76 (0.34) | EtOH: $F_{(1,493)}$=7.33<br>MVM: $F_{(1,493)}$ <1<br>E&M: $F_{(1,493)}$=1.12 | 0.007<br>NS<br>NS |
| Transverse Cerebellar Diameter (mm) | 40.46 (0.32) | 40.85 (0.34) | 40.86 (0.25) | 41.02 (0.26) | EtOH: $F_{(1,399)}$=8.86<br>MVM: $F_{(1,399)}$=1.02<br>E&M: $F_{(1,399)}$<1 | 0.003<br>NS<br>NS |
| Occipitofrontal Diameter (mm) | 105.67 (0.51) | 104.84 (0.51) | 105.25 (0.48) | 105.08 (0.50 | EtOH: $F_{(1,487)}$=1.03<br>MVM: $F_{(1,487)}$ <1<br>E&M: $F_{(1,487)}$<1 | NS<br>NS<br>NS |
| Caval-Calvarial Distance (mm) | 38.07 (0.37) | 38.49 (0.37) | 38.99 (0.31) | 38.72 (0.32) | EtOH: $F_{(1,491)}$=5.46<br>MVM: $F_{(1,491)}$ <1<br>E&M: $F_{(1,491)}$=1.08 | 0.020<br>NS<br>NS |
| Frontothalamic Distance (mm) | 59.20 (0.54) | 60.03 (0.54) | 59.26 (0.50) | 59.58 (0.52) | EtOH: $F_{(1,491)}$ <1<br>MVM: $F_{(1,491)}$=1.22<br>E&M: $F_{(1,491)}$<1 | NS<br>NS<br>NS |
| Orbital Diameter (mm) | 15.31 (0.15) | 15.67 (0.15) | 15.77 (0.16) | 15.82 (0.17) | EtOH: $F_{(1,485)}$=5.72<br>MVM: $F_{(1,485)}$=1.71<br>E&M: $F_{(1,485)}$<1 | 0.017<br>NS<br>NS |

*Sample size may vary due to missing values

Values are mean (SD) for univariate ANOVA, or mean (SE) for univariate ANCOVA.

MVM=Multivitamin supplementation groups; GA=Gestational Age; EtOH=alcohol exposure groups; E&M=Interaction between alcohol exposure groups and multivitamin supplementation groups.

A simple linear regression analysis of the association between absolute ounces of alcohol per day at conception and second and third trimester fetal growth outcomes adjusted for gestational age are presented in Table 6. Absolute ounces of alcohol per day at conception is not significantly associated with any second trimester fetal growth outcomes; however, there was a significant negative association between absolute ounces of alcohol per day and several third trimester fetal growth outcomes. Significant regression equations predicting the effect of absolute ounces of alcohol per day on estimated fetal weight (EFW), abdominal circumference (AC), biparietal diameter (BPD), occipitofrontal diameter (OFD), and caval-calvarial distance (CCD) were found. Estimated fetal weight at third trimester was significantly associated with absolute ounces of alcohol per day ($R^2$=0.655, $F_{(2,489)}$=464.887, p<0.001), with a reduction in estimated fetal weight with increased alcohol consumption (β=-54.223, p=0.046). Abdominal circumference at third trimester was significantly associated with absolute ounces of alcohol per day ($R^2$=0.689, $F_{(2,491)}$=544.372, p<0.001), with a reduction in abdominal circumference with increased alcohol consumption (β=-3.078, p=0.010). Biparietal diameter at third trimester was significantly associated with absolute ounces of alcohol per day ($R^2$=0.532, $F_{(2,491)}$=278.516, p<0.001), with a reduction in biparietal diameter with increased alcohol consumption (β=-1.131, p=0.002). Occipitofrontal diameter at third trimester was significantly associated with absolute ounces of alcohol per day ($R^2$=0.493, $F_{(2,485)}$=235.510, p<0.001), with a reduction in occipitofrontal diameter with increased alcohol consumption (β=-1.169, p=0.010). Caval-calvarial distance at third trimester was significantly associated with absolute ounces of alcohol per day ($R^2$=0.326, $F_{(2,489)}$=118.435, p<0.001), with a reduction in estimated fetal weight with increased alcohol consumption (β=-0.812, p=0.009).

Table 6: Simple linear regression analysis to evaluate the association of absolute ounces of alcohol per day at conception on second and third trimester fetal growth outcomes adjusted for gestational age.

| Outcomes | Second Trimester | | | Third Trimester | | |
|---|---|---|---|---|---|---|
| | β | SE | p-value | β | SE | p-value |
| Head Circumference (mm) | 0.605 | 0.955 | NS | -2.828 | 1.472 | NS |
| Estimated Fetal Weight (g) | 17.214 | 9.680 | NS | -54.223 | 27.044 | 0.046 |
| Femur Length (mm) | -0.132 | 0.246 | NS | -0.466 | 0.279 | NS |
| Abdominal Circumference (mm) | 0.216 | 0.921 | NS | -3.078 | 1.195 | 0.010 |
| Biparietal Diameter (mm) | 0.016 | 0.284 | NS | -1.131 | 0.355 | 0.002 |
| Transverse Cerebellar Diameter (mm) | -0.351 | 0.401 | NS | -0.470 | 0.265 | NS |
| Occipitofrontal Diameter (mm) | 0.101 | 0.359 | NS | -1.169 | 0.454 | 0.010 |
| Caval-Calvarial Distance (mm) | 0.052 | 0.218 | NS | -0.812 | 0.311 | 0.009 |
| Frontothalamic Distance (mm) | -0.281 | 0.407 | NS | -0.833 | 0.475 | NS |
| Orbital Diameter (mm) | 0.080 | 0.111 | NS | -0.231 | 0.142 | NS |

Birth growth outcomes, adjusted for gestational age, and infant development outcomes for alcohol exposed and unexposed pregnancies by multivitamin supplementation group are presented in Table 7. Alcohol exposure group was significantly associated with a decrease in gestational age at birth ($R^2$=0.017, $F_{(1,620)}$=10.32, p=0.001), a decrease in birth weight ($R^2$=0.437, $F_{(1,619)}$=17.56, p<0.001), a decrease in birth length ($R^2$=0.437, $F_{(1,619)}$=9.19, p=0.003), and a decrease in birth head circumference ($R^2$=0.385, $F_{(1,619)}$=6.41, p=0.012). Multivitamin supplementation was not significantly associated with birth weight, birth length, or birth head circumference. There were no significant interactions between alcohol exposure groups and multivitamin supplementation groups for birth growth outcomes.

Alcohol exposure group was significantly associated with a decrease in Bayley Scales of Infant Development-II MDI scores at 6 months of age ($R^2$=0.020, $F_{(1,379)}$=7.69, p=0.006), a decrease in MDI scores at 12 months of age ($R^2$=0.022, $F_{(1,292)}$=7.74, p=0.006), and a decrease in PDI scores at 12 months of age ($R^2$=0.035, $F_{(1,292)}$=11.69, p=0.001). Multivitamin supplementation was not significantly associated with Bayley Scales of Infant Development-II scores at 6 or 12 months. There was a significant interaction between alcohol exposure groups and multivitamin supplementation groups for MDI at 12 months of age ($F_{(1,292)}$=5.60, p=0.019). This interaction indicates that MDI at 12 months of age, among multivitamin supplementation groups, is

significantly different in each alcohol exposure group. Post-hoc analysis, shown in Figure 1, indicates that mean MDI score at 12 months in the alcohol exposure group was higher in the multivitamin supplementation group; however, in the alcohol unexposed group, MDI at 12 months of age was lower in the multivitamin supplementation group.

Figure 1: Mean Mental Development Index (MDI) score at 12 months of age for multivitamin supplementation groups by alcohol exposure.

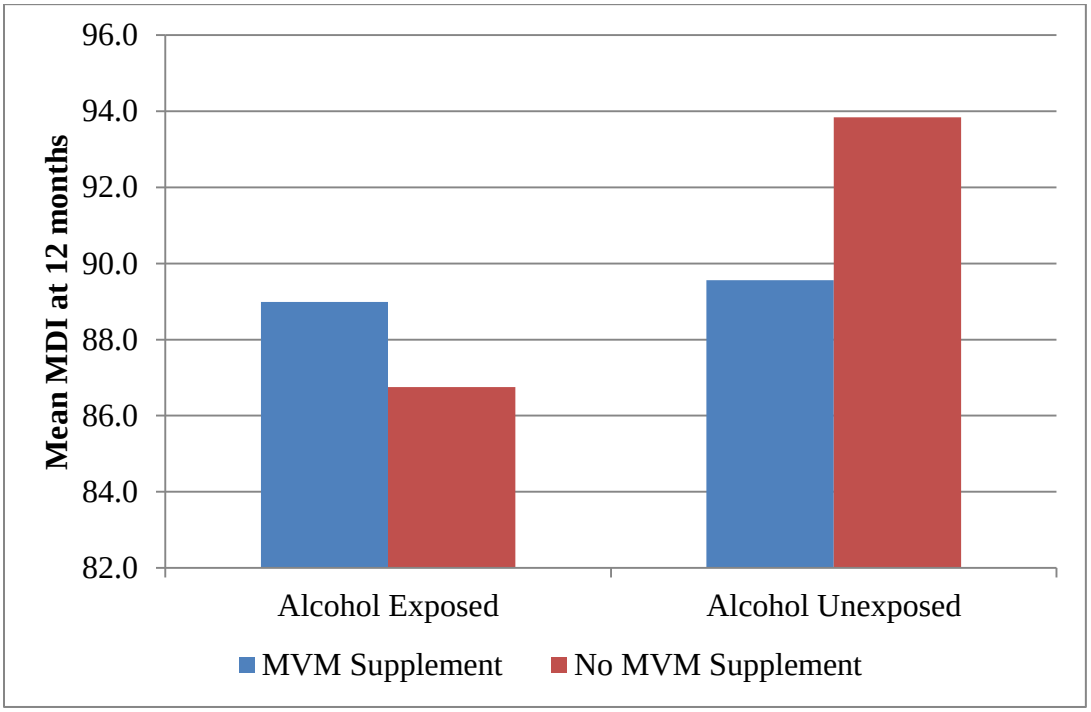


Table 7: Birth growth outcomes, adjusted for gestational age, and infant development outcomes for alcohol exposed and unexposed pregnancies by multivitamin supplementation group.

| Outcomes | Alcohol Exposed (n=305) | | Alcohol Unexposed (n=315) | | Statistic | p-value |
|---|---|---|---|---|---|---|
| | MVM supplement (n=142) | No supplement (n=163) | MVM supplement (n=159) | No supplement (n=156) | | |
| Child Sex (% Male) | 51.0% | 50.3% | 57.2% | 51.9% | $X^2(1)<1$ | NS |
| GA at birth (weeks) | 39.26 | 38.80 | 39.56 | 39.52 | EtOH: $F_{(1,620)}=10.32$ | 0.001 |
| | (1.93) | (2.40) | (1.74) | (1.80) | MVM: $F_{(1,619)}=2.45$ | NS |
| | | | | | E&M: $F_{(1,619)}<1$ | NS |
| Birth Weight (g) | 3136.42 | 3120.20 | 3379.88 | 3369.22 | EtOH: $F_{(1,619)}=17.56$ | <0.001 |
| | (39.54) | (36.89) | (32.05) | (32.36) | MVM: $F_{(1,619)}<1$ | NS |
| | | | | | E&M: $F_{(1,619)}<1$ | NS |
| Birth Length (cm) | 50.65 | 50.70 | 51.86 | 51.81 | EtOH: $F_{(1,619)}=9.19$ | 0.003 |
| | (0.23) | (0.21) | (0.17) | (0.17) | MVM: $F_{(1,619)}<1$ | NS |
| | | | | | E&M: $F_{(1,619)}<1$ | NS |
| Birth OFC (cm) | 33.85 | 33.78 | 34.58 | 34.29 | EtOH: $F_{(1,619)}=6.41$ | 0.012 |
| | (0.14) | (0.14) | (0.11) | (0.11) | MVM: $F_{(1,619)}=2.12$ | NS |
| | | | | | E&M: $F_{(1,619)}<1$ | NS |
| Palpebral Fissure Length (cm) | 2.06 | 2.08 | 2.12 | 2.10 | EtOH: $F_{(1,316)}=2.77$ | NS |
| | (0.21) | (0.22) | (0.18) | (0.21) | MVM: $F_{(1,316)}<1$ | NS |
| | | | | | E&M: $F_{(1,316)}<1$ | NS |
| | | | Bayley Scales of Infant Development-II | | | |
| MDI (6 months) | 89.18 | 87.83 | 91.54 | 90.56 | EtOH: $F_{(1,379)}=7.69$ | 0.006 |
| | (11.00) | (10.03) | (6.31) | (8.18) | MVM: $F_{(1,379)}=1.60$ | NS |
| | | | | | E&M: $F_{(1,379)}<1$ | NS |
| PDI (6 months) | 88.34 | 88.13 | 89.69 | 91.17 | EtOH: $F_{(1,378)}=3.04$ | NS |
| | (13.75) | (13.89) | (10.48) | (10.15) | MVM: $F_{(1,378)}<1$ | NS |
| | | | | | E&M: $F_{(1,378)}<1$ | NS |
| MDI (12 months) | 88.99 | 86.75 | 89.56 | 93.84 | EtOH: $F_{(1,292)}=7.74$ | 0.006 |
| | (13.48) | (12.60) | (9.15) | (11.89) | MVM: $F_{(1,292)}<1$ | NS |
| | | | | | E&M: $F_{(1,292)}=5.60$ | 0.019 |
| PDI (12 months) | 95.38 | 94.83 | 98.36 | 102.30 | EtOH: $F_{(1,292)}=11.69$ | 0.001 |
| | (15.06) | (14.20) | (10.98) | (11.93) | MVM: $F_{(1,292)}<1$ | NS |
| | | | | | E&M: $F_{(1,292)}=2.16$ | NS |

Values are %, mean (SD) for univariate ANOVA, or mean (SE) for univariate ANCOVA.
MVM=Multivitamin supplementation groups; EtOH=alcohol exposure groups; E&M=Interaction between alcohol exposure groups and multivitamin supplementation groups; GA=Gestational Age; OFC=Occipitofrontal Head Circumference; MDI=Mental Development Index; PDI=Psychomotor Development Index

A simple linear regression analysis of the association between absolute ounces of alcohol per day at conception and birth growth adjusted for gestational age, and infant development outcomes are presented in Table 8. A significant negative association between absolute ounces of alcohol per day at conception and birth weight, length, and head circumference was found, as well as a significant negative association between absolute ounces of alcohol per day at conception and Bayley Scales of Infant Development-II MDI and PDI scores at 6 and 12 months of age. Birth weight was significantly associated with absolute ounces of alcohol per day ($R^2$=0.437, $F_{(2,617)}$=239.196, p<0.001), with a reduction in birth weight with increased alcohol consumption (β=-136.78, p<0.001). Birth length was significantly associated with absolute ounces of alcohol per day ($R^2$=0.439, $F_{(2,617)}$=241.108, p<0.001), with a reduction in birth length with increased alcohol consumption (β=-0.621, p=0.001). Birth head circumference was significantly associated with absolute ounces of alcohol per day ($R^2$=0.388, $F_{(2,617)}$=195.563, p<0.001), with a reduction in birth head circumference with increased alcohol consumption (β=-0.347, p=003). MDI at 6 months of age was significantly associated with absolute ounces of alcohol per day ($R^2$=0.069, $F_{(1,378)}$=28.166, p<0.001), with a reduction in scores with increased alcohol consumption (β=-4.226, p<0.001). PDI at 6 months of age was significantly associated with absolute ounces of alcohol per day ($R^2$=0.028, $F_{(1,377)}$=10.730, p=0.001), with a reduction in scores with increased alcohol consumption (β=-3.581, p=0.001). MDI at 12 months of age was significantly associated with absolute ounces of alcohol per day ($R^2$=0.052, $F_{(1,291)}$=15.983, p<0.001), with a reduction in scores with increased alcohol consumption (β=-4.579, p<0.001). PDI at 12 months of age was significantly associated with absolute ounces of alcohol per day ($R^2$=0.071, $F_{(1,291)}$=22.384, p<0.001), with a reduction in scores with increased alcohol consumption (β=-5.964, p<0.001).

Table 8: Simple linear regression analysis to evaluate the association of absolute ounces of alcohol per day at conception on birth growth outcomes adjusted for gestational age and infant development outcomes.

| Outcomes | β | SE | p-value |
|---|---|---|---|
| Birth weight (g) | -136.78 | 33.02 | <0.001 |
| Birth length (cm) | -0.621 | 0.184 | 0.001 |
| Birth head circumference (cm) | -0.347 | 0.114 | 0.003 |
| Palpebral fissure length (cm) | -0.027 | 0.023 | NS |
| **Bayley Scales of Infant Development-II** | | | |
| Mental Development Index (6 months) | -4.226 | 0.726 | <0.001 |
| Psychomotor Development Index (6 months) | -3.581 | 1.093 | 0.001 |
| Mental Development Index (12 months) | -4.579 | 1.145 | <0.001 |
| Psychomotor Development Index (12 months) | -5.964 | 1.261 | <0.001 |

Generalized linear regression models were used to evaluate the impact of alcohol use, multivitamin supplementation, and serum folate concentration on fetal and birth growth outcomes and Bayley Scales of Infant Development-II measures significantly associated with either alcohol exposure group or absolute ounces of alcohol per day at conception. Using a factorial design, the interaction of these factors and their direct effects on outcomes was examined. Covariates tested for inclusion in the model were gestational age, study site, SES, maternal age, child sex, maternal smoking, gravidity, number of prenatal visits, and multivitamin use in early pregnancy.

For fetal growth outcomes at second trimester, estimated fetal weight and orbital diameter were analyzed separately. For estimated fetal weight, the overall model was significant ($X^2_{(8)}$=203.863, p<0.001). Only gestational age at interview, number of prenatal visits, and gestational age at exam contributed significant variances. Alcohol exposure groups were included in the analysis due to the significant association indicated with ANOVA with estimated fetal weight at second trimester. Alcohol exposure groups, multivitamin supplementation, and serum folate concentration were not significantly associated with estimated fetal weight at second trimester.

For fetal growth outcomes at third trimester, estimated fetal weight, abdominal circumference, biparietal diameter, transverse cerebellar diameter, occipitofrontal diameter, caval-calvarial distance, and orbital diameter were analyzed separately. A generalized linear regression analysis to evaluate the impact of alcohol use, multivitamin supplementation, and serum folate concentration (ng/mL) on estimated fetal weight is presented in Table 9. For estimated fetal weight at third trimester, gestational age adjusted percentiles were included in the analysis as response variable and absolute ounces of alcohol per day at conception was included in the analysis as predictor. The overall model was significant ($X^2_{(7)}$=18.044, p=0.012), multivitamin supplementation group was significantly associated with an increase in estimated fetal weight percentile during third trimester ($X^2_{(1)}$=5.554, p=0.018), and absolute ounces of alcohol per day was significantly associated with a decrease in estimated fetal weight percentile ($X^2_{(1)}$=5.680, p=0.017). Serum folate concentration at second and third trimester was not significantly associated with estimated fetal weight percentile. A significant interaction between absolute ounces of alcohol per day at conception and serum folate concentration during 2$^{nd}$ trimester was found ($X^2_{(1)}$=4.468, p=0.035); however, the interaction was insignificant after reducing to the model to include the interaction and the main effects of absolute ounces of alcohol per day and serum folate concentration at second trimester ($X^2_{(1)}$=3.786, p=0.052). Post-hoc examination of the interaction indicate that absolute ounces of alcohol per day at conception is significantly associated with high levels of serum folate ($X^2_{(1)}$=8.123, p=0.004), but not significantly associated with low levels of serum folate. The interaction is plotted in Figure 2; estimated fetal weight during 3$^{rd}$ trimester tends to be higher at low levels of alcohol exposure and lower at high levels of alcohol exposure with high levels of serum folate, indicating that folate has a greater effect at low levels of alcohol exposure.

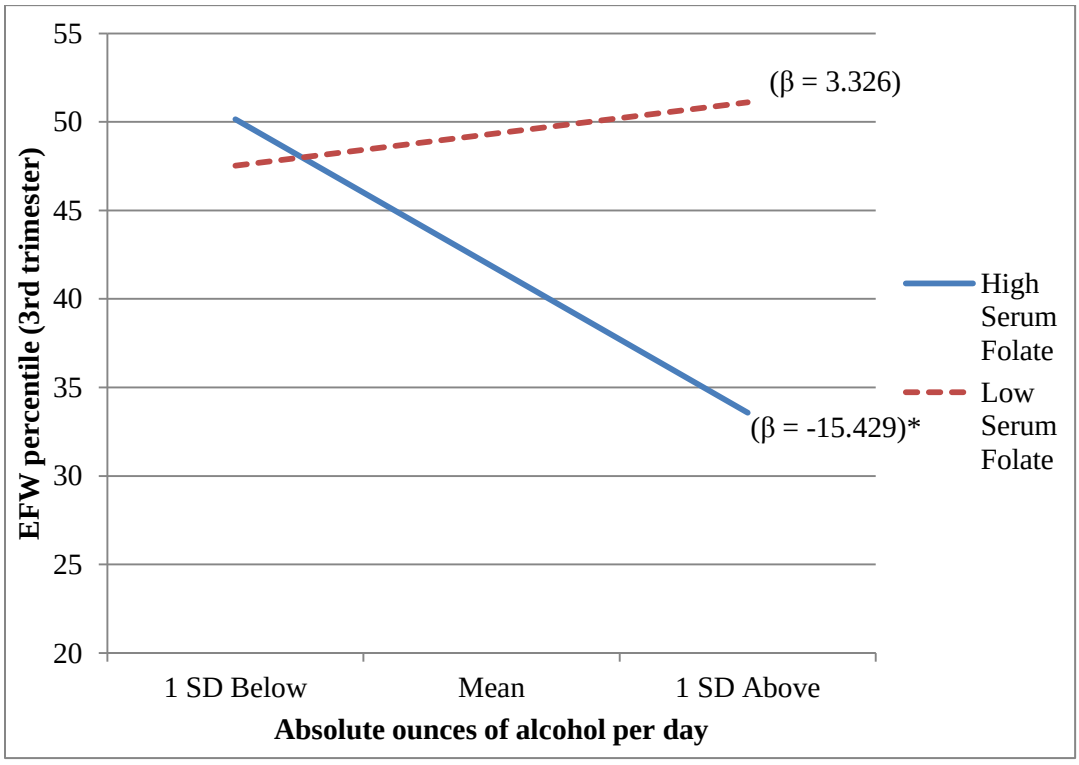
Table 9: Generalized Linear Regression Model for estimated fetal weight percentile at third trimester ultrasound ($X^2_{(7)}$=18.044, p=0.012).

| Variable | β | SE | $X^2$ | p-value |
|---|---|---|---|---|
| MVM Group[1] | 10.588 | 4.235 | 5.554 | 0.018 |
| AA/Day at conception | -18.694 | 7.844 | 5.680 | 0.017 |
| Serum folate 2nd trimester (ng/mL) | -0.259 | 0.165 | 2.488 | NS |
| Serum folate 3rd trimester (ng/mL) | 0.260 | 0.187 | 1.932 | NS |
| AA/Day*Folate 2nd trimester[2] | 0.898 | 0.425 | 4.468 | 0.035 |
| AA/Day*Folate 3rd trimester[3] | -0.155 | 0.461 | 0.113 | NS |

SE=Standard Error; MVM=Multivitamin Supplementation Group; AA/Day at conception=absolute ounces of alcohol per day at conception

[1] No MVM> MVM; [2] AA/Day*Folate 2nd trimester=Interaction between absolute ounces of alcohol per day at conception and serum folate concentration at 2nd trimester; [3] AA/Day*Folate 3rd trimester=Interaction between absolute ounces of alcohol per day at conception and serum folate concentration at 3rd trimester

Figure 2: Regression lines for relationships between absolute ounces of alcohol per day at conception and estimated fetal weight percentile as moderated by serum folate concentration during 2nd trimester.




β=unstandardized regression coefficient; SD=Standard Deviation; EFW=Estimated Fetal Weight
*p<0.05

A generalized linear regression analysis to evaluate the impact of alcohol use, multivitamin supplementation, and serum folate concentration (ng/mL) on abdominal

circumference is presented in Table 10. For abdominal circumference at third trimester, gestational age adjusted percentiles were included in the analysis as response variable and absolute ounces of alcohol per day at conception was included in the analysis as predictor. The overall model was significant ($X^2_{(5)}$=17.955, p=0.003), multivitamin supplementation group was significantly associated with an increase in abdominal circumference percentile during third trimester ($X^2_{(1)}$=7.222, p=0.007). Increased absolute ounces of alcohol per day resulted in a decrease in abdominal circumference percentile ($X^2_{(1)}$=5.489, p=0.019). Serum folate concentration during second and third trimester was not significantly associated with abdominal circumference percentile during third trimester. There were no significant interactions between absolute ounces of alcohol per day at conception and serum folate concentration during second and third trimester.

Table 10: Generalized Linear Regression Model for abdominal circumference percentile at third trimester ultrasound ($X^2_{(5)}$=17.955, p=0.003).

| Variable | B | SE | $X^2$ | p-value |
|---|---|---|---|---|
| MVM Group[1] | 12.062 | 4.489 | 7.222 | 0.007 |
| AA/Day at conception | -9.072 | 3.872 | 5.489 | 0.019 |
| Serum folate 2nd trimester (ng/mL) | 0.018 | 0.128 | 0.021 | NS |
| Serum folate 3rd trimester (ng/mL) | -0.001 | 0.146 | 0.000 | NS |

SE=Standard Error; MVM=Multivitamin Supplementation Group; AA/Day at conception=absolute ounces of alcohol per day at conception
[1]No MVM> MVM.

For biparietal diameter at third trimester, gestational age adjusted percentiles were included in the analysis as response variable and absolute ounces of alcohol per day at conception was included in the analysis as predictor. The overall model was significant ($X^2_{(8)}$=33.533, p<0.001) with child sex, gestational age at exam, and gravidity contributing significant variance. Multivitamin supplementation group, absolute ounces of alcohol per day, and serum folate concentration during second and third trimester were not significantly

associated with biparietal diameter percentile. There were no significant interactions between absolute ounces of alcohol per day at conception and serum folate concentration during second and third trimester.

For transverse cerebellar diameter at third trimester, alcohol exposure groups were included in the analysis as predictor. The overall model was significant ($X^2_{(8)}$=185.781, p<0.001) with child sex, study site, and gestational age at exam contributing significant variance. Multivitamin supplementation group, alcohol exposure group, and serum folate concentration during second and third trimester were not significantly associated with transverse cerebellar diameter. There were no significant interactions between alcohol exposure groups and serum folate concentration during second and third trimester.

For occipitofrontal diameter at third trimester, absolute ounces of alcohol per day at conception were included in the analysis as predictor. The overall model was significant ($X^2_{(6)}$=140.231, p<0.001) with gestational age at exam contributing significant variance. Multivitamin supplementation group, absolute ounces of alcohol per day at conception, and serum folate concentration during second and third trimester were not significantly associated with occipitofrontal diameter. There were no significant interactions between absolute ounces of alcohol per day at conception and serum folate concentration during second and third trimester.

For caval-calvarial distance at third trimester, absolute ounces of alcohol per day at conception were included in the analysis as predictor. The overall model was significant ($X^2_{(8)}$=115.329, p<0.001) with maternal smoking, study site, and gestational age at exam contributing significant variance. Multivitamin supplementation group, absolute ounces of alcohol per day at conception, and serum folate concentration during second and third trimester

were not significantly associated with caval-calvarial distance. There were no significant interactions between absolute ounces of alcohol per day at conception and serum folate concentration during second and third trimester.

For orbital diameter at third trimester, alcohol exposure groups were included in the analysis as predictor. The overall model was significant ($X^2_{(6)}$=51.545, p<0.001) with gestational age at exam contributing significant variance. Multivitamin supplementation group, absolute ounces of alcohol per day at conception, and serum folate concentration during second trimester and third were not significantly associated with orbital diameter. There were no significant interactions between alcohol exposure groups and serum folate concentration during second and third trimester.

For birth growth outcomes birth weight, length, and head circumference were analyzed separately. Absolute ounces of alcohol per day at conception were included in the analysis as predictor. A generalized linear regression analysis to evaluate the impact of alcohol use, multivitamin supplementation, and serum folate concentration (ng/mL) on birth weight is presented in Table 11. For birth weight, the overall model was significant ($X^2_{(6)}$=75.058, p<0.001) with gestational age at birth contributing significant variance. Multivitamin supplementation was associated with a significant increase in birth weight ($X^2_{(1)}$=5.073, p=0.024). Absolute ounces of alcohol per day at conception and serum folate concentration during second and third trimester were not significantly associated with birth weight. There were no significant interactions between absolute ounces of alcohol per day at conception and serum folate concentration during second and third trimester.

Table 11: Generalized Linear Regression Model for birth weight ($X^2_{(6)}$=75.058, p<0.001).

| Variable | β | SE | $X^2$ | p-value |
|---|---|---|---|---|
| Gestational age at birth | 156.640 | 18.682 | 70.304 | <0.001 |
| MVM Group[1] | 190.446 | 84.552 | 5.073 | 0.024 |
| AA/Day at conception | -103.300 | 73.745 | 1.962 | NS |
| Serum folate 2nd trimester (ng/mL) | 1.421 | 2.460 | 0.333 | NS |
| Serum folate 3rd trimester (ng/mL) | -2.666 | 2.749 | 0.941 | NS |

SE=Standard Error; MVM=Multivitamin Supplementation Group; AA/Day at conception=absolute ounces of alcohol per day at conception
[1]No MVM> MVM.

For birth length, the overall model was significant ($X^2_{(7)}$=77.845, p<0.001) with child sex and gestational age at birth contributing significant variances. There were no significant effects of absolute ounces of alcohol per day at conception, multivitamin supplementation, or serum folate concentration on birth length. There were no significant interactions between absolute ounces of alcohol per day at conception and serum folate concentration during second and third trimester.

For birth head circumference, the final model was significant ($X^2_{(7)}$=65.460, p<0.001) with child sex and gestational age at birth significantly contributing to the outcome. There were no significant effects of absolute ounces of alcohol per day at conception, multivitamin supplementation, or serum folate concentration on birth head circumference. There were no significant interactions between absolute ounces of alcohol per day at conception and serum folate concentration during second and third trimester.

For infant development outcomes only study site, child sex, SES, and gestational age at birth contributed significant variance. Bayley Scales of Infant Development-II MDI scores at 6 and 12 months and PDI scores at 12 months were analyzed separately. A generalized linear regression analysis to evaluate the impact of alcohol use, multivitamin supplementation, and serum folate concentration (ng/mL) on Bayley Scales of Infant Development-II MDI at 6 months

of age is presented in Table 12. For MDI at 6 months of age, the final model was significant ($X^2_{(11)}$=63.051, p<0.001) with gestational age at birth, study site, SES, and child sex contributing significant variance. There were no significant effects of absolute ounces of alcohol per day at conception, multivitamin supplementation, or serum folate concentration during second trimester on MDI at 6 months of age. Serum folate concentration during 3rd trimester was significantly associated with an increase in Bayley Scales of Infant Development-II MDI scores at 6 months of age. There were no significant interactions between absolute ounces of alcohol per day at conception and serum folate concentration during second and third trimester.

Table 12: Generalized Linear Regression Model for Bayley Scales of Infant Development-II Mental Development Index (MDI) at 6 months of age ($X^2_{(11)}$=63.051, p<0.001).

| Variable | β | SE | $X^2$ | p-value |
|---|---|---|---|---|
| SES Category[1] | 17.810 | 4.842 | 13.531 | <0.001 |
| Study Site[2] | -5.692 | 1.511 | 14.196 | <0.001 |
| Child Sex[3] | -3.850 | 1.373 | 7.862 | 0.005 |
| GA at birth | 1.258 | 0.458 | 7.554 | 0.006 |
| MVM Group[4] | 2.317 | 1.418 | 2.672 | NS |
| AA/Day at conception | -1.586 | 1.542 | 1.058 | NS |
| Serum folate 2nd trimester (ng/mL) | -0.070 | 0.049 | 2.043 | NS |
| Serum folate 3rd trimester (ng/mL) | 0.135 | 0.062 | 4.780 | 0.029 |

SE=Standard Error; GA=Gestational Age; SES=Socioeconomic Status; MVM=Multivitamin Supplementation Group; AA/Day at conception=absolute ounces of alcohol per day at conception
[1] Category 1=Highest SES through Category 5=Lowest SES; [2] Rivne>Khmelnytsky; [3] Female>Male; [4] No MVM> MVM

A generalized linear regression analysis to evaluate the impact of alcohol use, multivitamin supplementation, and serum folate concentration (ng/mL) on Bayley Scales of Infant Development-II PDI at 6 months of age is presented in Table 13. For PDI at 6 months of age, the final model was significant ($X^2_{(13)}$=45.637, p<0.001) with SES, study site, and gestational age at birth contributing significant variances. There were no significant effects of absolute ounces of alcohol per day, multivitamin supplementation, or serum folate concentration. There was a significant interaction between absolute ounces of alcohol per day at conception and

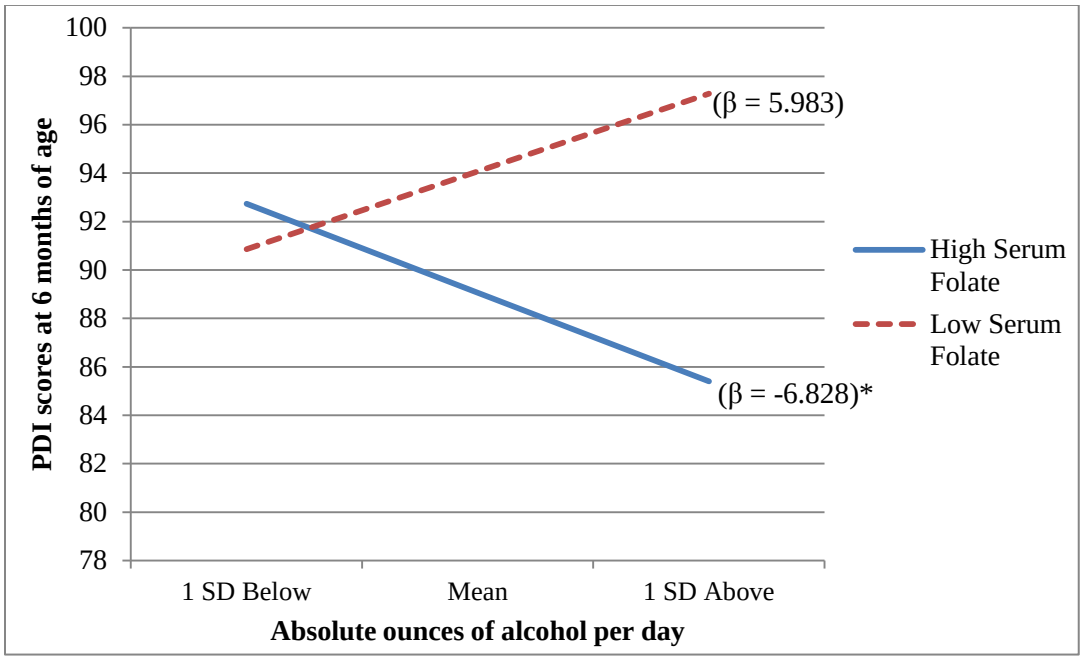
serum folate concentration during third trimester ($X^2_{(1)}$=5.674, p=0.017). The interaction remained significant after reducing the model to include the interaction and main effects of absolute ounces of alcohol per day and serum folate concentration at third trimester ($X^2_{(1)}$=4.460, p=0.035). Post-hoc examination of the interaction indicate that absolute ounces of alcohol per day at conception is significantly associated with high levels of serum folate ($X^2_{(1)}$=3.939, p=0.047), but not significantly associated with low levels of serum folate. The interaction is plotted in Figure 3; PDI scores at 6 months of age tend to be higher at low levels of alcohol exposure and lower at high levels of alcohol exposure with high levels of serum folate, indicating that folate has a greater effect at low levels of alcohol exposure.

Table 13: Generalized Linear Regression Model for Bayley Scales of Infant Development-II Psychomotor Development Index (PDI) at 6 months of age ($X^2_{(13)}$=45.637, p<0.001).

| Variable | β | SE | $X^2$ | p-value |
|---|---|---|---|---|
| Study Site[1] | -8.853 | 2.360 | 14.076 | <0.001 |
| SES Category[2] | 22.170 | 7.682 | 8.329 | 0.004 |
| GA at birth | 1.959 | 0.686 | 8.156 | 0.004 |
| MVM Group[3] | 2.751 | 2.843 | 0.939 | NS |
| AA/Day at conception | -5.333 | 4.740 | 1.266 | NS |
| Serum folate 2nd trimester (ng/mL) | -0.109 | 0.099 | 1.223 | NS |
| Serum folate 3rd trimester (ng/mL) | -0.026 | 0.112 | 0.055 | NS |
| AA/Day*Folate 2nd trimester[4] | -0.092 | 0.244 | 0.144 | NS |
| AA/Day*Folate 3rd trimester[5] | 0.637 | 0.267 | 5.674 | 0.017 |

SE=Standard Error; GA=Gestational Age; SES=Socioeconomic Status; MVM=Multivitamin Supplementation Group; AA/Day at conception=absolute ounces of alcohol per day at conception
[1] Rivne>Khmelnytsky; [2] Category 1=Highest SES through Category 5=Lowest SES; [3] No MVM> MVM;
[4] AA/Day*Folate 2nd trimester=Interaction between absolute ounces of alcohol per day at conception and serum folate concentration at 2nd trimester; [5] AA/Day*Folate 3rd trimester=Interaction between absolute ounces of alcohol per day at conception and serum folate concentration at 3rd trimester.

Figure 3: Regression lines for relationships between absolute ounces of alcohol per day at conception and PDI scores at 6 months of age as moderated by serum folate concentration during 3rd trimester.




β=unstandardized regression coefficient; SD=Standard Deviation; PDI=Psychomotor Development Index
*p<0.05

For MDI at 12 months of age, the final model was not significant ($X^2_{(4)}$=5.057, p=0.282) with no predictors or factors contributing significant variances. For PDI at 12 months of age, the final model was not significant ($X^2_{(4)}$=1.758, p=0.780) with no predictors or factors contributing significant variances. There were no significant effects of absolute ounces of alcohol per day at conception, multivitamin supplementation, or serum folate concentration on MDI or PDI at 12 months of age. There were no significant interactions between absolute ounces of alcohol per day at conception and serum folate concentration during second and third trimester.

### 3.3 Discussion

The lifelong implications of prenatal alcohol exposure are widely studied, with adverse effects to physical development, cognition, and behavior. The teratogenic effect of prenatal alcohol exposure on the brain can be extensive with a wide range of possible outcomes. Fetal

growth and brain size, obtained through ultrasound measurements, have not been widely studied in alcohol exposed pregnancies. The impact of micronutrient supplementation and maternal folate levels on prenatal alcohol effects has been the focus of animal studies and should be analyzed in human studies to determine clinical significance. This study examined the association between alcohol exposure and prenatal and postnatal growth, and infant development outcomes as well as the impact of micronutrient supplementation, specifically maternal folate levels, on the effects of prenatal alcohol exposure.

The results from this study support previous research indicating increased risk of growth retardation and impaired neurodevelopment with moderate-to-heavy prenatal alcohol exposure. The dose and timing of exposure necessary to produce detrimental effects is not clearly understood, and the results of this study only lead to further questions. Alcohol exposure in this sample led to prenatal and postnatal growth retardation, as well as impaired neurodevelopment at infancy. Appropriate interventions are necessary to reduce the teratogenic effects of prenatal alcohol exposure. The results of this study provide evidence for the potential benefit of multivitamin supplementation during pregnancy, as well as the benefit of high doses of folate during pregnancy. We hypothesized that increased levels of alcohol exposure will lead to a decrease in head circumference, fetal weight, abdominal circumference, femur length, and individual brain measurements during second and third trimester ultrasounds. We also hypothesized that fetal growth measurements will be greater in the micronutrient supplementation group and higher maternal folate levels will lead to greater fetal growth measurements. We hypothesized that birth weight, length, and head circumference will be lower in the alcohol exposure group, that micronutrient supplementation will lead to an increase in these growth measurements, and that higher maternal folate levels will lead to greater birth

growth measurements. We also hypothesized that Bayley Scales of Infant Development-II MDI and PDI scores will be lower in the alcohol exposure group compared to controls, that micronutrient supplementation will lead to an increase in scores on these measures, and that higher maternal folate levels will lead to greater scores.

Micronutrient supplementation groups were assigned during initial maternal interview at an average of 18.57 weeks gestation. Blood folate levels during second trimester among alcohol exposure groups adjusted for micronutrient supplementation group are not significantly different. However, there is a significant difference between blood folate levels in alcohol exposure and micronutrient supplementation groups during third trimester. The lack of effect during second trimester is likely due to the fact that the second trimester blood samples were taken at approximately the same time as assignment into supplementation groups. This indicates that micronutrient supplementation is having a positive effect on blood folate levels during third trimester. The greater percentage of deficient blood folate levels in the alcohol exposure group among those receiving micronutrient supplementation supports previous research indicating reduced uptake of folate with alcohol use.[86] Absolute ounces of alcohol per day at conception in the alcohol exposure group were consistently in the moderate-to-heavy range. This alcohol variable was found to be a greater predictor of outcomes compared to alcohol consumption patterns reported during the first trimester of pregnancy. This finding is consistent with previous research indicating a greater willingness of women to report alcohol use prior to pregnancy recognition.[95]

The results of this study indicate that a number of routine ultrasound measurements during third trimester of pregnancy may be markers for prenatal alcohol effects in moderate-to-heavily exposed fetuses. Estimated fetal weight, abdominal circumference, and biparietal

diameter are standard ultrasound measurements obtained during both second and third trimester of pregnancy. Reduced estimated fetal weight during third trimester with increased alcohol exposure is consistent with previous findings of reduced birth weight with increased levels of alcohol exposure. Additionally, abdominal circumference was reduced in third trimester ultrasound indicating a reduction in overall fetal growth with increased alcohol exposure. Smaller biparietal diameter during third trimester is consistent with a previous pilot study in Ukraine by Kfir *et al*, which demonstrated a significant reduction in biparietal diameter during third trimester ultrasound.[20] Biparietal diameter measurements are used to calculate estimated fetal weight and gestational age along with head circumference, abdominal circumference and femur length.[96] The use of biparietal diameter to estimate head size and brain volume is widely debated; however, previous MRI studies have shown an association between reductions in biparietal diameter and delayed cognitive development.[97,98] The association between alcohol exposure and estimated fetal weight, abdominal circumference, and biparietal diameter is a potentially important finding due to the availability of these measurements in routine prenatal examinations. With further study, these measurements could be used as markers for the adverse effects of prenatal alcohol exposure.

Additional study specific brain and facial growth measurements including transverse cerebellar diameter, occipitofrontal diameter, caval-calvarial distance, and orbital diameter showed significant reduction with increased alcohol exposure during third trimester. The effect of alcohol exposure on occipitofrontal diameter indicates an overall reduction in brain size, the reduction in caval-calvarial distance indicates a reduction in frontal lobe size, and the reduction in transverse cerebellar diameter indicates an effect on size of individual brain structures.[99] This is an interesting finding due to the relationship between the frontal lobe of the brain and many of

the cognitive deficits seen in patients with FASD's. The effect of alcohol exposure on orbital diameter indicates a reduction in eye size during third trimester. These effects should be further studied in a larger sample to determine the clinical significance of this finding, and the potential for including these measurements on routine ultrasound examinations.

The effect of micronutrient supplementation on estimated fetal weight and abdominal circumference are also interesting findings. In this study, the micronutrient supplementation group had larger estimated fetal weight and abdominal circumference compared to controls; this effect did not extend to biparietal diameter, which was not significantly affected by multivitamin supplementation. There was a significant interaction between estimated fetal weight at third trimester, absolute ounces of alcohol per day at conception and serum folate concentration during $2^{nd}$ trimester. Estimated fetal weight tends to be higher at low levels of alcohol exposure and lower at high levels of alcohol exposure high levels of serum folate, indicating that folate has a greater effect at low levels of alcohol exposure. This could be due to the impaired uptake of folate into the bloodstream and impaired transport of folate across the placenta with high levels of alcohol exposure. Serum folate concentration did not significantly affect any of the fetal growth measurements. There were no significant effects of micronutrient supplementation or folate levels on transverse cerebellar diameter, occipitofrontal diameter, caval-calvarial distance, and orbital diameter. This could be a result of the lack of clinically recognized gestational-age adjusted growth norms for these measurements. Gestational-age adjusted growth norms are available for standard ultrasound measurements including estimated fetal weight, abdominal circumference, femur length, biparietal diameter, and head circumference; there is also limited growth norm data for transverse cerebellar diameter, orbital diameter measurements, and frontothalamic distance from the 1980's and early 1990's. For frontothalamic distance, the

reference values are for fetuses with Down syndrome between 15 and 21 weeks gestation.[100] It is important to establish gestational-age adjusted growth norms for all fetal growth measurements in order to correct for rapid growth during second and third trimester.

The results of this study support previous research indicating a relationship between moderate-to-heavy prenatal alcohol exposure, preterm birth, and small for gestational age. Alcohol exposure during pregnancy was significantly associated with lower gestational age at birth, smaller birth weight, shorter birth length, and smaller birth head circumference. When adjusting for gestational age, micronutrient supplementation was significantly associated with an increase in birth weight. This is similar to results in this study of estimated fetal weight during third trimester. Birth length and head circumference were not significantly associated with micronutrient supplementation and serum folate concentration was not significantly associated any of these outcomes. Additional research should be conducted to identify individual micronutrients to provide the greatest benefit to birth weight.

In this study, alcohol exposure significantly decreased infant development scores at 6 months and 12 months of age. Serum folate concentration during 3$^{rd}$ trimester was significantly associated with an increase in MDI scores at 6 months of age. Micronutrient supplementation was not significantly associated with MDI or PDI scores at 6 months of age. However, there was a significant interaction between PDI at 6 months of age, absolute ounces of alcohol per day at conception and serum folate concentration during third trimester, indicating that PDI scores at 6 months of age at high levels of serum folate tend to be higher at low levels of alcohol exposure and lower at high levels of alcohol exposure. High levels of folate had a greater positive effect on PDI at 3$^{rd}$ trimester at low levels of alcohol exposure. These findings support previous research indicating reduced uptake and transport across the placenta of folate with high levels of

alcohol exposure. Micronutrient supplementation and serum folate concentration were not significantly associated with MDI and PDI scores at 12 months of age. A significant interaction between MDI scores at 12 months of age, alcohol exposure groups, and micronutrient supplementation groups was also found. The interaction indicated that MDI scores at 12 months of age were higher in the multivitamin supplementation group in the alcohol exposed group, but lower in the multivitamin supplementation group in the alcohol unexposed group. These findings are consistent with a previous study with the CIFASD consortium in Ukraine which found significant reduction in Bayley MDI and PDI scores with increased alcohol exposure. The study also found a significant increase in Bayley MDI scores with multivitamin supplementation.[101]

Folate supplementation during pregnancy in alcohol exposed mice has been the focus of many studies; however, few human studies exist. With further study, the use of maternal folate supplementation may have a beneficial effect in alcohol exposed pregnancies. In addition to folate, other micronutrients should be studied to determine the possible benefit of micronutrient supplementation in alcohol exposed pregnancies.

### 3.4 Limitations and implications

There are several limitations to this study. The absolute amount of alcohol consumed throughout pregnancy in the alcohol exposure group is unknown, although the frequency and pattern of alcohol use during the month around conception and first trimester was collected during maternal interview. The use of maternal self-report of alcohol and tobacco use may not represent true consumption levels. Additionally, maternal alcohol consumption during pregnancy may vary between trimesters, leading to inconsistent exposure patterns. The use of absolute ounces of alcohol per day at conception may have provided a more accurate picture of

alcohol consumption patterns in the alcohol exposure group; however, it is not possible to know if this exposure pattern was consistent throughout pregnancy. There is no known safe level or timing for alcohol exposure during pregnancy, therefore, the use of moderate to heavy amounts of alcohol at any time point during pregnancy may produce teratogenic effects.

The maternal characteristics of women who returned for follow-up ultrasound scans and blood samples may lead to bias in sampling. The sample size varied for follow-up examinations, which may impact comparability of measurements assessed during second and third trimester. The lack of clinically accepted gestational-age adjusted percentiles for study specific fetal brain growth measurements may lead to errors in interpretation of the analysis. Adjustment for gestational age is necessary to accurately compare the sample; however, there are few growth charts for fetal brain growth measurements available with little reliability. The growth norm data for these ultrasound measurements that is available is outdated and in many cases does not account for rapid growth during third trimester. In order to include these measurements in standard clinical practice, clinically accepted gestational-age and gender adjusted percentiles must be established.

This study also has several strengths. The relatively large sample size provides increased statistical power for comparison of alcohol exposed pregnancies and unexposed pregnancies. The prospective cohort design provides a temporal sequence between alcohol exposure and prenatal outcomes. The standardized ultrasound measurements are routinely used in a clinical setting allowing for reproducibility, and translatability to clinical practice.

One major implication of this study is that the findings could be used to develop a prenatal screening process for pregnancies with known alcohol exposure. Further study is

needed to determine the reproducibility of these findings; however, the consistency of the results with a previous pilot study suggests that biparietal diameter may be a marker for prenatal alcohol effects. The use of maternal micronutrient supplementation as a possible preventive measure for the lasting effects of prenatal alcohol exposure is also an important implication. Additional studies should be conducted to identify specific micronutrients and the levels necessary to produce the greatest effect. Early interventions are necessary to improve outcomes in children prenatally exposed to alcohol. The results of this study provide evidence for the need for additional early intervention methods, including potential benefits to ultrasonography and micronutrient supplementation.

### 3.5 Conclusion

The results of this study suggest that the effects of prenatal alcohol exposure may be detectable in late pregnancy; however, additional studies are needed to determine the reliability of these measures. The data do not presently support adopting clinical standards for prenatal ultrasound measurements for early diagnosis of FASD's. Additional research into the biological mechanism by which alcohol inhibits brain growth is needed to fully understand which areas of the brain are most affected by alcohol exposure, and at which level of consumption does this effect occur. The results also support previous research indicating moderate-to-heavy alcohol exposure leads to prenatal and postnatal growth restriction as well as impaired neurodevelopment. The data suggest that micronutrient supplementation during pregnancy, including high doses of folate, may be an early intervention to reduce the harmful effects of prenatal alcohol exposure. Further studies are needed to identify the specific micronutrients producing these effects, and assess the appropriate level of these micronutrients necessary to provide the greatest benefit without exceeding the safe limit. Currently research is being

conducted with the CIFASD consortium to examine the effect of prenatal alcohol exposure on fetal growth, as well as the potential benefits of maternal micronutrient supplementation on FASD's.

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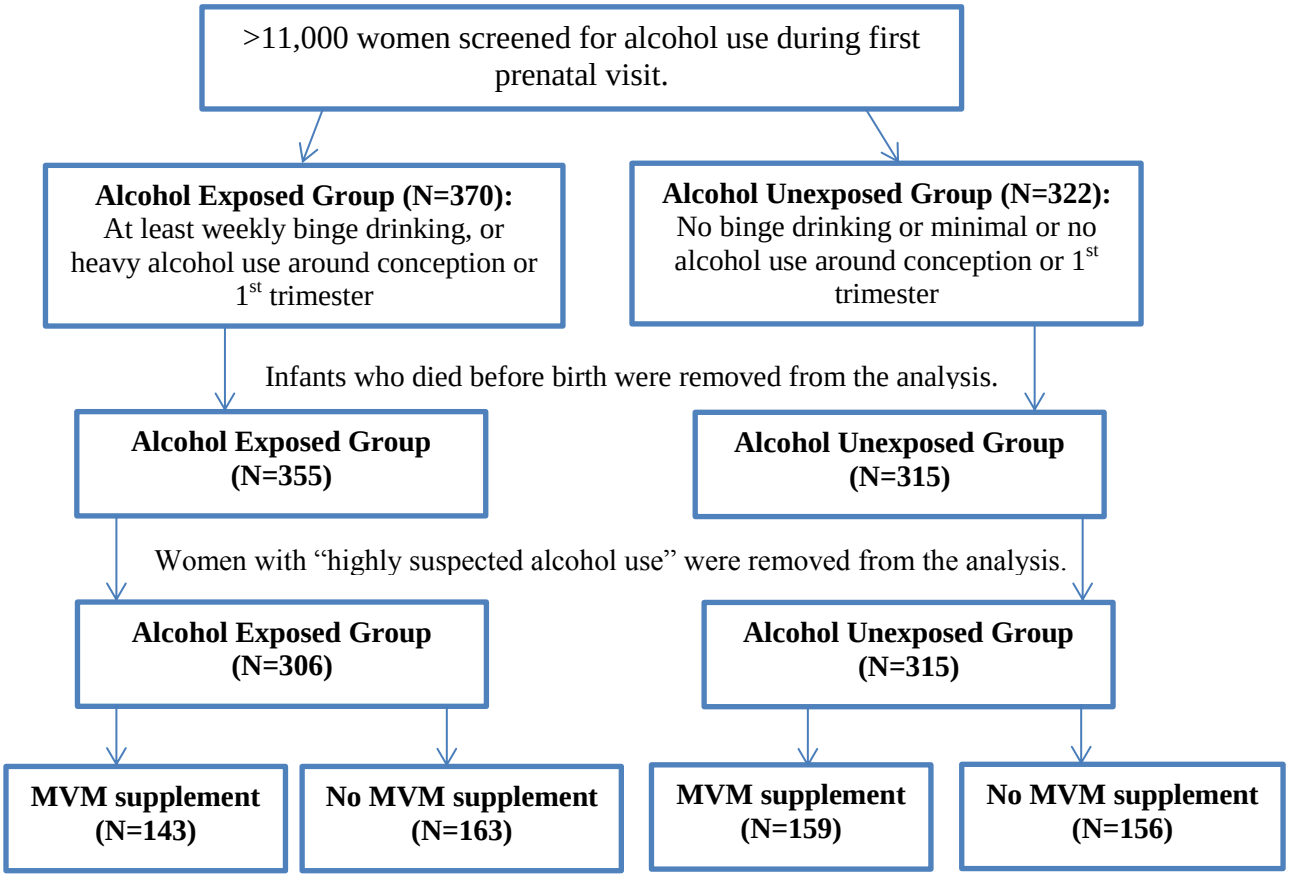
**Appendix 1:** Study sample characteristics.



**Appendix 2:** Regression equations for fetal growth measurements at third trimester, birth growth measurements, and Bayley Scales of Infant Development-II.

Predicted estimated fetal weight during third trimester is equal to -3378.466 + 166.520(Gestational age at exam) – 54.223(absolute ounces of alcohol per day). Estimated fetal weight increased 166.520 g for every week gestation, and decreased 54.223 grams for every absolute ounce of alcohol consumed ($F_{(2,489)}$=464.887, p<0.001, $R^2$ = 0.655).

Predicted abdominal circumference during third trimester is equal to 17.769 + 8.189(Gestational age at exam) – 3.078(absolute ounces of alcohol per day). Abdominal circumference increased 8.189 millimeters for every week gestation, and decreased 3.078 millimeters for every absolute ounce of alcohol consumed ($F_{(2,491)}$=544.372, p<0.001, $R^2$ = 0.689).

Predicted biparietal diameter during third trimester is equal to 26.749 + 1.726(Gestational age at exam) – 1.131(absolute ounces of alcohol per day). Biparietal diameter increased 1.726 millimeters for every week gestation, and decreased 1.131 millimeters for every absolute ounce of alcohol consumed ($F_{(2,491)}$=278.516, p<0.001, $R^2$ = 0.532).

Predicted occipitofrontal diameter during third trimester is equal to 37.190 + 2.067(Gestational age at exam) – 1.169(absolute ounces of alcohol per day). Occipitofrontal diameter increased 2.067 millimeters for every week gestation, and decreased 1.169 millimeters for every absolute ounce of alcohol consumed ($F_{(2,485)}$=235.510, p<0.001, $R^2$ = 0.493).

Predicted caval-calvarial distance during third trimester is equal to 6.388 + 0.981(Gestational age at exam) – 0.812(absolute ounces of alcohol per day). Caval-calvarial distance increased 0.981 millimeters for every week gestation, and decreased 0.812 millimeters for every absolute ounce of alcohol consumed ($F_{(2,489)}$=118.435, p<0.001, $R^2$ = 0.326).

Predicted birth weight is equal to -3914.216 + 183.587(Gestational age at birth) – 136.783(absolute ounces of alcohol per day). Birth weight increased 183.587 grams for every week gestation, and decreased 136.783 grams for every absolute ounce of alcohol consumed ($F_{(2,617)}$=239.196, p<0.001, $R^2$ = 0.437).

Predicted birth length is equal to 10.591 + 1.041(Gestational age at birth) – 0.621(absolute ounces of alcohol per day). Birth length increased 1.041centimeters for every week gestation, and decreased 0.621centimeters for every absolute ounce of alcohol consumed ($F_{(2,617)}$=241.108, p<0.001, $R^2$ = 0.439).

Predicted birth head circumference is equal to 11.368 + 0.582(Gestational age at birth) – 0.347(absolute ounces of alcohol per day). Birth head circumference increased 0.582 centimeters for every week gestation, and decreased 0.347 centimeters for every absolute ounce of alcohol consumed ($F_{(2,617)}$=195.563, p<0.001, $R^2$ = 0.388).

Predicted MDI score at 6 months of age is equal to 91.208 – 4.226(absolute ounces of alcohol per day). MDI score at 6 months of age decreased 4.226 points for every absolute ounce of alcohol consumed ($F_{(1,378)}$=28.166, p<0.001, $R^2$ = 0.069).

Predicted PDI score at 6 months of age is equal to 90.516 – 3.581 (absolute ounces of alcohol per day). PDI score at 6 months of age decreased 3.581 points for every absolute ounce of alcohol consumed ($F_{(1,377)}$=10.730, p=0.001, $R^2$ = 0.028).

Predicted MDI score at 12 months of age is equal to 91.311 – 4.579(absolute ounces of alcohol per day). MDI score at 12 months of age decreased 4.579 points for every absolute ounce of alcohol consumed ($F_{(1,291)}$=15.983, p<0.001, $R^2$ = 0.052).

Predicted PDI score at 12 months of age is equal to 99.760 – 5.964(absolute ounces of alcohol per day). PDI score at 12 months of age decreased 5.964 points for every absolute ounce of alcohol consumed ($F_{(1,291)}$=22.384, p<0.001, $R^2$ = 0.071).

**Appendix 3:** Post-hoc analysis of interactions

Regression equation for PDI at 6 months of age with main effects and interaction:

PDI6mo = 93.764 – 8.009 (AA/day) – 0.127 (Folate2) + 0.502 (AA/day*Folate2)

Regression equation for PDI at 6 months of age with main effects and interaction (with centered variables):

PDI6mo = 91.705 – 0.423 (AA/day) + 0.034 (Folate2) + 0.502 (AA/day*Folate2)

High Folate 3rd trimester (with centered variables):

PDI6mo = 91.265 – 6.828 (AA/day) + 0.034 (Folate2above) + 0.502 (AA/day*Folate2above)

Overall model: $X^2_{(3)}$=4.831, p=0.185. AA/day: $X^2_{(1)}$=3.939, p=0.047.

With 0 for Folate 2 above 1 SD:

PDI6mo = 91.265 – 6.828 (AA/day)

PDI6mo = 91.265 – 6.828 (0.859363) = 85.39726944

PDI6mo = 91.265 – 6.828 (0.32221) = 89.0649501

PDI6mo = 91.265 – 6.828 (-0.214943) = 92.7326308

Low Folate 3rd trimester (with centered variables):

PDI6mo = 92.145 + 5.983 (AA/day) + 0.034 (Folate2below) + 0.502 (AA/day*Folate2below)

Overall model: $X^2_{(3)}$=4.831, p=0.185. AA/day: $X^2_{(1)}$=1.921, p=0.166.

With 0 for Folate 2 below 1 SD:

PDI6mo = 92.145 + 5.983 (AA/day)

PDI6mo = 92.145 + 5.983 (0.859363) = 97.2865688

PDI6mo = 92.145 + 5.983 (0.32221) = 94.0727824

PDI6mo = 92.145 + 5.983 (-0.214943) = 90.858996

Regression equation for Estimated Fetal Weight at 3rd trimester with main effects and interaction:

EFW = 52.237 – 17.148 (AA/day) – 0.170 (Folate1) + 0.690 (AA/day*Folate1)

Regression equation for Estimated Fetal Weight at 3rd trimester with main effects and interaction (with centered variables):

EFW = 47.546 – 6.051 (AA/day) + 0.052 (Folate1) + 0.690 (AA/day*Folate1)

High Folate 2nd trimester (with centered variables):

EFW = 46.841 – 15.429 (AA/day) + 0.052 (Folate1above) + 0.690 (AA/day*Folate1above)

Overall model: $X^2_{(3)}$=8.041, p=0.045. AA/day: $X^2_{(1)}$=8.123, p=0.004.

With 0 for Folate 1 above 1 SD:

EFW = 46.841 – 15.429 (AA/day)

EFW = 46.841 – 15.429 (0.859363) = 33.58188827

EFW = 46.841 – 15.429 (0.32221) = 41.86962191

EFW = 46.841 – 15.429 (-0.214943) = 50.15735555

Low Folate 2nd trimester (with centered variables):

EFW = 48.252 + 3.326 (AA/day) + 0.052 (Folate1below) + 0.690 (AA/day*Folate1below)

Overall model: $X^2_{(3)}$=8.041, p=0.045. AA/day: $X^2_{(1)}$=0.237, p=0.626.

With 0 for Folate 1 below 1 SD:

EFW = 48.252 + 3.326 (AA/day)

EFW = 48.252 + 3.326 (0.859363) = 51.44024134
EFW = 48.252 + 3.326 (0.32221) = 49.32367046
EFW = 48.252 + 3.326 (-0.214943) = 47.53709958