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Association between Folate Levels and Preterm Birth in Tampa, Florida

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by

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DEDICATION

I dedicate my thesis work to my mother, Camille and to my siblings, Liz and Miguel. Thank you for keeping me close while so far away.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Background: Preterm birth is one of the leading causes of perinatal mortality and morbidity and poses extensive economic liability. The rate of preterm births globally is approximately 11.1%, and in the US, the preterm birth rate has been estimated to be 12-13%. Folate and B_{12} requirements increase during pregnancy as a result of increased cell division to accommodate maternal and fetal growth; inadequate levels can result in placental abnormalities and thus present implications for preterm birth.

Objective: To investigate the association between red blood cell (RBC) folate and B_{12} concentrations with the risk of preterm birth.

Methods: Study participants were recruited from Tampa General Hospital between January 2011 and May 2013. Women with a singleton delivery occurring less than 37 weeks gestation were classified as cases and those with a singleton delivery occurring between 37 and 42 weeks gestation were classified as controls. The study had a final sample size of 227 women, including 36 cases and 191 controls. Maternal blood was collected in order to measure folate and B_{12} concentrations. The association between folate/B_{12} and preterm birth was assessed using logistic regression; odds ratio (OR), 95% confidence interval (CI) and p values are reported. A power analysis was also performed using the available sample as well as imputation for missing values in the B_{12} variable.

Results: Although not statistically significant, the mean concentration of folate and B_{12} levels were higher in the cases than in the controls, 894 ± 158.1 vs. 869.2 ± 169.6 and

245.2 ± 102.2 vs 238.3 ± 81.5, respectively. No significant associations were found between folate or B_{12} and the risk of preterm birth.

Conclusions: This study did not detect a significant association between folate or B_{12} and preterm birth; however, due to the small sample size this analysis was underpowered. Additional studies are needed, preferably using a randomized control study design, in order to elucidate the relationship between folate/B_{12} and preterm births.

INTRODUCTION

Preterm birth (PTB) has been implicated as one of the leading causes of perinatal morbidity and mortality globally. Preterm birth is characterized according to the World Health Organization (WHO) as births occurring prior to 37 weeks of gestation or less than 259 days since the first day of a woman's last menstrual period (WHO, 1977). According to 2010 estimates, 14.9 million babies were born preterm around the world, which estimates to 11.1% of all live births globally and >1 in 10 of all live births (Blencowe et al., 2012). The United States experiences a frequency of 12-13% of preterm births, while many other developed countries have a 5-9% outcome of PTBs. Even more alarming is that these rates are estimated to be considerably higher in developing countries (Goldenberg, Culhane, Iams & Romero, 2008). Preterm births have also been dubbed the single largest direct cause of neonatal deaths, accounting for over 1 million of the 3.1 million neonatal deaths occurring globally (Blencowe et al., 2013). Despite the survival of many preterm infants (Goldenberg, Culhane, Iams & Romero, 2008; Slattery & Morrison, 2002), they are often at a higher risk for adverse outcomes later in life such as medical and social disabilities, including cerebral palsy, learning impairment and even visual disorders (Moster, Lie, & Markestad, 2008; Rogers & Velten, 2011). Additionally, the economic burden of preterm birth is extensive, both in early life intervention and transfers into adulthood across the globe (Clements, Barfield, Ayadi & Wilber, 2007; Petrou & Khan, 2012). Specifically, in the US, the cost of preterm birth has been estimated to be

$26.2 billion per year (Behrman & Butler, 2007). Despite much advancement in medical technology and public health interventions, preterm births still account for about 75% of perinatal mortality and over 50% of morbidity transcending across life (Goldenberg, Culhane, Iams & Romero, 2008). As a result, the prevention of preterm births has continued to be a significant perinatal priority in public health with the goal of reducing mortality, morbidity and its economic burden.

The exact causes and mechanisms by which preterm birth occurs remain elusive but due to extensive research, much is now known about its multifactorial etiology. Though preterm birth is defined by time, it manifests itself as two major subtypes, either spontaneous, which includes spontaneous onset of labor with intact membranes and preterm premature rupture of the membranes (pPROM) or provider-indicated preterm birth, which is defined as induction of labor or caesarean birth prior to 37 weeks of gestation (Goldenberg, Culhane, Iams & Romero, 2008; Blencowe et al., 2013). Additionally, more than 70% of preterm births happen spontaneously, while less than 30% account for medically indicated preterm births (Goldenberg, Culhane, Iams & Romero, 2008). An extensive catalogue of risk factors of preterm birth has been elucidated in the literature to date with some variation among the aforementioned types of preterm birth. For spontaneous preterm births, risk factors include the age at pregnancy and its spacing, multiple pregnancy, infections such as HIV, urinary tract infections, malaria; maternal chronic medical conditions including diabetes, hypertension, anemia; poor nutrition; stress, smoking/tobacco use, alcohol consumption genetic predispositions and depression or violence. For provider-indicated preterm birth, maternal induction of labor or a cesarean section is employed as a result of maternal or fetal distress; moreover,

many of the same risk factors for spontaneous preterm birth apply here (Blencowe et al., 2013). For both provider-indicated and spontaneous preterm birth, a history of preterm birth has been implicated as a crucial risk factor for recurrence in future pregnancies; accounting for a 15-50% increase in the risk of recurrence (Goldenberg, Culhane, Iams & Romero, 2008).

Nutritional demands escalate during pregnancy due to fetal development, which involves rapid cell growth and division in both the mother and fetus. As a result, nutritional factors have been studied extensively for its relation to adverse pregnancy outcomes, one of which is preterm birth. More specifically, one of B vitamins, folate (B_9) has been investigated substantially as a risk factor for preterm births due to its increased requirements during pregnancy and its proven efficacy in preventing neural tube defects (NTDs) in babies (Molloy, Kirke, Brody, Scott & Mills, 2008). Folate is an essential molecule known for its central role in one-carbon metabolism as related to nucleic acid production, DNA methylation and synthesis (Chen et al., 2014, Scholl & Johnson, 2000; Crider, Yang, Berry & Bailey, 2012). Under this premise, low folate concentrations can result in unfavorable cell division and subsequently fetal and placental development anomalies (Scholl & Johnson, 2000). Due to its modifiable nature, folate's association with preterm births has peaked researchers' interests over the years. However, despite the extensive research, the available literature on the relationship between folate/folic acid and preterm birth has been inconclusive to date. The majority of studies have found that folate has a negative association with preterm births in that lower concentrations of folate during pregnancy increases the risk of preterm birth (Scholl, Hediger, Schall, Khoo & Fischer, 1996; Siega-Riz, Savitz, Zeisel, Thorp, & Herring, 2004; Catov, Bodnar, Ness,

Markovic, & Roberts, 2007; Furness, Yasin, Dekker, Thompson, & Roberts, 2011; Bergen et al., 2012; Chen et al., 2014). However, several studies have found that there is no association between folic acid or folate concentrations and the risk of preterm births (Ronnenberg, 2002; Timmermans, Jaddoe, Hofman, Steegers-Theunissen, & Steegers, 2009; Nilsen et al.; 2010; Yamada et al., 2012; Sengpiel et al., 2014), including a recent Cochrane review that encompassed all of the existing randomized control trials (RCTs) as well as cross-over controlled trials (Lassi, Salam, Haider & Bhutta, 2013). Folate is mainly stored in the liver but throughout the years, scientists have developed several tests that are reliable indicators of folate status. These include serum, plasma or red blood cell folate measurements (WHO, 2012). Serum folate is a non-specific test and is the most commonly used measure of folate status though it provides a measure of short term folate status. Red blood cell folate measurements are more reflective of long term folate status and can be more useful in pregnancy studies (WHO, 2012; Furness, Yasin, Dekker, Thompson, & Roberts, 2011). Noteworthy is that fact that mean folate intake and circulating folate concentrations are highly correlated (Scholl, Hediger, Schall, Khoo & Fischer, 1996; Kim et al., 2011) and thus folic acid supplementation has been used extensively as a proxy for investigating the association between folate and preterm births. Furthermore, the literature shows that there is a significant positive correlation between serum folate and red blood cell folate measurements, thus providing comparable results in the assessment of folate deficiency (De Bruyn, Gulbis, & Cotton, 2014; Galloway, 2003). Therefore, dietary folate, folic acid supplementation, red blood cell folate and serum folate have all been used to assess folate status and to investigate this association. (Scholl & Johnson, 2000).

Another B vitamin that has also garnered some attention in the literature, though less extensive than folate is B_{12} due to their metabolic links in the DNA synthesis pathway (Moores, Fenech, & O'Callaghan, 2011) and its correlation with folate (Ronnenberg, 2002). However, there are limited studies on this association and similar to that of folate and preterm birth, the results are equivocal. A study in Chinese women showed that there was an increased risk of preterm birth in those who were B_{12} deficient (Ronnenberg, 2002) but other studies have not replicated these results (Scholl, Hediger, Schall, Khoo & Fischer, 1996; Chen et al., 2014; Bergen et al., 2012).

Folate requirements increase in pregnant women in conjunction with maternal and fetal growth and B_{12} is metabolically related to folate, providing premise for their widespread investigation as risk factors in preterm birth. Additionally, the current literature on these associations report conflicting results. Many studies have examined these associations using either folate supplementation or dietary folate as a measure of folate concentrations. Few studies have used red blood cell folate concentrations as a measure of folate and its status in pregnant women. Therefore, using red blood cell folate and B_{12} concentrations, this study seeks to explore their relationship with preterm birth in mothers by analyzing data from a case control study in Tampa, Florida.

METHODS

Study Population

This was a case-control study that recruited 316 women who had either a spontaneous preterm birth or women who delivered at term at Tampa General Hospital (TGH) in Tampa, Florida. Research staff recruited study participants as they were admitted to the hospital for delivery. Faculty of the Department of Obstetrics and Gynecology at the University of South Florida (USF) provided the obstetrical care (prenatal, labor and delivery) services at TGH. Cases were defined as mother-infant dyads with spontaneous live preterm birth from 20 to 36 weeks 6 days gestation. Controls were defined as mother-infant dyads with spontaneous live birth at ≥ 37 completed weeks gestation. To be eligible for the study, pregnant women had to satisfy the following criteria: (1) Mothers that were at least 18 years of age with a singleton pregnancy and live birth. (2) Non-eligible were women with medical illnesses (e.g., hypertension, pre-existing diabetes mellitus, cardiac disease, etc.) requiring long-term or intermittent drug therapy; (3) Women with fetuses that showed evidence of chromosomal anomalies were excluded; (4) Women with congenital uterine anomaly were excluded; (5) Women with large leiomyomata distorting the uterine cavity were also excluded as well as those with in-place cervical cerclage during pregnancy. Eighty-one (n=81) women dropped out of the study or were discontinued due to incomplete information, leaving 235 women who were enrolled in the

study. Those with incomplete folate data were excluded from analysis, resulting in a final study sample size of 227, inclusive of 36 cases and 191 controls. The University of South Florida Institutional Review Board and ethics committee approved this study on July 29, 2010. Informed consent was received from all the women who agreed to participate in the study.

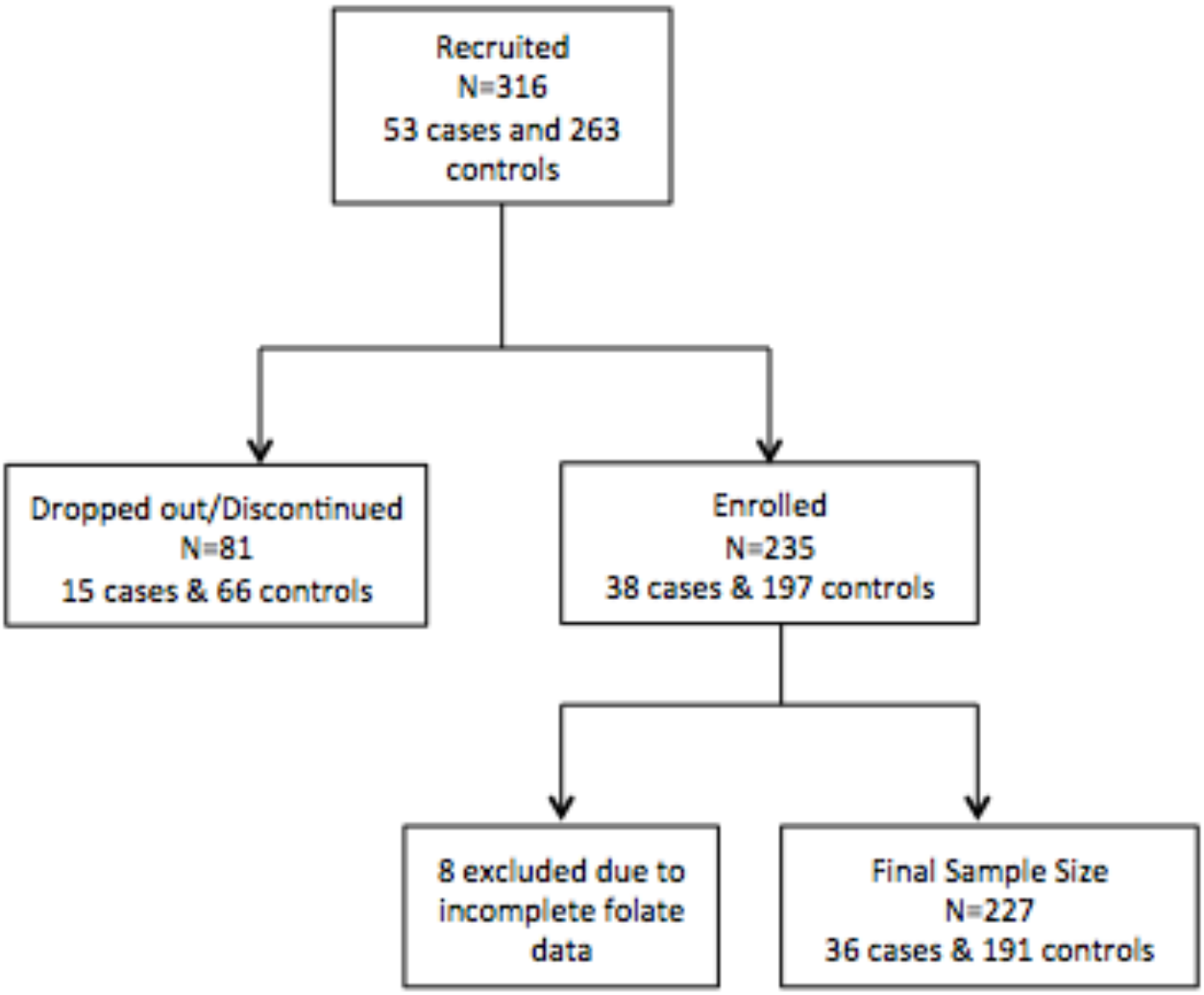

Figure 1: Study Protocol Flowchart

Data Collection

Medical records were used in order to attain information on sociodemographic characteristics and newborn measures including parental race/ethnicity, markers of socioeconomic status, clinical data including gestational age, pre-pregnancy body mass index, age and maternal substance abuse. Additionally, data regarding parity, history of preterm and history of abortion were also extracted from the patients' medical records. Data on smoking status of the participants was collected via oral questions related to passive/active smoking in pregnancy as well as the frequency and intensity of smoking. Maternal blood was collected when the patient was no longer under duress and/or in active labor. 10ml (with no EDTA) of blood was sent to Quest Diagnostics in order to measure folate and B12 concentrations. These measures were recorded and used for analysis. For the folate test, the normal range was defined as a red blood cell folate concentration of >280 ng/ml; and for B_{12}, the normal range was a serum concentration of 200-1100 pg/ml.

Statistical Analysis

Descriptive statistics among the entire cohort and then a comparison of cases vs. controls were ascertained using the frequency procedure. For continuous variables, mean ± SD was calculated for the entire cohort as well as separately among cases and controls. A two-sample t-test was used to compare the means of cases and controls in the cohort.

The p-value corresponding to Satterthwaite was used to compare the cases and controls. Satterthwaite is used when the assumption that the two populations have equal variances seem unreasonable. For categorical variables, variables were dichotomized in preparation for analysis. Parity was sectioned into two categories where primiparous was assigned for first time mothers and multiparous for mothers with at least one (1) previous pregnancy. Race was categorized into two (2) where whites were placed into one category and all other races were put into the "other" category for comparison. Participants who selected "single" for marital status were placed in one category whereas those who were separated, divorced or married were placed into the "other" category, which was used as the reference. History of preterm was collapsed into two categories of 'Yes' and 'No'. History of abortion was also collapsed from the different types of abortion into a dichotomous 'Yes'/'No' for analysis. The continuous variables including folate concentration and B_{12} level were dichotomized using median cut-off values. The number and % of participants in the entire cohort that possessed a certain characteristic was determined. N and % were also calculated among cases and controls for all the characteristics. A chi-squared test was used to compare cases and controls for each variable.

In order to determine the association between maternal folic acid levels and spontaneous preterm birth, a logistic regression was used. Our dependent variable is preterm births and our independent variables are folate concentration and B_{12}, where high concentrations were used as the reference for the association. This is as our outcome (preterm birth) is dichotomous and we want to determine its relationship with an independent variable(s). Crude and adjusted odds ratios as well as 95% confidence

intervals and a p value to assess significance was calculated using this regression technique. Additionally, adjustment for confounders (socio-demographic and behavioral habits, lifetime stressors, socio-economic status as well as community-level factors) was done in order to estimate the association between maternal-infant dyad folate level/ B12 levels and spontaneous preterm birth. A p-value of 0.05 was considered to be statistically significant. All analyses will be done using SAS ® version 9.4 (SAS Institute Inc., Cary, NC, USA).

Study power is the probability of rejecting a false null hypothesis and as result, it should be close to 1. This will allow us to determine our ability to detect a difference between the case and control proportions and whether the observed results are purely due to chance or are we observing a real effect. In this instance, the sample sizes and proportions in the case and control groups for both the associations of folate and preterm birth as well as B_{12} and preterm birth was used to calculate actual study power. A 'two independent proportions' power analysis was utilized where the null hypothesis is that there is no difference between the two observed proportions and the alternative hypothesis states that there is a difference in the two observed proportions. The test statistic used was a two-sided Z test with pooled variance. The analysis was done using PASS, 2008.

A multiple imputation method was used to deal with the missing values for the B12 variable using the MI procedure in SAS ® version 9.4 (SAS Institute Inc., Cary, NC, USA). The continuous form of B_{12} was first used in the imputation step and then dichotomized just as in the original analysis. In order to determine the exact imputation method, the pattern of missingness was first determined to be arbitrary. Due to the nonmonotone missing data pattern and the fact that B_{12} concentration is a continuous variable, the

Markov Chain Monte Carlo (MCMC) was used. MCMC is possibly the most common multiple imputation method and allows us to impute all the missing values or to impute enough values so that our missing data pattern changes to a monotone one (Yuan, 2011; Horton & Kleinman, 2007). MCMC performs under the assumption that the variables in the imputation model display a joint multivariable normal distribution. However, this method has been shown to be robust to the normality assumption if our proportion of missing data is low and the sample size is sufficient (Lee & Carlin, 2010); in our case the missing data is approximately 4%. Analysis was done in three phases; first proc MI was used to impute the missing values using a single chain, 100 imputations and the expectation-maximization (EM) algorithm to compute the maximum likelihood estimate (MLE). In proc MI, we have also produced the trace plot in order to assess convergence and the autocorrelation plot for B_{12}. A logistic regression was then performed using each of the imputed datasets using preterm birth as the outcome and a dichotomized B_{12} variable as the exposure. Finally, the results of the logistic regressions were pooled using the MI analyze procedure in order to get the parameter estimates.

RESULTS

Study Population Characteristics

Out of the 316 women recruited, 81 women were loss to follow up or discontinued the study, while 8 additional women had incomplete data for folate levels resulting in a sample size of 227 (36 cases and 191 controls). Characteristics of the study sample are presented in Table 1 and 2. The age of the entire cohort ranged from 18 to 43 years old with a mean of 26.2 years (± 6.00 SD). The race distribution among the 227 women was 37.8% white females and the remainder was Black, Asian or Hispanic. Among the entire cohort, the mean folate level was 873.2 (± 167.8 SD) and the mean B12 level was 239.4 ± 84.9, 22.9% were smokers and 7.9% engaged in illicit drug use, 17.3% had a history of preterm births and 46.4% had at least one previous abortion. The infants in the cohort displayed a mean birth weight of 3214.2g (± 673.9 SD) and a mean length at birth of 50.6cm (± 3.8 SD). 69% of the women were experiencing their first pregnancy. Among the cases, the mean age was 26.9 (± 5.7 SD), 41.7% were white and 58.3% were Black, Asian or Hispanic. 40.1% of these women were smokers, 52.8% had a history of abortion and 86.4% had a history of preterm birth. In the controls, the mean age was 26.1 (± 6.1 SD), 36.5% were white females and the remainder were Blacks, Asian or Hispanic. 20.1% of the women with full term births were smokers, 6.8% had a history of preterm birth and

46.1% had a history of abortion. Folate and B_{12} levels were higher in the mothers with preterm births than those with full term births. However, this difference between cases and controls was not significant ($p>0.05$) (Table 1). Also from Table 1, birth weight was significantly lower in cases than in controls, 2304.3 ± 567.9 in cases vs. 3385.5 ± 542.9 in controls, $p < 0.01$. Additionally, as expected length at birth was also significantly lower in preterm babies than in full term babies, 47.6 ± 3.5 in cases vs. 51.1 ± 3.6 in controls, $p < 0.01$. There were also significant differences between cases and controls for maternal tobacco use during pregnancy, $p=0.0308$ and history of preterm births, $p<0.01$ (Table 2).

Folate and Preterm Birth

From the simple logistic model, higher folate levels were associated with greater risk of preterm birth (Crude OR= 0.76, 95% CI: 0.37 – 1.55, p=0.45). Upon adjusting for maternal age, there was no change in the odds ratio and a miniscule change in the 95% CI (Table 3). Adjusting for history of preterm birth increases the odds ratio (Adjusted OR= 0.84, 95% CI: 0.24 – 3.01, p=0.79) and the OR increases even further when maternal smoking is included in the model with folate (OR = 0.92, 95% CI: 0.37 – 2.30, p=0.86). However, none of these associations were found to be significant.

B_{12} and Preterm Birth

Similar results were seen when looking at the relationship between B12 concentration and preterm birth. In the simple regression model, higher B12 levels were associated with (Crude OR= 0.86, 95% CI: 0.42 – 1.77, p = 0.67). After adjusting for maternal age or race, the association remained insignificant and the ORs about the same (Table 3). Adjusting simultaneously for smoking and a history of preterm births, the OR decreased by about 33%, and shockingly conferring an even higher risk for preterm birth in women with high B12 (Adjusted OR= 0.57, 95% CI: 0.15 – 2.20, p=0.42). These associations were also found to be insignificant just as in folate and preterm birth.

After adjustments for a single covariate including maternal age, history of preterm birth, smoking, race in both the folate and B12 logistic models, all the associations remained negative and insignificant. The model that included multiple covariate adjustment, i.e. smoking and a history of preterm birth, also continued to show a negative association for both B12/folate and preterm births. These results also rendered insignificant p values.

Power Analysis

For the folate and preterm birth association, there were 114 people with “low” folate and 113 people with “high” folate (reference group). These sample sizes were able to achieve a very low 12% power in detecting a difference of -0.0370 (table 4) between the two proportions.

Investigating the relationship between B12 and preterm birth, the group sample sizes were able to achieve a power of 7% to detect the difference between the group proportions of -0.0210 (table 4). The power for this data is even lower than that in the folate and preterm birth relationship.

Multiple Imputation

MCMC was used as the multiple imputation method. Following the MI procedure, the between variance was 1.401, the within variance was 31.83 and the total variance was 33.25 for B_{12} as a continuous variable. The mean of B_{12} variable after imputation is 239.5 (figure 2), almost exactly the same as our sample with the missing values (table 2).

The trace plot to assess convergence (figure 3) shows that the chain converged to its stationary distribution and that it is mixing well. It displays a recognizably constant mean and variance with the center of the chain at around 240 where there are slight fluctuations around this value.

The autocorrelation plot for B_{12} from the MI procedure (figure 4) shows no significant positive or negative autocorrelation.

Following 100 imputations, a pooled analysis of the parameter estimates was carried out using the imputed data sets (figure 5). The B_{12} parameter estimate was -0.138 and when exponentiated, provides us with an OR of 0.87 (95% CI: 0.42, 1.80), which is quite similar to the original results of the data.

Variance Information							
Variable	Variance			DF	Relative Increase in Variance	Fraction Missing Information	Relative Efficiency
	Between	Within	Total				
B12	1.400736	31.832918	33.247661	213.66	0.044443	0.042587	0.999574

Parameter Estimates									
Variable	Mean	Std Error	95% Confidence Limits		DF	Minimum	Maximum	Mu0	t for H0: Mean=Mu0	Pr > |t|
B12	239.487546	5.766078	228.1219	250.8532	213.66	236.948294	242.302786	0	41.53	<.0001

Figure 2: Variance information and parameter estimates from the MI procedure

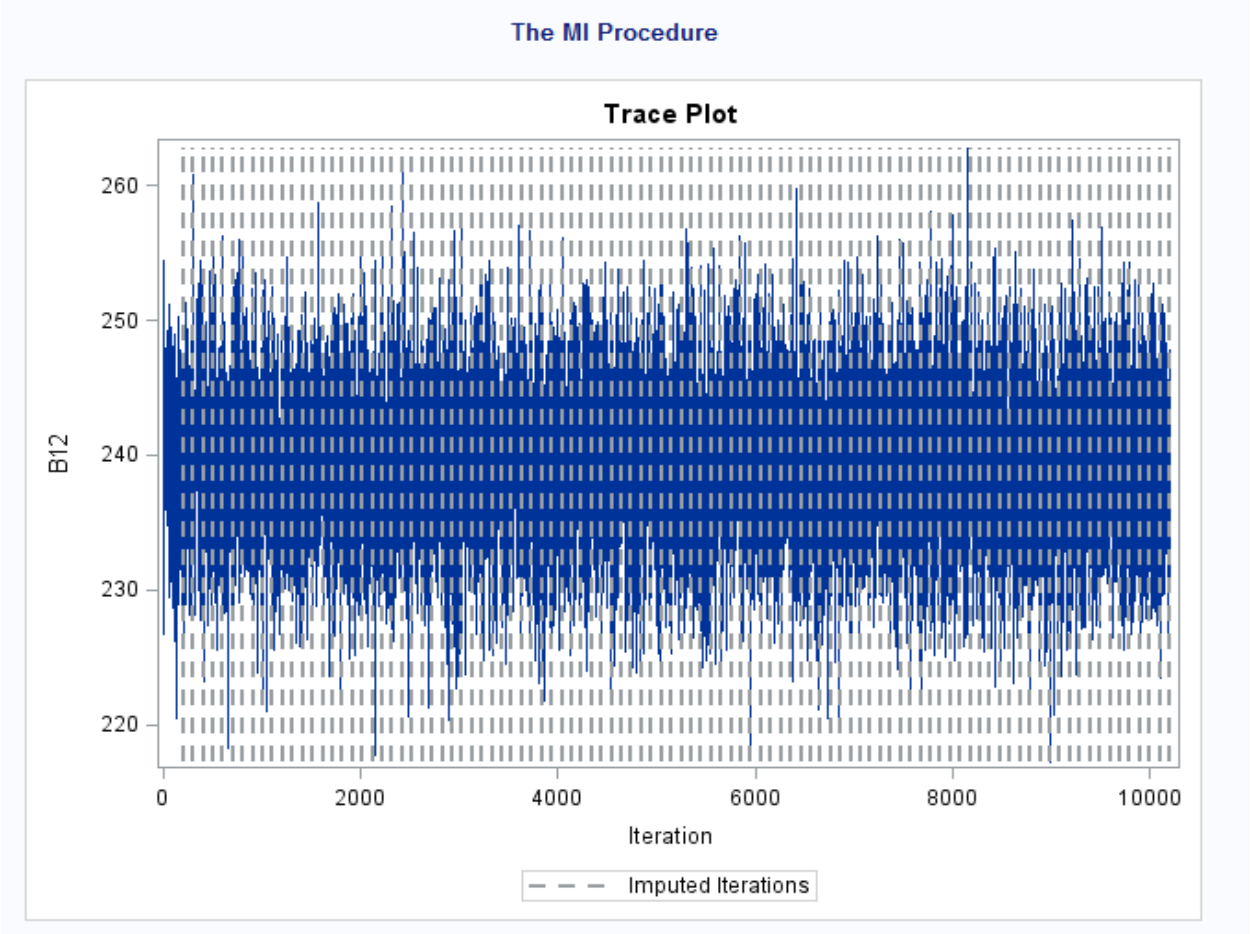

Figure 3: Trace Plot for the B_{12} variable from the MI procedure to assess convergence

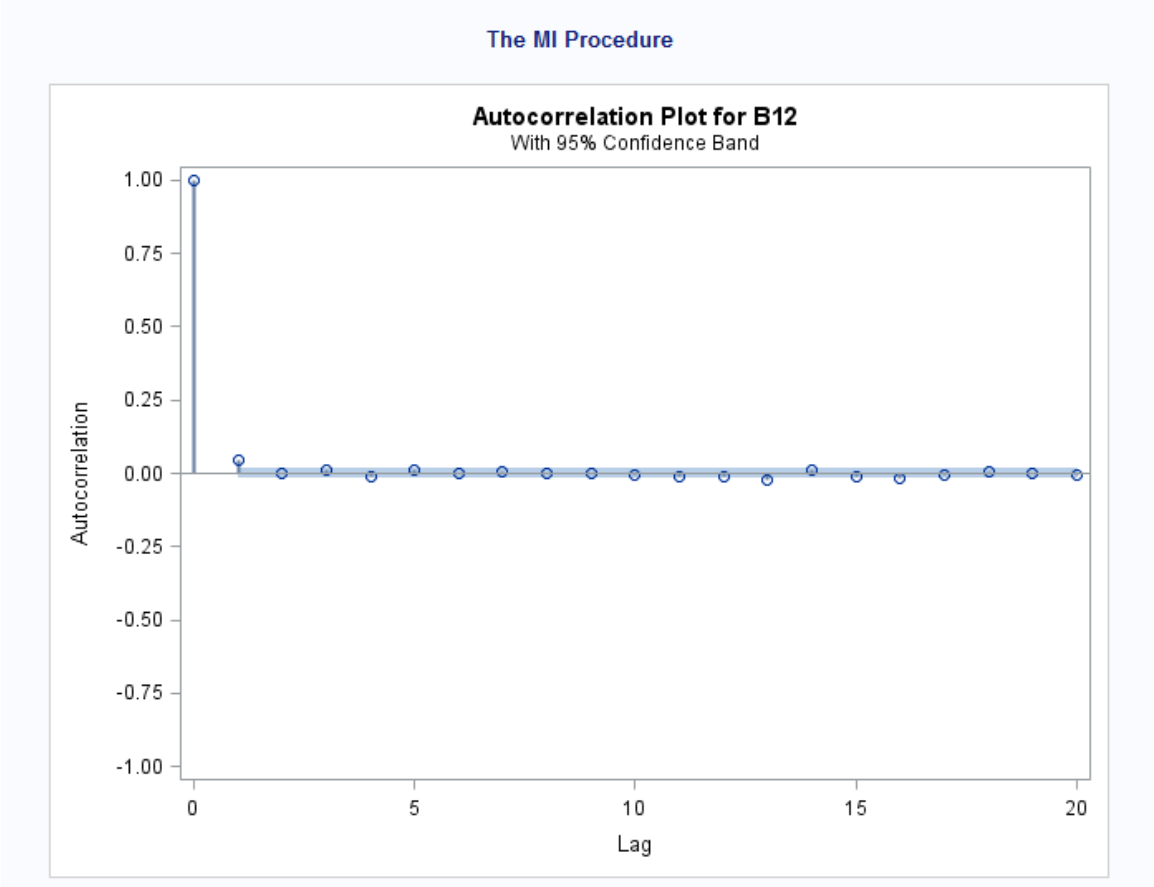

Figure 4: Autocorrelation plot for B_{12} from the MI procedure

PARMS Data Set	WORK.LGPARMS
Number of Imputations	100

Variance Information

Parameter	Variance			DF	Relative Increase in Variance	Fraction Missing Information	Relative Efficiency
	Between	Within	Total				
Intercept	0.001036	0.065089	0.066136	395276	0.016080	0.015831	0.999842
b12_new	0.004240	0.132260	0.136543	100639	0.032380	0.031383	0.999686

Parameter Estimates

Parameter	Estimate	Std Error	95% Confidence Limits		DF	Minimum	Maximum	Theta0	t for H0: Parameter=Theta0	Pr > |t|
Intercept	-1.599348	0.257169	-2.10339	-1.09530	395276	-1.652837	-1.544188	0	-6.22	<.0001
b12_new	-0.138274	0.369517	-0.86252	0.58598	100639	-0.247562	-0.031419	0	-0.37	0.7083

Figure 5: Pooled analysis using MI Analyze procedure

Table 1: Continuous characteristics of cases and controls

Characteristics	Entire Cohort	Cases (20 ≤ GA < 37 weeks)	Controls (GA ≥ 37 weeks)	
	Mean ± SD	Mean ± SD	Mean ± SD	P-value[a]
Maternal Age N=225	26.2 ± 6.00	26.9 ± 5.7	26.1 ± 6.1	0.42
Maternal BMI N=166	32.8 ± 9.1	31.8 ± 7.2	32.9 ± 9.4	0.51
Birthweight (g) N=183	3214.2 ± 673.9	2304.3 ± 567.9	3385.5 ± 542.9	**<0.01**
Length at Birth (cm) N=177	50.6 ± 3.8	47.6 ± 3.5	51.1 ± 3.6	**<0.01**
Folate N=227	873.2 ± 167.8	894.4 ± 158.1	869.2 ± 169.6	0.39
B12 N= 218	239.4 ± 84.9	245.2 ± 102.2	238.3 ± 81.5	0.71

[a] P value estimated by t test under Satterthwaite approximation

Table 2: Categorical characteristics of cases and controls

Characteristics	Entire Cohort	Cases (20 ≤ GA < 37 weeks)		Controls (GA ≥ 37 weeks)		
	N (%) 227	N 36	% 15.9	N 191	% 84.4	P-value[a]
Maternal Tobacco Yes No Missing=61	38 (22.9) 128 (77.1)	9 13	40.1 59.9	29 115	20.1 79.9	**0.031**
Illicit Drug Use Yes No Missing=61	13 (7.9) 152 (92.1)	3 19	13.6 86.4	10 134	69 93.1	0.28
Parity Primiparous Multiparous Missing=58	52 (69) 116 (31)	7 15	31.8 68.2	45 102	30.6 69.4	0.91
Race White Other Missing=2	84 (37.8) 138 (62.2)	15 21	41.7 58.3	69 120	36.5 63.5	0.56
Marital Status Single Other Missing=1	136 (61) 87 (39)	2 11	69.4 30.6	114 76	60.0 40.0	0.29
History of Preterm Yes No Missing=58	29 (17.3) 139 (82.7)	19 3	86.4 13.6	10 137	6.8 93.2	**<0.01**
History of Abortion Yes No	104 (46.4) 120 (53.6)	19 17	52.8 47.2	88 103	46.1 53.9	0.46
Folate Level[b,c] ≤952 >952	114 (50.2) 113 (49.8)	16 20	44.4 55.6	98 93	51.3 48.7	0.45
B12 Levels[b,d] ≤229 >229 Missing=9	113 (51.8) 105 (48.2)	17 18	48.6 51.4	96 87	52.5 47.5	0.67

[a] P-value estimated by chi-square test
[b]The cut off values were determined by the median of the variable; [c] Unit – ng/ml; [d] Unit – pg/ml

Table 3: Logistic regression with preterm birth as the outcome

Folate	0.76 (0.37, 1.55) p = 0.45	0.76 (0.37, 1.56) p= 0.45	0.84 (0.24, 3.01) p= 0.79	0.92 (0.37, 2.30) p = 0.86	0.86 (0.24, 3.13) p = 0.82	0.75 (0.37, 1.54) p = 0.44
B12	0.86 (0.42, 1.77) p = 0.67	0.87 (0.42, 1.80) p = 0.71	0.64 (0.17, 2.39) p = 0.49	0.73 (0.29, 1.84) p = 0.51	0.57 (0.15, 2.20) p = 0.42	0.85 (0.41, 1.76) p = 0.66

[a]Adjusted for maternal age; [b]Adjusted for history of preterm births; [c]Adjusted for smoking; [d]Adjusted for smoking and history of preterm births; [e]Adjusted for race

Table 4 : Two independent proportions power analysis

	Power	N1[a]	N2[a]	P1[b]	P2[c]	D1[d]	Target Alpha	Beta
Folate	0.119	114	113	0.140	0.177	-0.037	0.05	0.881
B12	0.072	114	113	0.150	0.171	-0.021	0.05	0.929

[a] N1 and N2 are the sizes of the samples from the case and control groups respectively

[b] P1 is the proportion for group 1 (cases) under H1

[c] P2 is the proportion of group 2 (control group)

[d] Difference between the 2 proportions

DISCUSSION

The entire cohort displayed adequate levels of folate, with a mean of 873.2 ± 167.8; a similar absence of clinical deficiency of folate was also reported in a Pittsburgh cohort study (Bodnar et al., 2010). Surprisingly, but not unique to this study, the cases of preterm births were characterized by higher mean folate concentrations than the controls. Yamada et al. (2012) also reported similar results in a Japanese population where women who delivered babies at 28-36 weeks of gestation had significantly higher serum folate levels than mothers who delivered term babies. However, this study measured folate levels in the first trimester, while in our study, folate measurements were collected following delivery. Several possible explanations for our results of such high folate concentrations in the cohort studied exist. Interestingly, despite the fact that the US has mandatory folate fortification of food, there is still a high prevalence of preterm births, and this fortification could contribute to the folate-replete population status. Furthermore, mothers who have a history of preterm birth and even a history of abortion are more likely to have a preterm birth in the future; in this cohort, almost 87% of the cases of preterm births had a history of preterm birth. These mothers may have been more health-conscious and this could have prompted them to undergo supplementation in order to try to prevent poor birth outcomes, reflected in the high folate levels seen. Another possible explanation is that women could have undergone supplementation in the third trimester of pregnancy, which could have led to higher RBC folate levels at the time of the sample

collection. One study in a cohort of British women reported that multivitamin-mineral supplementation in the third trimester of pregnancy was associated with a higher risk of preterm birth, (adjusted OR = 3.4, 95% CI 1.2, 9.6, P = 0.02) compared to supplementation in the first and second trimester of pregnancy (Alwan, Greenwood, Simpson, McArdle & Cade, 2010). B_{12} levels were also in the normal range in the entire cohort studied with the exception of a couple outliers. Furthermore, the B_{12} levels displayed similar pattern to folate levels in that the mean serum concentration was higher in the cases than in the controls. These observations may also be attributed to the aforementioned reasons about the folate levels. Finally, we should not exclude the role of chance in these results as our calculated study power was minimal (below).

Additionally, we found no association between maternal RBC folate level and preterm birth. The crude OR displayed non-significant results as the null was embedded in the 95% confidence interval and p values were calculated to be greater than 0.05. Several models were included where individual adjustments for maternal age, a history of preterm births, smoking and race were performed; these results were also non-significant ($p > 0.05$). A final model with simultaneous adjustment for smoking and a history of preterm births also rendered non-significant results. Both crude and adjusted odds ratios displayed non-significant confidence intervals and p values. Investigating the crude and adjusted ORs for B_{12} and preterm births, similar results to the folate relationship were seen in that there was no significant association between preterm births and serum B_{12} concentrations (Table 3). Similar to this study, several studies have found no association between B_{12} and the risk of preterm birth (Bergen et al., 2012; Scholl, Hediger, Schall, Khoo & Fischer; Chen et al., 2014). This may be due to the fact that B_{12} is a cofactor in

the metabolic pathway and thus is rarely a limiting factor. However, both the results for the folate/preterm birth and B_{12}/preterm birth associations should be interpreted with caution. Firstly, our study was underpowered to investigate the effect sizes calculated (table 4). The calculated power for the folate/preterm birth association was approximately 12%, while for the B_{12}/preterm birth association, we experienced an even lower power of 8%. These low study powers can potentially be one of the reasons for our lack of significant associations. Furthermore, we cannot exclude the role of chance in our results as our power was almost negligible.

However, the literature that currently exists on the relationship between folate and preterm births remains equivocal. Several studies have found no association between folate levels and the risk of preterm birth (Ronnenberg, 2002; Bodnar et al., 2010; Nilsen et al., 2010, Yamada et al., 2012; Timmermans, Jaddoe, Hofman, Steegers-Theunissen, & Steegers, 2009; Sengpiel et al., 2014). However, many studies have also shown that higher folate levels are associated with lower risk of preterm births and longer gestational age (Bergen et al., 2012; Parazzini et al., 2011, Chen et al., 2014; Scholl, Hediger, Schall, Khoo & Fischer, 1996; Siega-Riz, Savitz, Zeisel, Thorp, & Herring, 2004; Catov, Bodnar, Ness, Markovic, & Roberts, 2007; Furness, Yasin, Dekker, Thompson, & Roberts, 2011; Bukowski et al., 2009). Barriers to the comparability of these results in the studies include the method of folate level assessments, the time at which it is performed, the dose of supplementation and the fact that many of these studies are performed in populations that are folate-deficient, unlike the US. For instance, many of the randomized control trials use folic acid supplementation as a proxy for folate measurements and there is even variation in the timing of this, i.e. preconceptional more/less than a year in advance,

periconceptional or later on in pregnancy. Additionally, some studies measure serum folate concentrations, while others use dietary intake or supplementation as a proxy for folate concentrations. Nevertheless, many researchers have shown that supplementation and/or dietary intake is positively correlated with circulating levels of folate and even RBC folate concentrations, thereby postulating legitimacy for the results seen (Scholl, Hediger, Schall, Khoo & Fischer, 1996; Kim et al., 2011; Furness, Yasin, Dekker, Thompson, & Roberts, 2011).

It is well established that folate requirements increase throughout pregnancy in order to facilitate the rapid cellular division and growth of the mother and fetus (Chen et al., 2014). The exact mechanisms by which inadequate folate levels influence the risk of preterm birth have not yet been elucidated. However, it is thought that low folate concentration is linked to elevated homocysteine levels, which in turn is related to placental vasculopathy and evidently preterm birth (Chen et al, 2014; Bergen et al., 2012). Moreover, folate, B_{12} and homocysteine are all metabolically linked in the nucleotide and DNA synthesis pathways, through one-carbon metabolism and consequently have all been implicated as risk factors for preterm birth.

There is an extensive array of literature available on this subject as it is of great public health significance due to the high rates of preterm births globally. This study, though underpowered can contribute to this widespread research on preterm births. One of the key observations in this study is the high concentrations of folate and B_{12} concentrations in the cohort. Yet despite this, the preterm birth rate is still above the national and city prevalence of 9.6% and 10.9% respectively. (“2015 PREMATURE BIRTH REPORT CARD PRETERM BIRTH RATES & GRADES BY STATE,” 2014). It will be interesting

then to conduct future studies to see what other factors are responsible for these alarming rates of preterm births if not these nutritional factors. The severely underpowered nature of the study makes it very difficult to draw inferences and conclusions about the associations observed. There are quite a few more limitations that should be acknowledged in the study, many of which are related to the substandard study power. Firstly, the data was subject to many missing entries and consequently our sample size and power fell short as a result. This may have impeded our ability to detect a significant association between folate/B_{12} and a risk of preterm birth in the cohort. Moreover, this limited study power has hampered our capacity to adjust for multiple potential confounders. Being an observational study, it is fundamental to adjust for confounding factors, of which we could have only included up to two covariates in the model and even then there still may be residual confounding. Also related to sample size, any subtype analysis was impossible due to the limited numbers in the dataset. It has been shown that the risk of preterm birth as relate to folate concentration may vary by subtype, which includes early vs late preterm or iatrogenic vs spontaneous preterm (Mantovani, Filippini, Bortolus & Franchi, 2014; Liu et al., 2016). Additionally, our limited sample size in the study only allowed us to look at preterm birth as less than 37 weeks of gestation and more than 37 weeks of gestation, but the associations can vary based on the weeks of gestation, ranging from early to late preterm (Yamada et al., 2012). Bukowski et al. (2009) also reported that there was no association between folate supplement groups and preterm births at 32-37 gestational weeks. Thus, if we could have investigated the association by a separation of the different subtypes of preterm birth and/or early vs late preterm births, we may have found varying results.

Folate and B_{12} levels were measured only once throughout the study, i.e. at delivery and levels could be substantially different at varying time points throughout the pregnancy and as such our study is subject to exposure misclassification. Despite RBC folate measurements being an indicator of long-term folate status, it's only measured once and results can potentially vary at the start of pregnancy for instance and at the end. Furthermore, the recruitment period was at delivery and therefore subject to selection bias. More specifically, some women may have had no/late prenatal care and those excluded due to maternal complications and/or high risk for adverse outcomes could have introduced bias into our study. For example, what if these exclusions were associated with the measured parameters, thereby posing selection bias by their exclusion. Thirdly, these results may only be generalizable to populations with adequate levels of folate as the study was done in a folate-replete population, unlike many areas outside of the US. Additionally, these results are also only generalizable to women of low socioeconomic status (SES) as many of the women who deliver at Tampa General Hospital are of low SES background. Fourthly, there was no data collection on multi-vitamin use and dietary folate in the population. The timing and dose of supplementation could drastically alter our results, especially if the vitamins contain folic acid. Finally, folate was measured once and using red blood cell concentrations, which is a non-specific test and only provides us with overall folate levels. However, Bodnar et al (2010) found that there is a difference in preterm birth risk based on the type of folate species measured from the metabolic pathway and their interaction. When looking at women with a low concentration of 5-methyltetrahydrofolate (5MeTHF), a positive and linear association between 5-formyltetrahydrofolate (5FoTHF) and the preterm births was observed. No association

was seen when 5MeTHF was at its median levels and the association became negative when 5MeTHF levels were high. They also found significant interaction between these folate metabolites (p=0.01). This study only measured overall folate concentration, and in light of our high preterm birth rates in the background of a folate-replete population, it may be more valuable to measure individual folate metabolites rather than overall concentration. Also a limitation is that another key metabolite, homocysteine serves as a functional biomarker for folate metabolism and has been extensively researched for its role in preterm births was not assessed. A study in Chinese women showed that the risk of preterm births was four times higher in those with elevated homocysteine concentrations than those with low/normal levels (Ronnenberg, 2002). Noteworthy is folate's central role in one-carbon metabolism in a complex pathway that is responsible for DNA repair and synthesis, as well as DNA methylation. As such, interest has peaked more recently about the role of genetics in this pathway and its association with preterm birth (Wang et al., 2015; Yangrong & Hongmei, 2015). Several single nucleotide polymorphisms (SNPs) have been shown to have an association with preterm birth, two of which are in the methylene tetrahydrofolate reductase (MTHFR) gene, i.e. C677T and A1298C. MTHFR is responsible for the production of the active form of folate, i.e. 5-Methyltetrahydrofolate (5-MTHF), which is responsible for the conversion of homocysteine to methionine (Zeisel, 2009). The aforementioned SNPs result in reduced enzymatic activity and subsequently increased levels of homocysteine, a functional indicator of folate deficiency (Zeisel, 2009). Several studies have shown that these SNPs result in an increased demand for folic acid (Wang et al., 2015; Yangrong & Hongmei, 2015). Moreover, research has shown that these SNPs affect red blood cell

concentrations whereby the C677T results in decreased concentration and A1298C results in an increased concentration (DiLuglio, 2015). In future studies, these genetic factors should be taken into consideration and its implications for our measurements.

Despite the limitations, this study still adds to the growing body of research about the relationship between folate and/or B_{12} and preterm births. More specifically, it is interesting that preterm birth rates are so high in a folate-replete population. For the future, since this is only an observational study and we cannot infer causation, more randomized controlled trials need to be carried out; though the ethics involved in this area of research are in question. Since this is a folate-replete population, it would be interesting to conduct another study that measures the concentrations of individual folate metabolites and look at these associations with the risk of preterm births. Finally, it will be advantageous to gather longitudinal information on both folate and B_{12} concentrations before, during and following pregnancy as folate has a half-life of 100 days (Liu et al., 2016). This may help us to further elucidate its dynamic nature and role of folate and B_{12} in preventing preterm births.

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APPENDIX A: Literature Review on Folate/B_{12} and Preterm Birth

Author (Year of Publication)	Study Design	Population	Relevant Findings
Scholl et al. (1996)	Prospective cohort	730 pregnant women who had their samples taken at 28 weeks of gestation	• Mean folate intake was significantly correlated with circulating folate concentrations (P < 0.001) • Lower concentrations of serum folate were associated with an increased risk of preterm birth • Serum vitamin B-12 concentration was not associated with the risk of preterm delivery.
Ronnenberg et al. (2002)	Case-control	29 preterm cases (babies born before 37 weeks of gestation); 405 controls (babies born on or after 37 weeks of gestation)	• There was a negative association between vitamin B_{12} levels and preterm births (Adjusted OR 0.4, 95% CI: 0.2, 0.9) • There was no association between folate levels and preterm birth (Adjusted OR 1.0, 95% CI: 0.4, 2.9)

Siega-Riz et al. (2004)	Prospective cohort	2,468 lower to middle income women at 24-29 weeks of gestation	• Mean dietary and serum folate was significantly higher in women who delivered term than those who delivered preterm (P = 0.01 and P = 0.04 respectively) • Lower serum folate levels were associated with a higher risk of preterm birth (Adjusted RR 1.8, 95% CI: 1.3, 2.5)
Catov et al. (2007)	Prospective cohort	1,823 women recruited at <16 weeks of gestation and followed through the postpartum visit	• There was a negative association between periconceptional multivitamin use and the risk of preterm births (Adjusted OR 0.29, 95% CI: 0.13, 0.64)
Bukowski et al. (2009)	Prospective cohort	34,480 women who delivered singleton pregnancies at 20-42 weeks of gestation	• There was a 50% decrease in the risk of spontaneous preterm birth at 28-32 gestational weeks in those who had preconceptional folate supplementation compared to no supplements (Adjusted HR 0.53, 95% CI: 0.28, 0.99, P = 0.046). • There was no association between preconceptional folate supplementation and preterm birth after 32 weeks of gestation.
Timmermans et al. (2009)	Population-based	6,353 low-risk singleton pregnancies	• No significant association was found between preconceptional or

	prospective cohort		periconceptional folate supplementation and preterm birth
Nilsen et al. (2010)	Population-based prospective cohort	2934 singleton births	• Dietary folate intake, supplemental folic acid use and maternal plasma folate concentrations were not associated with gestational age, infant birth weight, head circumference or crown-heel length; p values > 0.05 for all associations.
Bodnar et al. (2010)	Prospective cohort	313 women enrolled at <16 weeks of gestation	• Women who had serum total folate concentrations in the highest tertile experienced a 60% reduction in the risk of preterm birth (Adjusted RR 0.4, 95% CI: 0.1, 0.9) • Insignificant results garnered after further adjustment for unmeasured confounding by regular periconceptional multivitamin use (RR 0.4, 95% CI: 0.1, 1.0) • Significant interaction between folate metabolites 5MeTHF and 5FoTHF (P = 0.05), showing a positive and negative association between folate and risk of preterm birth
Alwan et al. (2010)	Prospective birth cohort and nested-case control	Women 18-45 years old at 8-12 weeks of gestation; 425	• Association with any type of daily supplement (including folic acid) and an increased risk of preterm birth (Adjusted OR 3.0, 95% CI:

		women in 2:1 ratio for nested case-control	1.2, 7.4, P = 0.02) in the 3rd trimester; not statistically significant in the 2nd trimester (P = 0.2) and marginally significant in the 1st trimester (p=0.05)
Parazzini et al. (2011)	Prospective cohort	244 singleton pregnant women at 8-10 weeks of gestation	• Mean week of gestation at birth was higher in women who were in the 2nd and 3rd tertiles of red cell folate levels at baseline and at 16 weeks of gestation
Furness et al. (2011)	Retrospective case-control	400 primiparous women at their first antenatal visit (10-12 weeks of gestation)	• Women who were folate-deficient in early pregnancy had a higher risk of preterm delivery (OR 5.4, 95% CI: 1.4, 21.2)
Bergen et al. (2012)	Population-based birth cohort	5805 total singleton live births; 5774 with folate data	• Women in the lowest quantile of folate levels had twice the risk of preterm birth compared to those in the highest quantile (Adjusted OR 2.17, 95% CI: 1.34, 3.57, P = 0.002). • No associations were found with B_{12}
Yamada et al. (2012)	Prospective cohort	5,075 pregnant women at 5 to 13 weeks of gestation	• The mean serum folate level was significantly higher in women who had preterm births than those who didn't • No association between preterm births and folate status

Chen et al. (2014)	Population-based birth cohort	999 maternal blood samples collected at 26-28 weeks of gestation	• Higher plasma folate concentrations were associated with lower risk of all preterm birth (Adjusted OR 0.79, 95% CI: 0.63, 1.00) and spontaneous preterm birth (Adjusted OR 0.76, 95% CI: 0.56, 1.04) • Higher maternal vitamin B_{12} concentrations were not significantly associated with lower preterm birth risk (Adjusted OR 0.81, 95% CI: 0.64, 1.03)
Li et al. (2014)	Population-based cohort	207,936 singleton live births delivered between 20 and 42 weeks of gestation	• Incidence of overall preterm births was significantly lower in folic acid users than in non-users (5.28% vs 6.10%) • There was a protective association between folic acid use and the risk of overall preterm birth (Adjusted RR 0.86, 95% CI: 0.82, 0.90)
Sengpiel et al. (2014)	Population-based prospective cohort	66,014 women with singleton live births	• Dietary folate intake was not associated with the risk of preterm birth (Adjusted HR 1.00, 95% CI: 0.65,1.65) • Supplemental folate intake was also not associated with the risk of preterm birth (Adjusted HR 1.00, 95% CI: 1.00-1.00) • Folic acid supplementation more than 8 weeks prior to conception

			was associated with higher risk of preterm birth compared to no folic acid supplementation (Adjusted HR 1.18, 95% CI: 1.05, 1.32) • No association observed between folic acid supplementation within 8 weeks of conception (Adjusted HR 0.99, 95% CI: 0.87, 1.13)
Liu et al. (2015)	Birth cohort	10,179 women with singleton live births	• Folic acid supplement users had a lower risk of preterm births compared to non-users (OR 0.80, 95% CI: 0.68, 0.98) • Higher estimated intake of dietary folate was also associated with lower risk of preterm birth (OR 0.68, 95% CI: 0.56, 0.83)

APPENDIX B: IRB Approval

USF
UNIVERSITY OF
SOUTH FLORIDA

RESEARCH INTEGRITY AND COMPLIANCE
Institutional Review Boards, FWA No. 00001669
12901 Bruce B. Downs Blvd., MDC035 • Tampa, FL 33612-4799
(813) 974-5638 • FAX(813)974-7091

8/4/2016

Ronee Wilson, PhD
Epidemiology and Biostatistics
13201 Bruce B. Downs Blvd
MDC 56
Tampa, FL 33612

RE: **Expedited Approval for Continuing Review**
IRB#: CR6_Pro00001616
Title: Role of Folic Acid in preventing spontaneous preterm birth

Study Approval Period: 8/26/2016 to 8/26/2017

Dear Dr. Wilson:

On 8/4/2016, the Institutional Review Board (IRB) reviewed and **APPROVED** the above application and all documents contained within including those outlined below.

Approved Item(s):
Protocol Document(s):
Revised Study Protocol_8-20-15_clean

The IRB determined that your study qualified for expedited review based on federal expedited category number(s):

(8) Continuing review of research previously approved by the convened IRB as follows: (a) where (i) the research is permanently closed to the enrollment of new subjects; (ii) all subjects have completed all research-related interventions; and (iii) the research remains active only for long-term follow-up of subjects; or (b) where no subjects have been enrolled and no additional risks have been identified; or (c) where the remaining research activities are limited to data analysis.

As the principal investigator of this study, it is your responsibility to conduct this study in accordance with USF HRPP policies and procedures and as approved by the USF IRB. Any changes to the approved research must be submitted to the IRB for review and approval by an amendment. Additionally, all unanticipated problems must be reported to the USF IRB within five (5) calendar days.

We appreciate your dedication to the ethical conduct of human subject research at the University of South Florida and your continued commitment to human research protections. If you have any questions regarding this matter, please call 813-974-5638.

Sincerely,

V Jorgensen MD

E. Verena Jorgensen, M.D., Chairperson
USF Institutional Review Board