

FOLIC ACID SUPPLEMENTATION IN PREGNANCY: MATERNAL OUTCOMES

by

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(Under the Direction of Dorothy B. Hausman)

ABSTRACT

The present study reports an interim analysis of serum and RBC folate response to supplementation in a subset of participants with available baseline and 28 week blood samples (n=22). This analysis is part of an on-going double blind randomized control trial in pregnant women comparing the effects of 400 µg and 800 µg daily folic acid on folate biomarkers from the first prenatal visit (<12 weeks gestation) through delivery. Mixed effects analysis indicated a significant time effect for RBC folate concentration (p = 0.035) and this time response was significantly impacted by length of folic acid supplementation prior to enrollment (p = 0.047) and race/ethnicity (p = 0.016). No treatment effect was seen for serum folate, but both race/ethnicity (p = 0.007) and length of previous supplementation (p = 0.038) had significant overall effects on this variable. Overall, there was no significant effect of BMI on folate status. Completion of the study and further analysis is needed to analyze the effects of folic acid dose on additional outcome parameters such as oxidized folic acid in the serum and changes in maternal and cord blood DNA methylation.

INDEX WORDS: Pregnancy, Folate, Folic acid, Serum Folate, RBC Folate, Folic acid supplementation, BMI

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CHAPTER 1

INTRODUCTION

Folate, or vitamin B9, is an essential water-soluble vitamin found naturally in beans, legumes, leafy green vegetables, and orange juice, while folic acid is the fully oxidized, more stable form of the vitamin that is added to fortified foods and supplements. Since folic acid is more bioavailable than natural food folate, dietary recommendations are made in dietary folate equivalents (DFEs). One DFE is the equivalent of 1 µg natural food folate or 0.6 micrograms of folic acid (Sutor and Bailey 2000). Upon absorption, dihydrofolate reductase (DHFR) must reduce folic acid into the most biologically active form, 5-methyltetrahydrofolate (5-methylTHF) (Shane 2010). With larger boluses of folic acid, DHFR becomes saturated and unmetabolized (or oxidized) folic acid remains in the blood (Patanwala et al 2014, Kelly et al 1997).

In the body, folate plays a key role in one-carbon metabolism making it an essential component of amino acid metabolism, purine and pyrimidine synthesis, and the formation of *S*-adenosylmethionine (SAM) (Bailey and Gregory 1999). Because of these biological functions, folate is necessary for the production of red blood cells (RBCs) and sufficient cell division and growth during periods of rapid development, such as pregnancy (Bailey and Gregory 1999, Gropper et al 2009). Folate is important throughout the entirety of pregnancy to support blood volume expansion, increased cell division, and the rapid growth and development of the fetus (Picciano 2003), but is particularly important during the first trimester of pregnancy when the neural tube of the fetus is forming. Although the exact

mechanism is not known, randomized control trials have shown a strong association between inadequate blood folate concentrations and increased risk for neural tube defects (NTDs) (Czeizel and Dudas 1992, Wald et al 1992), a group of potentially fatal birth defects characterized by the failure of the neural tube to close at the base of the spine or at the brain (Medline Plus 2014).

In order to help identify the RDA for pregnancy, Caudill et al (1997) conducted a controlled feeding study in pregnant women to compare the effects of 330 μg and 730 μg supplemental folic acid in addition to 120 μg dietary folate on folate biomarkers. At the end of the 12-week intervention, there was no significant difference in RBC folate concentrations between the two groups, indicating that both doses were sufficient to maintain adequate blood folate concentrations during pregnancy. This study led to the current recommended dietary allowance (RDA) from the Institute of Medicine (1998), which recommends that all women of childbearing age consume at least 400 μg DFE daily and pregnant women consume 600 μg DFE, or approximately 400 μg folic acid, daily to meet the increased demands of pregnancy and prevent negative birth outcomes.

Although the benefits of 400 μg supplemental folic acid on pregnancy outcomes are well studied (Ellison et al 2004, McNulty et al 2013), most over-the-counter prenatal vitamins available in the United States contain 800-1000 μg folic acid, over twice the recommendation. This raises the concern that some pregnant women may be exceeding the current folic acid tolerable upper limit (UL) of 1000 μg folic acid/ d set for pregnancy (Institute of Medicine 1998). In the Newborn Epigenetics Study (NEST), consisting of 539 pregnant women, over 10% of the participants were exceeding the UL for folic acid (Hoyo et al 2011). Most of the potential side effects of surpassing the UL identified

through animal studies, such as reproductive effects and carcinogenicity, are controversial, but there is an emerging concern that higher doses of folic acid can alter epigenetic regulation causing unforeseen long-term effects in the offspring of mothers taking high doses of folic acid (Burdge and Lillycrop 2012).

Another factor of concern is the increasing prevalence of pregnant women who are obese (Fisher et al 2013). Almost 32% of women of childbearing age are obese (Ogden et al 2014), yet there are a limited number of studies in which the impact of body mass index (BMI) on folate status during pregnancy has been investigated. Studies conducted in women of childbearing age have found that a higher BMI is associated with lower concentrations of serum folate when folic acid supplements are not regularly consumed (Tinker et al 2012, da Silva et al 2013). Stern et al (2011) concluded that body composition and metabolic differences between normal weight and obese individuals may cause obese individuals to need a higher dose of folic acid compared to normal weight women. Additionally, a higher dose of folic acid may be beneficial for obese individuals since there is a higher prevalence of negative birth outcomes, including NTDs, in this population (Ray et al 2005, Gao et al 2013).

For this study, 400 µg/d and 800 µg/d folic acid doses were selected to compare the RDA for pregnant women (Institute of Medicine 1998) to the dose commonly found in over-the-counter prenatal vitamins. Serum folate and RBC folate biomarkers were used to evaluate the effect of folic acid supplementation since serum folate is a reliable marker of short term folate status (Chanarin 1986, O'Keefe 1995), while RBC folate is a better marker of long-term (>120 d) folate status (Chanarin 1986, Shane 2011).

This study is significant because it is the first double blind randomized control trial to compare these two doses of folic acid in pregnant subjects from the first prenatal visit (~6-12 wk gestation) through delivery. In addition, to our knowledge, it is the first folic acid intervention study in pregnant women to take pre-pregnancy BMI into consideration.

The research question asks if there is a difference in serum folate and RBC folate concentrations in response to 400 or 800 µg folic acid supplementation throughout pregnancy, after adjusting for BMI and other potential confounders. Based on the finding of Caudill et al (1997), it is hypothesized that maternal serum folate concentrations will be significantly higher in the 800 µg group, but there will be no difference in RBC folate concentrations. Additionally, it is hypothesized that serum and RBC folate concentrations will be impacted by pre-pregnancy BMI.

Following the introduction, this thesis will contain additional chapters. Chapter 2 is a review of the literature on folate and pregnancy. Topics covered on folate include its forms and bioavailability, metabolism and biological function, fortification in the United States, oxidized folic acid, folate biomarkers, and factors influencing folate status. Additionally, topics related to pregnancy include pregnancy physiology and folate metabolism, folate requirements and recommendations for pregnancy, and folic acid supplementation during pregnancy. Chapter 3 is a manuscript on serum folate and RBC folate response to folic acid supplementation throughout pregnancy in healthy pregnant women. Chapter 4 is the conclusion, which summarizes the findings of this research study, presents conclusions, and offers suggestions for future research.

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CHAPTER 2

LITERATURE REVIEW

Folate forms and bioavailability

Folate is the generic term used for the water-soluble B vitamin that includes naturally occurring folate in food and synthetic folic acid. The molecular structure of folate includes a pteridine bicyclic ring system, *p*-aminobenzoic acid, and at least one glutamic acid residue. Folate refers to the polyglutamate form that is naturally found in beans, legumes, leafy green vegetables, and orange juice. Folic acid is the more stable oxidized monoglutamate form found in supplements and fortified foods.

Bioavailability is defined as the amount of consumed nutrient that is absorbed and is either available to take part in metabolic processes or is stored (McNulty and Pentiva 2009). With regard to folate, the varying composition and stability of folate forms results in differences in bioavailability and it is well recognized that folic acid is more bioavailable than natural food folate (Colman et al 1975). Although it is difficult to measure the exact bioavailability of different folate forms, a review of studies shows that folic acid containing foods may be anywhere for 37-153% more bioavailable compared to natural food folate (Gregory 2001, Ohrvik and Witthoft 2011). It is generally recognized that there is almost 100 percent bioavailability from folic acid supplements while fasting (Gregory 1997) and roughly 85 percent bioavailability from fortified foods (Pfeiffer et al 1997). Because of differences in availability, the Recommended Dietary Allowances (RDA) uses Dietary Folate Equivalents (DFEs) to determine adequacy of folate intake. One DFE is equivalent to 1 µg of natural food

folate or approximately 0.6 µg folic acid (Suitor and Bailey 2000) and total folate intake is comprised of micrograms natural food folate plus 1.7 times the micrograms of folic acid consumed.

Folate metabolism and biological function

When folate or folic acid is absorbed into the body, it undergoes a series of reduction reactions to create 5,10-methylenetetrahydrofolate (5,10-methylene THF). This molecule then undergoes further reduction by methylene tetrahydrofolate reductase (MTHFR) to create the most biologically active form of folate, 5-methyltetrahydrofolate (5-methyl THF) (Shane 2010). This is the form primarily responsible for accepting one-carbon units from amino acid metabolism in the mitochondria and cytosol and donating them in a variety of biological reactions (Shane 2010).

One-carbon metabolism is the term used to describe all the reactions that require folate to accept and subsequently donate one-carbon units. This biological function makes folate a key component of amino acid metabolism, purine and pyrimidine synthesis, and the formation of *S*-adenosylmethionine (SAM) (Bailey and Gregory 1999). In order to produce SAM, a key methylating agent, 5-methyl THF donates a methyl group to convert homocysteine to methionine, which is further converted to SAM (Bailey and Gregory 1999). SAM plays a role in over 100 methyltransferase reactions in the body, including the methylation of DNA, RNA, and histones (Bailey and Gregory 1999, Stover 2010).

During pyrimidine synthesis, 5,10-methylene THF is used to convert deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), an essential component of DNA synthesis (Gropper et al 2009). For the formation of purine ring

structure, 10-formyl THF is needed to donate a formyl unit to 5-phosphoribosylglycinamide (glycinamide ribotide) to create 5-phosphoribosyl formylglycinamide ribotide.

Additionally, 10-formyl THF also donates a formyl unit to 5-phosphoribosyl 5-amino 4-imidazole 4-carboxamide ribonucleotide (AICAR) (Gropper et al 2009).

Because of the essential role folate plays in these reactions, folate is necessary for proper cell division and growth, particularly for the relatively quick turnover of red blood cells (RBCs) and during periods of rapid growth, such as pregnancy (Bailey and Gregory 1999, Gropper et al 2009). Although the exact mechanism is not known, randomized control trials have shown a clear link between insufficient folic acid intake and increased risk for neural tube defects (NTDs) (Czeizel and Dudas 1992, Wald et al 1992). NTDs are a group of birth defects that occur when the neural tube does not close during the first month of gestation (Medline Plus 2014a). Spina bifida occurs when the neural tube does not close at the base of the spine, typically resulting in minor to severe paralysis or even death. Anencephaly is the improper development of the brain or skull, resulting in early postnatal death (Medline Plus 2014a). Through an unknown biological mechanism, folic acid supplementation can reduce the risk for NTDs, even in women who have previously had NTD affected pregnancies (Laurence 1981).

Folic acid fortification in the United States

Fortification is the addition of vitamins and minerals to a food that are not naturally found in the food (Academy and Nutrition and Dietetics). This method of vitamin supplementation has been used in the United States since 1924 and has been shown to be a cost effective way to prevent nutrient deficiencies (Backstrand 2002, Berry et al 2010a). In

the United States, grain flour and ready to eat cereals are among the most commonly fortified products, while there is no requirement for the fortification of corn flour or corn based products (Food and Drug Administration 1996).

By 1992, randomized control trials showed a clear link between insufficient folic acid intake and increased risk for NTDs (Czeizel and Dudas 1992, Wald et al 1992). After a thorough review of the data and in an effort to reduce risk for NTDs, the US Food and Drug Administration mandated that all enriched cereal grain products be fortified with folic acid by January 1, 1998 (Food and Drug Administration 1996). The enriched grain products are required to contain between 0.43 mg to 1.4 mg folic acid per pound to provide women of childbearing age an additional 100 to 200 µg of folic acid per d.

Mandatory folic acid fortification in the United States has increased consumption of folic acid, which has been associated with increased blood folate concentrations (Berry et al 2010a). Nationally, NHANES data shows that since mandatory folic acid fortification serum folate concentrations increased by 136% (11.4 nmol/L to 26.9 nmol/L) and RBC folate increased 57% (375 nmol/L to 590 nmol/L) (Dietrich et al 2005).

The increase in folate concentration is associated with a decline in the rates of NTDs (Berry et al 2010b). Williams et al (2002) looked at the prevalence of NTDs prior to fortification, during optional fortification, and after folic acid fortification became mandatory. The researchers identified 5,630 NTD cases from 24 different population-based surveillance systems. Overall, there was a 31% decrease in spina bifida and a 16% decrease in anencephaly after mandatory fortification. Similar trends were found in the CDC's National Center for Health Statistics (NCHS) reports between 1991 and 2001. Mathews et al

(2002) reviewed the reports on spina bifida and anencephaly and found a 23% decrease in the occurrence of all NTDs, a 24% decrease in spina bifida, and a 21% decline in anencephaly.

Oxidized folic acid

Folic acid, from fortified foods and supplements, must be reduced to dihydrofolate and then reduced again by dihydrofolate reductase (DHFR) in order to become tetrahydrofolate and be converted to 5-methylTHF (Shane 2010). While some folic acid can be converted to 5-methylTHF on its first pass through the intestinal mucosa and liver (Shane 2010), it appears that the DHFR may be a rate limiting step, causing unmetabolized (or oxidized) folic acid to remain in the blood with large doses (Patanwala et al 2014, Kelly et al 1997). The effects of continuous exposure to high concentrations of oxidized folic acid are not well understood (Berry et al 2010a, Smith et al 2008).

Circulating concentrations of oxidized folic acid have been reported for multiple studies in both non-pregnant and pregnant subjects. In Dublin, Ireland, where folic acid fortification is voluntary, Sweeny et al (2009) collected blood samples from non-fasting blood donors (n = 50) at the Irish Blood Transfusion Service. Detectable concentrations of oxidized folic acid were found in 49 of the blood donors. In addition, an association was observed between plasma folate and plasma oxidized folic acid. Serum folate forms were also recently measured in the 2007-2008 NHANES cohort (Pfeiffer et al 2015). In this nationally representative sample of the US population, over 95% of individuals had detectable concentrations of oxidized folic acid in the serum and 33% of the population had concentrations of oxidized folic acid greater than 1 nmol/L.

Kelly et al (1997) conducted an intervention trial to determine a threshold of folic acid intake that causes oxidized folic acid to appear in the blood. Two experiments were conducted in subjects ages 18-42 years old. In the first experiment, healthy subjects (n = 14) were given folic acid in fortified cereals and bread for a total daily intake of 90, 400, 800, 900, 1000, 1100, or 1200 μg for 4 d. Blood draws were performed 2.25 h after the last intake of fortified cereal to measure serum folate and folic acid. All subjects had a significant increase in serum folate, but only subjects consuming 800 $\mu\text{g}/\text{d}$ folic acid or more had measureable concentrations of oxidized serum folic acid. In the second experiment, subjects (n = 6) were given a single bolus of 200, 300, or 400 μg folic acid in Dioralyte two weeks apart. Fasting and postprandial blood samples were drawn for measurement of total folate and folic acid. None of the individuals had detectable fasting concentrations of oxidized folic acid, whereas the majority of individuals that consumed the 300 and 400 μg doses of folic acid had detectable postprandial concentrations of oxidized folic acid (Kelly et al 1997).

Studies have found detectable concentrations of oxidized folic acid in the serum of pregnant women taking supplements and in pregnant women only consuming fortified foods and dietary folate (Berry et al 2010a). Obeid et al (2010) looked at the folate biomarker concentrations at delivery of women who took 400 $\mu\text{g}/\text{d}$ folic acid during pregnancy (n = 25) and pregnant women who took no folic acid supplement (n = 61). They found that women taking supplements had significantly higher concentrations of total folate and 5-methylTHF, but both groups had similar concentrations of oxidized folic acid in the blood. The effects of higher doses of folic acid during pregnancy are not known.

Folate biomarkers

A landmark paper by Herbert (1962) first showed an effect of a folate-depleted diet (5 µg/d food folate) on red blood cell (RBC) and serum folate biomarkers. A single male physician was enrolled in this study. Serum folate concentrations fell after only 3 wks on the folate depleted diet, but RBC folate concentrations did not fall until 17 wks into the study. A culmination of studies during this time period led to the conclusion that serum folate is a reliable marker of short-term folate status and should be measured while fasting, if possible, as it can be affected by recent intakes of folate and folic acid (Chanarin 1986, Shane 2011, O'Keefe 1995). Red blood cells only take up folate during erythropoiesis, and retain it for the 120-day lifespan of the cell. Therefore, RBC folate status changes more slowly with the turnover of RBCs making it a good marker of long-term folate status (Chanarin 1986, Shane 2011). In a controlled folate feeding study by O'Keefe et al (1995), it was demonstrated that with inadequate dietary folate intake serum folate can significantly drop in a relatively short amount of time (<70 d), while RBC folate values will trend downward but not reach statistical significance within this time period.

Homocysteine is an amino acid created in the methionine cycle. 5-methylTHF is required for the re-methylation of homocysteine to methionine, so with a drop in available folate, homocysteine builds up. In 1987, Kang et al conducted a cross-sectional study observing the relationship between total serum folate and homocysteine concentrations. In the study, 84% of individuals with subnormal serum folate (<2 ng/mL) and 56% of individuals with low normal serum folate (4.0-17.9 ng/mL) had serum homocysteine concentrations over two standard deviations above the normal mean (7.05 nmol/mL). A third of the individuals with low folate status with elevated homocysteine levels had homocysteine

values over three standard deviations above the normal mean (Kang et al 1987).

Homocysteine is often used as an indicator of folate status, but alone cannot indicate a folate deficiency (Stabler 2010). The conversion of homocysteine to methionine also requires vitamin B12, therefore homocysteine concentrations are influenced by vitamin B12 status as well. In addition to folate and vitamin B12 status, homocysteine is also influenced by other physiological indicators, such as liver and kidney function and cannot be used as a sole indicator of low folate status. (Hannibal et al 2010, Stabler 2010, Savage 1994).

Factors influencing folate status

Diet and supplement intake

Some of the best food sources of natural food folate include leafy green vegetables, legumes, and orange juice, although this form of folate is less bioavailable than folic acid found in fortified food and supplements (Gregory 1997, Pfeiffer et al 1997). Prior to 1998, when folic acid fortification became mandatory for cereal grain products, vegetables were the greatest source of folate (20% of folate intake) in the American diet, but total folate intake only averaged about 275 ± 3.2 $\mu\text{g}/\text{d}$ (Dietrich et al 2005). Because of the low dietary intake, average serum folate (11.4 ± 0.24 nmol/L) and RBC folate (375 ± 3.8 nmol/L) concentrations were also relatively low. After mandatory fortification, breads, rolls, and crackers became the largest source of folate in the American diet, making up approximately 15.6% of total folate intake. Due to increased availability of folic acid in the diet, total folate intake increased 351 ± 9.1 $\mu\text{g}/\text{d}$, as did serum folate (26.9 ± 0.49 nmol/L) and RBC folate (590 ± 11.6 nmol/L) concentrations (Dietrich et al 2005).

In addition to obtaining folate from foods naturally containing folate and folic acid fortified foods, dietary supplements are also a good source of folic acid. Based on data from the NHANES cohort from 2003-2006, it was estimated that 53% of the United States population was taking a supplement containing folic acid (Bailey et al 2010). Individuals taking a supplement in addition to fortified grain products and ready to eat breakfast cereals were consuming over twice as much folic acid as those just consuming fortified foods and breakfast cereals (Yang et al 2010). The individuals taking supplements had significantly higher RBC folate (329 ng/mL) and serum folate (16.9 ng/mL) compared to the RBC folate (273 ng/mL) and serum folate (12.1 ng/mL) values of individuals not taking supplements (Yang et al 2010).

Genetic polymorphisms

Methylenetetrahydrofolate reductase (MTHFR) is important for the conversion of methylene tetrahydrofolate to 5-methyl-tetrahydrofolate (5-methyl-THF). Single nucleotide polymorphisms in this gene have been linked to increased risk for vascular disease and NTDs (Christensen and Rozen 2009, van der Put et al 1995).

One of the most common MTHFR polymorphisms is the MTHFR 677C→T variant. A study by Rady et al (2002) looking at frequencies of MTHFR genetic polymorphisms of 507 individuals of various ethnic populations in Texas, found significant differences in the rate of occurrence of the 677TT polymorphism. Hispanics (26%) and Ashkenazi Jewish (26.5%) were the most likely to have the homozygous 677T polymorphism, followed by Caucasians (11.3%), and African-Americans (1%) who were the least likely to be homozygous for the variation. This polymorphism in the MTHFR gene leads to decreased

production of 5-methyl-THF and an increased in homocysteine. In a study of 6793 individuals of all races and ethnicities from the third NHANES, Yang et al (2008) found that persons with the 677TT variant had 22.1% lower serum folate and 25.7% higher homocysteine than individuals with the wild-type 677CC gene. This effect was more prominent when folate intake was low.

Race/Ethnicity

Folate status is known to vary by race. NHANES data from 2005-2006 in women of childbearing age indicated that non-Hispanic whites (272 ng/mL) and Mexican-Americans (252 ng/mL) had similar median RBC folate concentrations and non-Hispanic blacks (210 ng/mL) had a significantly lower RBC folate concentration (McDowell 2008). In addition, this study found that non-Hispanic black women (9.8 ng/mL) and Mexican-Americans (10.4 ng/mL) had significantly lower serum folate than non-Hispanic whites (11.8 ng/mL). Marchetta and Hamner (2014) analyzed NHANES data from 2001 to 2010 and found that the disparities in blood folate markers between non-Hispanic white women and Mexican American women were even more pronounced when the Mexican American women were born in Mexico, had been in the United States <15 years, and spoke Spanish as their primary language. While this discrepancy in folate status may partially be accounted for by genetics, cultural preferences may impact total dietary intake of folate and folic acid containing foods and supplements leading to lower concentrations of folate in the blood (Marchetta and Hamner 2014). Hamner et al (2011), analyzed NHANES data from 2001 to 2008 and found that Mexican Americans had significantly lower intakes of folate and folic acid. This relationship was mediated by how long an individual had resided in the United States,

whether she was born in Mexico or the United States, and whether Spanish or English was the preferred language spoken. Individuals that were born in Mexico, had resided in the US less than 15 years, and spoke Spanish as the primary language tended to have lower folate intakes than the more acculturated Mexican Americans (Hamner et al 2011).

Obesity

A recent NHANES report stated that almost 32% of US women aged 20-39 y are obese (Ogden et al 2014). This is a concern since a higher BMI was significantly associated with lower concentrations of serum folate in an analysis of NHANES data on women of childbearing age (Mojtabai 2004). da Silva et al (2013) reported both lower baseline concentrations of serum folate and a lower response to a single dose of folic acid in obese women as compared with normal weight women, but the obese subjects had significantly higher baseline RBC concentrations. Tinker et al (2012) assessed the effect of BMI on the relationship between folic acid supplement intake and folate status in women of childbearing age taking part in the NHANES. In the study, there was an inverse association between BMI and serum folate in women who did not take folic acid supplements, but this effect was not observed in women that took folic acid supplements. Additionally, there was a positive correlation between BMI and RBC folate regardless of folic acid supplement intake (or consumption) (Tinker et al 2012). Together, these findings suggest that a higher BMI is associated with lower concentrations of serum folate and higher concentrations of RBC folate when folic acid supplements are not regularly consumed (Tinker et al 2012, da Silva et al 2013).

In a pharmacokinetic study conducted by Stern et al (2011) to assess the impact of BMI on folate metabolism, 12 normal weight women randomly assigned to receive either 1.1 mg or 5 mg folic acid were paired with 12 obese women who received equivalent folic acid doses per kg of body weight. Serum folate concentrations in samples taken for 10 hours after the folic acid bolus were higher in the obese as compared with the normal weight women, suggesting that folic acid may not distribute into adipose tissue. In a follow up study by Stern et al (2012), it was noted that lean body weight rather than total body weight accounted for 90% of the variability in serum folate concentrations. Thus, the researchers concluded that obese women need a slightly higher dose of folic acid based on lean body weight (0.3 to 0.5 mg) compared to normal weight women (0.2 to 0.4 mg) to maintain adequate folate status.

The prevalence of obesity during pregnancy is rising (Fisher et al 2013). The rise in obesity is a concern because studies have found an association between obesity and negative birth outcomes. Ray et al (2005) found that even after mandatory folic acid fortification, there is an association between a higher maternal weight and an increased risk for NTDs. In a study in China looking at 459 NTD cases matched with 459 controls, there was a significant increase in the risk of NTDs for mothers with a BMI ≥ 28 kg/m², even after adjusting for age, education, income, folic acid supplementation, and other factors (Gao et al 2013). In the same intervention, the researchers found that folic acid supplementation did not reduce the risk of NTDs in overweight and obese women as significantly as it did in underweight and normal weight women (Wang et al 2013).

Other factors

Non-smokers tend to have significantly higher concentrations of RBC and plasma folate than current smokers (Piyathilake et al 1994) and secondhand smoke may have an impact as well. Using data from the third NHANES, Mannino et al (2003) found that non-smokers with low exposure to secondhand smoke had significantly higher RBC and serum folate concentrations compared to non-smokers exposed to high amounts of smoke and smokers.

Folate status is also influenced by several drugs, notably methotrexate, an anti-catabolite drug commonly used for the treatment of cancer, rheumatoid arthritis, and psoriasis (Medline Plus 2014b) and anti-epileptic drugs (AEDs; Gropper et al 2009). Methotrexate acts as a folic acid antagonist by inhibiting dihydrofolate reductase, preventing the conversion of hydrofolate to tetrahydrofolate and ultimately to 5-methyl tetrahydrofolate (Grosflam and Weinbatt 1991, Goldman and Matherly 1985). Patients receiving long-term methotrexate treatment have been reported to have significantly lower RBC folate and higher oxidized folate forms in the plasma (Hendel and Nyfors, 1985), effects which can be reversed through supplementation with low-doses of folic acid (Hornung et al 2004, van Ede et al 2002).

In a retrospective survey, Speidel and Meadow (1972) found a high prevalence of birth defects for infants of mothers with epilepsy and that the rate was twice as high when mothers were taking anti-epileptic drugs (AEDs). Low folate concentrations and high homocysteine concentrations are most commonly seen with AEDs that induce liver enzymes, namely cytochrome P450, a liver enzyme known to interfere with folate metabolism (Kishi et al 1997, Kampman 2007). Cytochrome P450 inducing AEDs include phenytoin,

carbamazepine, phenobarbital and primidone. Because of the decreased folate concentrations seen with these drugs, it is important for women of childbearing age taking these AEDs to receive adequate folic acid supplementation (Kampman 2007).

Pregnancy physiology and folate metabolism

Blood Volume

During pregnancy, blood volume begins to increase slightly in the first trimester, significantly during the second trimester, and then slightly rises further in the third trimester (Peck and Arais 1979). Total blood volume increases approximately 35-40% during pregnancy consisting of about a 15-20% increase in red blood cell mass and a 45-50% increase in plasma volume (Picciano 2003) although there have been reports that blood volume can increase anywhere from 20 to 100% (Pritchard 1965). Due to the disproportionate increase in plasma volume compared to red blood cell mass, there is a noticeable decrease in hemoglobin concentrations and hematocrit values (Picciano 2003). This increase in plasma volume during pregnancy is needed to account for an increase in blood flow to the placenta, uterus, breast, skin, and kidneys and the blood volume increase is influenced by BMI, parity, and birth weight (Peck and Arais 1979).

Nutrient Needs

Pregnancy is considered an anabolic state that changes throughout gestation due to the development of the placenta, changes in hormones, fetal development, and preparation for lactation, in addition to meeting the nutrient needs of the mother (King 2000). During pregnancy, recommended energy intakes vary by trimester. During the first trimester there is

not a recommended increase in caloric needs since energy expenditure does not significantly change and there is typically no significant weight gain during this time period (Picciano 2003). Due to the increased metabolic demands and weight gain needed for most individuals during the later stages of pregnancy, there is a recommended 340 kcal/d and 452 kcal/d increase for the second and third trimesters respectively. In addition, protein requirements increase to approximately 71 g/d during pregnancy compared to RDA of 46 g/d for non-pregnant females (Institute of Medicine 2002).

Like energy and macronutrients, many micronutrients have increased RDAs during pregnancy (Picciano 2003). Some of the key micronutrients that have increased needs during gestation include folate for proper growth and development, iron for the development of the placenta and the increase in RBCs, and iodine for brain development and growth. The increased needs of many micronutrients, particularly folate and iron, cannot always be met through diet alone, so supplements are often recommended in order to meet the increased nutrient demands of pregnancy (Picciano 2003).

Folate Metabolism

Folate is an important one-carbon donor for the synthesis of DNA and RNA, making it vital for proper fetal growth and development (Tamura et al 2006). Studies have found a significant increase in folate catabolism and urinary excretion during pregnancy, potentially adding to the increased needs during this time period (Caudill et al 1998, Higgins et al 2000, Tamura et al 2010). In a study by Higgins et al (2000) in Dublin, urine folate catabolites were analyzed in 24 pregnant women during each trimester of pregnancy and compared against 25 non-pregnant controls. Results indicated that the rate of folate catabolism increased during

each trimester and peaked during the third trimester at which time the catabolic rate was twice that of the non-pregnant controls. In another study by Caudill et al (1997), both pregnant and non-pregnant subjects were fed a controlled diet comprised of 120 µg folate and either 330 or 730 µg folic acid per d during the second trimester of pregnancy. Twenty-four hour urine collections were conducted during each of the 12 weeks of the study. Unlike the previous study, no difference was found in catabolite excretion between the pregnant and non-pregnant subjects. Tamura et al (2010) suggested that folate catabolism might only be significantly increased during the third trimester since this the time of maximal fetal growth.

Folate requirements and recommendations during pregnancy

The Institute of Medicine (1998) recommends that all women of childbearing age consume at least 400 µg DFE daily in order to maintain long-term RBC folate status (O'Keefe et al 1995). Due to blood volume expansion, increased cell division, and the rapid growth and development of the fetus, requirements for folate during pregnancy increase (Picciano 2003) and the RDA for pregnant women has been set at 600 µg DFE daily (Institute of Medicine 1998). The current RDA, set in 1998, was based in part on a controlled feeding study conducted by Caudill et al (1997). Pregnant women were fed a diet containing 120 µg dietary folate and a supplement containing either 330 or 730 µg folic acid for 12 weeks during their second trimester of pregnancy. These researchers found a difference in serum folate between the groups, but no significant difference in RBC folate, suggesting that the lower dose of folic acid was sufficient to maintain a healthy folate status during pregnancy.

Folic acid supplementation during pregnancy

Data from NHANES showed that pregnant women consuming folic acid supplements had higher RBC folate concentrations than those that did not use supplements in the past 30 d (Branum et al 2013). Ellison et al (2004) looked at the change in plasma homocysteine concentrations from 16 weeks gestation to 36 weeks gestation in pregnant women consuming no folic acid supplement or 400 µg folic acid per d. Results indicated that women consuming the supplement showed no significant change in plasma homocysteine while women not taking the supplement had a significant increase in homocysteine between 28 weeks and 36 weeks gestation. McNulty et al (2013) supplemented pregnant women with 400 µg folic acid during the first trimester of pregnancy and then randomized 119 participants to receive either 400 µg folic acid or a placebo during the second and third trimesters of pregnancy. They found that women supplemented during the second and third trimesters had significantly higher RBC folate concentration, higher serum folate concentrations, and lower homocysteine concentrations than those receiving the placebo. These data suggest that supplementation through the third trimester can help to maintain folate concentrations despite higher demands during pregnancy.

The effects of 400 µg supplemental folic acid on folate status during pregnancy have been well studied and shown to be sufficient to maintain folate status during this period of rapid growth (Ellison et al 2004, McNulty et al 2013). This dose is approximately equivalent to 600 µg DFEs, the dietary recommended intake for pregnancy (Institute of Medicine 1998), whereas most of the over-the-counter prenatal vitamins available in the United States contain 800 µg folic acid, twice the recommendation. Effects of the higher doses of folic acid on folate status have not been studied.

Although the benefits of folic acid supplementation during pregnancy are well understood, there is a public health concern that pregnant women are not always meeting the folate recommendation to support a healthy pregnancy and prevent negative birth outcomes. Branum et al (2013) analyzed the data of 1296 pregnant women that took part in the NHANES study between 1999 and 2006 and found that 74% of the participants reported taking folic acid containing supplements in the past 30 d. Both a higher education level and the third trimester of pregnancy were significantly associated with folic acid supplement use. Case et al (2007) additionally found that obese pregnant women were 24% less likely to use folic acid supplements than normal weight pregnant women.

Conversely, there is also a concern that some individuals may be getting too much folic acid during pregnancy. The Institute of Medicine (1998) has set a tolerable upper limit (UL) of 1000 µg folic acid per d for pregnant women over the age of 19 y. No adverse effects have been observed for natural food folate, so the UL is specific to folic acid supplements and foods fortified with folic acid (Butterworth and Tamura 1989). The biggest concern with over consumption of folic acid is the exacerbation of neurological damage in individuals with B12 deficiency, which in the US primarily occurs in older adults. Other potential effects of over consumption are controversial, but include reproductive effects, carcinogenicity, decreased zinc absorption, and general side effects, such as gastrointestinal distress (Butterworth and Tamura 1989).

Folate is a key donor for the one-carbon group needed to methylate DNA, which may impact epigenetic regulation through the methylation reactions (Burdge and Lillycrop 2012). Research is emerging that higher doses of folic acid taken during pregnancy can alter this epigenetic regulation causing unforeseen long-term effects in offspring (Burdge and

Lillycrop 2012). The review by Burdge and Lillycrop (2012), also included studies indicating that folic acid intake was positively associated with allergy-related respiratory impairment in both humans and mice offspring and was positively correlated with insulin resistance in human and rat offspring (Whitrow et al 2009, Hollingsworth et al 2008). In addition, research in rats has found an association between folic acid supplementation during pregnancy and changes in liver metabolism and vascular function in offspring (Burdge and Lillycrop 2012).

Since both under-supplementation and over-supplementation of folic acid can be a concern during pregnancy, Hoyo et al (2011) looked at folic acid supplement use of 539 pregnant women in the Newborn Epigenetics Study (NEST) in Durham, NC. The data showed that during pregnancy, almost 34% of participants still had no regular intake of folic acid; while greater than 10% of the participants were exceeding the UL of 1,000 µg/d. This study supports the idea that some women are not meeting the folic acid recommendation for pregnancy, while other women are well exceeding the recommendation.

Summary

The established folate recommendation for pregnant women is 600 DFEs, or approximately 400 µg folic acid (Institute of Medicine 1998), in order to support the increased demands of rapid cell division during pregnancy (Picciano 2003). Despite the evidence that 400 µg folic acid is sufficient to maintain adequate blood folate concentrations and prevent negative birth outcomes (Bailey 2000), most prenatal vitamins contain over twice the recommendation. This higher dose of folic acid may exceed the body's capacity to reduce it, causing oxidized folic acid to remain in the blood (Obeid et al 2010). The effects of the oxidized folic acid in the blood are not well understood. Yet, to our knowledge, there has

been no research comparing these two doses of folic acid throughout pregnancy, particularly taking BMI into consideration. To fill this research gap it would be most informative to determine the blood folate response to maternal supplementation with 400 or 800 μg folic acid in pregnant women across a range of BMI. It was hypothesized that maternal serum folate concentrations would be significantly higher in the 800 μg group, but there would be no significant difference in RBC folate concentrations between the two groups, even after adjustment for potential confounders such as dietary intake, genetic polymorphisms, and race/ethnicity (Caudill et al 1997). Additionally, it was hypothesized that serum folate concentration would be influenced by body weight because a higher BMI has been associated with lower concentrations of serum folate (Mojtabai 2004).

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CHAPTER 3

FOLIC ACID SUPPLEMENTATION IN PREGNANCY: MATERNAL OUTCOMES¹

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Abstract

Folate plays an essential role in one carbon metabolism, required for DNA and RNA synthesis and rapid cell division and growth during pregnancy. The current Recommended Dietary Allowance (RDA) for pregnant women is 600 μg Dietary Folate Equivalents (DFEs), or approximately 400 μg folic acid, yet most over-the-counter prenatal vitamins contain at least 800 μg to 1000 μg folic acid. Accordingly, an on-going double blind randomized control trial in pregnant women is being conducted to compare the effects of these two doses on folate biomarkers from the first prenatal visit (<12 weeks gestation) through delivery. The present study reports an interim analysis of serum and RBC folate response to supplementation in a subset of participants with available baseline and 28 week blood samples (n=22). Mixed effects analysis indicated a significant time effect for RBC folate concentration ($p = 0.035$) and this time response was significantly impacted by length of folic acid supplementation prior to enrollment ($p = 0.047$). In addition, there was a significant race/ethnicity effect on RBC folate ($p = 0.016$), with African Americans having significantly lower concentrations compared to their white counterparts. No treatment effect was seen for serum folate, but both race/ethnicity ($p = 0.007$) and length of previous supplementation ($p = 0.038$) had significant effects on this variable. Overall, there was no significant effect of BMI on folate status, but anticipated associations may have been confounded by the variability in previous folic acid supplement use in both weight classes. These preliminary data do not show significant differences in folate biomarker response to prenatal supplementation with 400 μg /d vs 800 μg /d folic acid. Completion of the study and further analysis is needed to analyze the effects of folic acid dose on additional outcome parameters such as oxidized folic acid in the serum and changes in maternal and cord blood DNA methylation. Results of this

study may provide information about the relationship between folic acid dose and biomarker response in pregnant women and contribute to the evidence on which future recommendations for pregnant women can be based.

Introduction

Folate, a water soluble B vitamin, is necessary for the production of red blood cells (RBCs) and sufficient cell division and growth during periods of rapid development, such as pregnancy (Bailey and Gregory 1999, Gropper et al 2009). Folate is particularly important during the first trimester of pregnancy when the neural tube of the fetus is forming (Czeizel and Dudas 1992). Even after the closure of the neural tube, adequate intake is important during the second and third trimesters of pregnancy to maintain maternal folate status in order to support blood volume expansion, increased cell division, and the rapid growth and development of the fetus (Picciano 2003).

Folate is available in the diet in two forms, the reduced form (folate) found naturally in foods such as beans, legumes, leafy green vegetables, and orange juice and the fully oxidized (folic acid) form added to fortified foods and supplements. To account for the greater bioavailability of synthetic folic acid, dietary intake recommendations are based on dietary folate equivalents (DFE), with total DFE determined as microgram intake of natural food folate plus 1.7 times the microgram intake of folic acid (Suitor and Bailey 2000). The current recommended dietary intake (RDA) of folate was set by the Institute of Medicine in 1998. The recommendation for pregnancy was based, in part, on a controlled feeding study by Caudill et al (1997) who found that 330 µg folic acid in addition to 120 µg dietary folate was sufficient to maintain adequate blood folate concentrations during the second trimester

of pregnancy. The recommendations for women of childbearing age are that all women consume at least 400 µg DFE daily and that pregnant women consume 600 µg DFE, or approximately 400 µg folic acid, daily to meet the increased demands of pregnancy and prevent negative birth outcomes.

Although the benefits of 400 µg supplemental folic acid on pregnancy outcomes are well studied (Ellison et al 2004, McNulty et al 2013), most over-the-counter prenatal vitamins available in the United States contain 800 µg folic acid, twice the recommendation. This raises the concern that some women may be getting too much folic acid during pregnancy. In the Newborn Epigenetics Study (NEST; Hoyo et al 2011), the daily folic acid intake of over 10% of the 539 pregnant participants exceeded 1000 µg folic acid, the current tolerable upper limit (UL) for pregnant women (Institute of Medicine 1998). Most of the potential side effects of surpassing the UL, such as reproductive effects and carcinogenicity, are controversial, but there is an emerging concern that higher doses of folic acid can alter epigenetic regulation causing unforeseen long-term effects in the offspring of mothers taking high doses of folic acid (Burdge and Lillycrop 2012).

Another factor of concern is the increasing prevalence of pregnant women who are obese (Fisher et al 2013). Almost 32% of women of childbearing age are obese (Ogden et al 2014). Studies conducted in women of childbearing age have found that a higher BMI is associated with lower concentrations of serum folate when folic acid supplements are not regularly consumed (Tinker et al 2012, da Silva et al 2013). Due to differences in body composition between normal weight and obese individuals, obese individuals may need a higher dose of folic acid compared to normal weight women (Stern et al 2011). Additionally, a higher dose of folic acid may be beneficial for obese individuals since there is a higher

prevalence of negative birth outcomes, including NTDs, seen in this population (Ray et al 2005, Gao et al 2013). To our knowledge, the impact of body mass index (BMI) on folate status and requirements during pregnancy has not been examined.

The aim of this double blind randomized control trial is to determine if there is a difference in serum folate and RBC folate concentrations in response to daily 400 or 800 µg supplemental folic acid throughout pregnancy, after adjusting for BMI and other potential confounders. Folic acid doses of 400 µg/d and 800 µg/d were selected to compare the RDA for pregnant women (Institute of Medicine 1998) to the dose commonly found in over the counter prenatal vitamins. Based on previous research findings from Caudill et al (1997), it was hypothesized that maternal serum folate concentrations would be significantly higher in the 800 µg group, but there would be no significant difference in RBC folate concentrations between the two groups.

Materials and Methods

Participants

For this randomized double blind intervention pilot, healthy adult pregnant women (18-40 years old) were recruited and screened by the midwives at Athens Regional Midwifery Clinic (ARMC; Athens, GA). Inclusion criteria were less than 12 weeks gestation, singleton pregnancy, prenatal care provided through ARMC, normal weight to class I obesity (BMI 18.5-35 kg/m²), and willingness to comply with study protocol. Exclusion criteria were use of prescription drugs, anemia, chronic disease, antibiotic use in past two weeks, vegan dietary regimen, current smoker, alcohol consumption of 2 or more drinks per day, in vitro fertilization treatment, and multiple-fetus pregnancy. Fifty-one participants were recruited for

this study between July 17 and December 30, 2014, with expected delivery dates between February 15 and August 15, 2015 (Figure 1). For purposes of this analysis, only participants with a 28-week gestation blood sample and an expected delivery date before July 11, 2015 (n=41) were included since individuals past this due date had not reached 28 weeks gestation and therefore did not have a second blood draw available for analysis. The University of Georgia (#STUDY00000506) and Athens Regional Medical Center Institutional Review Boards approved this protocol and all subjects provided written informed consent after being made aware of the purpose and design of the study. This study was registered at ClinicalTrials.gov (NCT02124642).

Intervention and vitamin supplement protocol

Upon enrollment into the study at the first prenatal visit (5-12 weeks gestation), participants were provided with a regimen consisting of three nutrient supplements and were instructed to take each of the supplements daily for the duration of pregnancy (Figure 2). The first supplement was a multivitamin/multimineral (MVM) tablet (One-a-Day® Women's; Bayer Healthcare, USA) containing 400 µg folic acid along with other essential vitamins and minerals. The second supplement contained 200 mg docosahexaenoic acid (DHA) (life'sDHA™ 200 mg vegetarian capsules; DSM Nutritional Products North America, Parsippany, NJ). DHA is present in many prenatal vitamins as a source of essential omega-3 fatty acids for fetal neurodevelopment (Greenberg et al 2008), and women being seen by the ARMC midwifery clinic are encouraged to take prenatal vitamins with DHA as the usual standard of prenatal care. For the third supplement, participants were randomly assigned to either receive 'supplement A' (n=8) or 'supplement B' (n=14). Both supplements were

specially formulated capsules prepared by a Pharmacy Compounding Accreditation Board (PCAB®) accredited pharmacy (Westlab Pharmacy; Gainesville, FL). One capsule contained only 10 mg iron to meet the higher iron recommendation for pregnant women and the other capsule contained 10 mg iron plus an additional 400 µg folic acid to create the 800 µg folic acid group. Folic acid and iron composition of the compounded capsules were verified at a third-party laboratory (Covance, Princeton, NJ). Since this an ongoing study, researchers are still blinded to which supplement contains the additional folic acid. Participants were given a 6-week supply of supplements at each prenatal visit. Compliance was measured by return pill count, and for the 12 participants completing the study to date, the compliance was determined as 90.2%, based on a return of less than 10% of dispersed supplements..

Demographic and health behavior information

Medical records were used to obtain information regarding race/ethnicity, height, pre-pregnancy weight, weight gain during pregnancy, current age, gestational age, parity, oral contraceptive use, prescription and over-the counter medication and drug usage, past diagnosis of chronic disease, and type of insurance. Use of health information, protected by the Health Insurance Portability & Accountability Act (HIPAA), was authorized through signature of the consent form. The Center for Disease Control's website BMI calculator tool (http://www.cdc.gov/healthyweight/assessing/bmi/adult_bmi/english_bmi_calculator/bmi_calculator.html) was used to calculate BMI from the height and pre-pregnancy weight obtained from medical records. Participants were contacted by telephone to complete a brief health behavior questionnaire to obtain additional information regarding previous and current folic

acid supplement use, regular consumption of cereals and other folic acid fortified foods, past and present smoking and alcohol consumption habits, and physical activity.

Dietary Assessment

A 3-day dietary record was obtained at 24 weeks gestation to estimate usual intake of calories, natural food folate, and food folic acid for the second trimester. For each recall, participants recorded dietary intake for three non-consecutive days, including at least one weekend day. Participants had the option to either directly input dietary data into the Automated Self-administered 24-hour Recall (ASA24) system (<http://riskfactor.cancer.gov/tools/instruments/asa24>) or write the information on the provided Dietary Data Collection Sheet and mail it to the research team. Hand written dietary recalls were reviewed by a single researcher who followed up with participants by phone to obtain any missing information, such as serving size, preparation methods, or missing condiments, before entering the data into the ASA24 system. The ASA24 system, developed by the National Cancer Institute, uses the USDA Automated Multiple-Pass Method (AMPM) technique to prompt further information about preparation method, portion size, and additional food items so as to decrease underreporting (Subar et al 2012). The ASA24 system has been validated as a research tool to accurately estimate intake of energy, macronutrients, vitamins, and minerals (Bjorge-Schohl et al 2013).

Blood collection and analysis

Trained personnel at ARMC collected blood samples at baseline and 28 wk gestation. As per the typical standard of care of the collaborating clinic, the participants in this study

were not required to fast prior to blood collection. The clinic routinely draws blood at these time points, so no additional needle sticks were necessary. Blood samples were wrapped in foil, stored on ice and processed within 1 hour after collection. After allowing the serum tube to clot for 30 minutes at room temperature, it was centrifuged at room temperature for 15 minutes at 1200 x g. A 1 ml sample of serum was carefully removed, combined with 71.4 μ l of a 7% ascorbic acid solution and distributed to make two 500 μ l aliquots for serum folate analysis. For RBC folate analysis, a 100 μ l sample of whole blood was added to 1.0 ml of 1% ascorbic acid, protected from light, mixed on a rotating platform for 30 minutes and divided into two 500 μ l aliquots. All samples were stored at -80° C and analyzed in a single assay run to prevent variation between assays. Serum and RBC folate concentrations were determined by microbiologic assay using *Lactobacillus rhamnosus* as previously reported by this research team (da Silva et al 2013). The inter- and intra-assay coefficients of variation for serum folate were 8.9 to 10.5% and 6.6 to 6.8%, respectively; whereas those for RBC folate were 3.1 to 3.8% and 3.3 to 4.5%, respectively. MTHFR genotype was determined through purification of polymerase chain reaction products (QIAquick PCR Purification kit; Qiagen) and sequencing the DNA templates with the Applied Biosystems Automated 3730 DNA analyzer (Applied Biosystems) at the Georgia Genomics Facility (Athens, GA).

Statistics

Characteristics of participants were analyzed and compared between treatment groups using descriptive statistics. SAS version 9.3 (Cary, NC) was used to determine means and standard deviations and to perform the student's t-test (continuous dependent variables) and X^2 analysis (categorical variables) to determine differences between the 400 and 800 μ g

groups at baseline and 28 wk gestation. BMI, serum folate, and RBC folate were not normally distributed, so a natural log transformation was performed for these parameters. Pretreatment and post treatment values were used to determine the dependent variable (serum folate and RBC folate) response to folic acid supplementation. The data were analyzed using a mixed effects model where correlations among repeated measures are taken into account in order to examine response to supplementation over time. Potential effects of confounding variables including race/ethnicity, MTHFR genotype, pre-pregnancy BMI (by weight classification), gestational age at enrollment (< 56 days or >55 days), and length of time for previous folic acid supplementation (0 days, <30 days, 30-60 days, or >60 days) were modeled individually by repeated measures analysis of covariance using mixed effect models. Impact of pre-pregnancy BMI on folate status was further explored through Spearman rank order and partial correlation analysis, adjusting for race/ethnicity and MTHFR genotype. The level of statistical significance was defined as $p < 0.05$.

Results

Baseline characteristics

A total of 41 participants were included in the analysis for this study. Of these participants, 19 dropped out of the study prior to the 28 week time point due to morning sickness (n =8), use of other prenatal supplements (n=2), changing clinics (n=2), multiple fetus pregnancy (n=1), miscarriage (n=1), poor compliance (n=1), and unspecified reasons (n=4). Although there were no statistically significant differences for age, BMI, race/ethnicity, marital status, parity, or insurance type between those that dropped out and those that were retained, individuals that dropped out were more likely to be recruited at a

later gestational age (63.58 ± 11.28 days, mean \pm SD) than retained participants (54.95 ± 10.73 days) ($p = 0.017$). Baseline serum and RBC concentrations did not significantly differ between retained participants and dropouts.

At baseline, there were no significant differences between group A and group B for age, race, marital status, insurance type, height, weight, pre-pregnancy BMI, gestational age at recruitment, MTHFR genotype, or parity (Table 1). Eighteen participants completed the Health Behavior Questionnaire at baseline. Group A ($n=7$) had a total of 5 participants taking prenatal vitamins (800-1000 $\mu\text{g/d}$ folic acid) prior to study enrollment and in group B ($n=11$), 6 were taking a prenatal vitamin (800-1000 $\mu\text{g/d}$ folic acid), 1 was taking a multivitamin (400 $\mu\text{g/d}$ folic acid), and 4 were taking no folic acid containing supplement (Table 2). There was no difference between groups for use of a folic acid containing supplement prior to enrollment in this study ($p = 0.633$).

Food record analysis

Dietary recall information for the second trimester was received from 14 of the 22 participants (63.6%). There was no significant difference in mean caloric intake between group A ($n=4$) and group B ($n=10$) (1818 ± 363.8 kcal and 2048 ± 669 kcal, respectively) ($p = 0.533$). The average dietary folate intake for group A and group B was 597.6 ± 106.4 μg DFE and 510.5 ± 144.4 μg DFE ($p = 0.300$). Neither natural food folate intake (303.6 ± 64.3 $\mu\text{g/d}$ and 218.8 ± 105.8 $\mu\text{g/d}$, respectively) ($p = 0.166$) nor folic acid intake (172.6 ± 49.3 $\mu\text{g/d}$ and 171.8 ± 57.8 $\mu\text{g/d}$) ($p = 0.980$) significantly differed between group A and group B.

Serum and RBC folate

Baseline serum folate concentrations were 70.55 ± 10.50 nmol/L, range 37.71 to 113.19 nmol/L (Group A) and 47.00 ± 25.77 nmol/L, range 18.07 to 95.18 nmol/L (Group B) and no significant differences ($p = 0.057$) were observed between the two treatments. Serum folate concentrations remained non-significant between the two groups at 28 weeks gestation ($p = 0.446$). A repeated measures analysis of variance of serum folate concentrations at baseline and 28-week gestation identified no significant group ($p = 0.091$), time ($p = 0.668$), or group*time ($p = 0.118$) effects (Figure 3). Mixed model effects are reported in Table 3. Inclusion of race/ethnicity in the mixed effects model identified a significant overall effect of race/ethnicity ($p = 0.007$) and a trend towards a significant group effect ($p = 0.055$). Adjusting for length of time of prior folic acid supplementation identified a significant overall effect of prior supplementation ($p = 0.038$) and a trend towards a significant group effect ($p = 0.072$). No significant effects were indicated in the mixed effects models adjusting for MTHFR genotype, pre-pregnancy BMI, or gestational age at enrollment.

At baseline, RBC folate concentrations were 1454.8 ± 620.8 nmol/L, range 707.5 to 2741.52 nmol/L (Group A) and 1100.8 ± 444 nmol/L, range 378.18 to 1929.65 nmol/L (Group B) and there was no difference between the two treatment groups ($p = 0.159$). At 28 weeks gestation, RBC folate concentration for participants from both group A (1620.2 ± 915 nmol/L) and group B (1309.1 ± 438.9 nmol/L) increased, but the between group comparison remained not significant ($p = 0.446$). Repeated measures analysis of variance indicated significant increase in RBC folate concentrations over time ($p = 0.035$), but no time by treatment effect ($p = 0.198$) (Figure 4). Mixed effects model results are reported in Table 4. Incorporating race/ethnicity in the mixed effects model identified a significant race/ethnicity

effect ($p = 0.016$), a significant time effect ($p = 0.032$) and a trend toward a group effect ($p = 0.099$). Adjusting for length of folic acid supplementation prior to enrollment indicated a significant time effect ($p = 0.032$) and significant previous supplementation*time interaction ($p = 0.047$). The time effect observed with the repeated measures analysis remained significant after adjusting for MTHFR genotype ($p = 0.019$) and gestational age at enrollment ($p = 0.037$), but not after adjusting for pre-pregnancy BMI ($p = 0.889$). Additionally, the Spearman rank order and partial correlation analysis, adjusting for race/ethnicity and MTHFR genotype indicated that pre-pregnancy BMI had no impact on serum or RBC folate concentrations (Table 5).

Discussion

The aim of this study was to assess changes in serum and RBC folate concentrations in pregnant women in response to prenatal supplementation with either the current RDA for folate (600 μg DFE or approximately 400 μg) or the dose of folic acid commonly found in over-the-counter prenatal vitamins (800 μg), taking BMI into consideration. Results indicated that both doses of folic acid increased RBC folate by 28 weeks gestation, but there was no significant difference between treatment groups. Additionally, there was a significant race/ethnicity effect on serum and RBC folate concentrations. Contrary to the hypothesis that body weight status would influence the response to folic acid supplementation, there was no effect of pre-pregnancy BMI on any outcome variables.

During erythropoiesis, folic acid is accumulated in the red blood cells and retained for the lifespan of the cell (Chanarin 1986, Shane 2011). Therefore, RBC folate is considered a marker of long-term folate concentrations and reflects folate status for the previous 120 days

(Shane 2011). Recruitment occurred during the first trimester (<12 weeks gestation), the critical period for neural tube development, requiring adequate folate concentrations for the prevention of neural tube defects (Czeizel and Dudas 1992, Wald et al 1992). At baseline, participants in this study had a higher average RBC folate concentration (1236 nmol/L) than the average RBC folate concentrations reported for women of childbearing age in the United States (1060 nmol/L) in NHANES 1999-2010 (Pfeiffer et al 2012). Six participants in the present study, however, fell below optimal concentrations (<906 nmol/L) (WHO 2015, Daly et al 1995) with baseline RBC concentrations of 4 participants within the RBC folate range for elevated risk for NTD affected pregnancies (defined as RBC folate concentration 700-905 nmol/L) and 2 participants in the range for high risk for an NTD affected pregnancy (defined as RBC folate concentration <700 nmol/L) (Daly et al 1995). Folic acid supplementation from enrollment through 28 weeks gestation increased RBC folate concentrations in 3 of these 6 participants to concentrations greater than 906 nmol/L. As the 400 µg and 800 µg doses of folic acid were both effective in increasing suboptimal RBC folate concentrations to optimal levels and in maintaining RBC folate concentrations for all other participants above the 905 nmol/L cut off for adequacy, this supports the findings of Caudill et al (1997) that a lower dose of folic acid (450 µg/d total folate) is able to maintain RBC folate concentration above 906 nmol/L.

Significant differences in response to folic acid supplementation were not detected between group A and group B at 28 weeks gestation, likely due to high RBC folate concentrations and variability in concentrations at baseline. Information on previous supplement use was collected on 18 participants using the Health Behavior Questionnaire at baseline. Based on self-report, prior to study enrollment 10 of 18 participants (55.6%) were

using prenatal vitamins (between 4 and 168 days), 1 participant (5.6%) used a multivitamin, and 7 participants (38.9%) used no folic acid containing supplement, which likely lead to the high variability in baseline RBC concentration. The percentage of participants reporting no use of a folic acid containing supplement is consistent with the findings of the Newborn Epigenetics Study, which found that 34% of pregnant individuals did not take a supplement (Hoyo et al 2011). This variability in previous supplement use and the length of time of previous supplementation likely explains the significant time*supplementation interaction on RBC folate concentrations, as individuals that supplemented for <30 days prior to study enrollment had a greater increase in RBC folate concentrations from the intervention than individuals that had taken folate supplements for ≥ 30 days prior to the intervention.

Despite the variation in previous folic acid supplementation, a significant time effect was identified for RBC folate concentrations. This effect remained significant after individual adjustment for race/ethnicity, MTHFR genotype, length of prior folic acid supplementation, and gestational age at recruitment, but not after adjustment for pre-pregnancy BMI. Additionally, the mixed effects model showed a significant effect for race/ethnicity with lowest overall RBC folate concentrations observed for African American participants and highest overall RBC concentrations detected in non-Hispanic whites. This finding supports the results of Pfeiffer et al (2012) that in the US population, non-Hispanic blacks have the lowest average RBC folate concentrations, while non-Hispanic whites have the highest average concentrations.

There are biological homeostatic mechanisms in place to avoid fluctuations in folate status with large increases or decreases in dietary folate or folic acid intake (Shane 2011, Stover and Field 2011). When folic acid is absorbed, it must be reduced by dihydrofolate

reductase (DHFR) in order to be converted to tetrahydrofolate (Shane 2010). When large doses of folic acid are consumed, the DHFR enzyme becomes saturated and oxidized folic acid becomes detectable in the serum (Patanwala et al 2014, Kelly et al 1997). Therefore, higher intakes of folic acid could lead to an increase in oxidized folic acid concentrations in the serum. Oxidized serum folic acid concentrations are not yet available for the current study, but will be measured upon study completion. Additionally, large doses of folic acid can lead to increased urinary excretion of folate and folate metabolites (Kownacki-Brown 1992), therefore a 24-hour urinary folate excretion can also be used to estimate average folate intake and utilization (West et al 2012). Although urinary excretion of folate was not measured in the current study, it is expected that the 800 µg/day folic acid group would have experienced increased metabolism and excretion of the higher folic acid dose without a significant increase in RBC folate concentrations because of the high baseline concentrations (Caudill et al 1998).

Serum folate is a marker of short-term folate status and is highly influenced by recent dietary intake (Herbert et al 1987). This biomarker is ideally measured while fasting (Shane 2011), but per the standard protocol of our collaborating clinic (ARMC), non-fasting blood samples were drawn for the current study. Use of non-fasting blood samples may have contributed to the high variability in serum folate concentrations and inability to detect a significant serum folate response to the supplementation in either group. There was a significant effect for race/ethnicity identified in the mixed-effects model. This effect was likely because the African American participants tended to have much lower serum folate concentrations at baseline and 28-weeks gestation. Pfeiffer et al (2012) found similar results in the NHANES data that indicated non-Hispanic Blacks have the lowest concentrations of

serum folate and non-Hispanic Whites have the highest concentrations. Additionally, there was a significant effect for length of previous supplementation on serum folate concentrations. Tinker et al (2012) reported a significant inverse association between BMI and serum folate concentrations in women of childbearing age who did not use supplements, but no association between BMI and serum folate status for women using supplements. This suggests that the influence of factors influencing folate status, such as obesity or folic acid dose, may be more readily detected in unsupplemented individuals with lower folate status than in supplemented individuals of higher status. Supplementation studies in pregnant women not taking supplements prior to enrollment, or of sufficient size so as to allow subanalysis by prior supplementation status, may be required to effectively compare the influence of prenatal folic acid dose on folate biomarker status.

If group A were the 400 $\mu\text{g}/\text{d}$ group, the observed decline in serum folate concentration, albeit non-significant, would be supported by the findings of Caudill et al (1997). Caudill and associates found that there was a decrease in serum folate concentrations when pregnant women take lower doses of total folate (450 $\mu\text{g}/\text{d}$), but this value normalizes once the body adjusts to the lower dose (Caudill et al 1997). Based on the study by Kelly et al (1997), serum folate should not significantly differ in response to supplements containing 400 μg and 800 μg folic acid, but it is expected that there will be a significant difference in serum oxidized folic acid between the two treatment groups.

In addition to the 400 μg and 800 μg treatment doses of folic acid consumed, participants in group A and group B consumed a mean food folate intake of 304 $\mu\text{g}/\text{d}$ and 219 $\mu\text{g}/\text{d}$, respectively and dietary folic acid of 173 $\mu\text{g}/\text{d}$ and 172 $\mu\text{g}/\text{d}$, respectively. These findings are consistent with those of Dietrich et al (2005), who found that non-pregnant

American adults consumed approximately 275 µg/d dietary folate prior to fortification, but after fortification consumed 351 µg total folate (µg natural food folate + µg folic acid) per day. When looking specifically at non-pregnant females age 20-39 years in NHANES 1999-2000, it was noted that this population only consumed an average of 294 µg total folate per day (Dietrich et al 2005), lower than the 415 µg total folate (535 µg DFE) reported in the present study. Although the mean folate intake in this study was greater than previous reports for non-pregnant women, the average DFE intake remained below the RDA from the Institute of Medicine. If this intake is representative of pregnant women in the United States, this finding suggests that despite mandatory fortification of cereal grain products, the majority of pregnant women may not be meeting folate requirements by diet alone.

It was hypothesized that there would be a significant effect of BMI on folate biomarker response to folic acid supplementation due to the growing body of evidence suggesting BMI is negatively correlated with serum folate concentration. Mojtabai (2004) analyzed NHANES data from 1988-1994 and 1999-2000 for women of childbearing age. The results of this analysis indicated a significant association ($p < 0.001$) between increased BMI and lower serum folate levels. da Silva et al (2013) also found that obese women (18-40 y) had significantly lower serum folate status than normal weight women at baseline. After a single 800 µg folic acid dose, the obese women had a lower serum folate response compared to normal weight women, suggesting obese women may have altered response to folate supplementation. The data from the current study show no effect of BMI on folate biomarkers, contradicting findings of previous studies. If there is an effect of BMI on folate status, a response may not have been identified due to a small sample size, supplementation of folic acid prior to study enrollment, or the use of non-fasting blood samples.

Our study had some limitations. Due to a small sample size, we were limited in the types of statistical analyses we could perform and could only adjust for one confounding variable at a time in the mixed effects models. Additionally, we did not receive dietary intake and health behavior questionnaires from all study participants, which limited our ability to adjust for previous folic acid supplementation and dietary intake of folate and folic acid. In addition, we were unable to account for the effects of folate and folic acid intake prior to non-fasting blood draws. Finally, compliance was not measured directly but rather was calculated from return pill count, which may have lead to an overestimation as this method assumes consumption of all non-returned supplements. There were many strengths to this study. To our knowledge this is the first double blind randomized control trial conducted during pregnancy from the first prenatal visit (~6-12 wk gestation) through delivery comparing folic acid supplementation at the current recommended intake (400 µg /d) with a higher dose (800 µg /d) commonly found in over-the-counter prenatal vitamins. This study shows the feasibility of conducting the study in a community setting, which allowed all participants to receive consistent pregnancy recommendations and standard of care. In addition all races/ethnicities and MTHFR genotypes were included in this study, which makes this sample generalizable to individuals receiving care at ARMC.

In conclusion, these data indicate prenatal supplementation with either 400 µg or 800 µg folic acid significantly increased RBC folate concentrations from baseline to 28 weeks. Analyses of these data did not identify an effect of BMI on RBC or serum folate concentrations, but there was a significant effect of race/ethnicity and previous supplementation on both RBC and serum folate. Further examination is needed to determine the impact of the two folic acid doses on serum oxidized folic acid concentrations and DNA

methylation. Future research to observe the effects of 400 µg and 800 µg folic acid in a larger cohort with no previous supplementation in order to control for this confounding variable is needed.

Table 1. Baseline characteristics of study subjects^a

	Group A (n=8)	Group B (n=14)	p-value ^b
Age (years)	25.25 ± 5.0	27.93 ± 6.6	0.329
Race (% (n)) ^c			0.844
Non-Hispanic White	25 (2)	35.7 (5)	
Hispanic	50 (4)	35.7 (5)	
African American	12.5 (1)	21.4 (3)	
Other	12.5 (1)	7.1 (1)	
Marital Status (% married) ^c	50	28.6	0.315
Insurance Type (% (n)) ^c			0.675
Commercial	50 (4)	30.8 (4)	
Medicaid	37.5 (3)	53.9 (7)	
Other	12.5 (1)	15.4 (2)	
Height (cm)	158.8 ± 4.5	165.2 ± 8.1	0.056
Weight (kg) ^d	64.9 ± 14.9	74.0 ± 15.1	0.188
Pre-pregnancy BMI (kg/m ²) ^d	25.7 ± 5.7	27.1 ± 5.0	0.529
Gestational Age (days)	55.6 ± 14.0	54.6 ± 9.0	0.831
RBC Folate (nmol/L)	1454.8 ± 620.8	1100.8 ± 444.0	0.159
Serum Folate (nmol/L)	70.6 ± 29.7	47.0 ± 25.8	0.057
MTHFR Genotype (% (n)) ^c			0.340
CC	50 (4)	57.1 (8)	
TC	37.5 (3)	14.3 (2)	
TT	12.5 (1)	28.6 (4)	
Parity	0.9 ± 0.6	1.4 ± 1.5	0.295

^aMean ± SD, unless otherwise noted. No significant differences at $p < 0.05$ for these parameters. BMI, body mass index; RBC, red blood cell; MTHFR, methyltetrahydrofolate reductase.

^bTwo-tailed t-test, unless otherwise noted

^cChi-squared analysis

^dPre-pregnancy

^eGroup A missing one baseline RBC folate concentration

Table 2: Use of a folic acid supplement prior to study enrollment^a

	Group A (n=7)	Group B (n=11)
Supplement Use (% (n))		
None	28.6 (2)	36.4 (4)
Multivitamin (400 µg folic acid)	0.0 (0)	9.1 (1)
Prenatal vitamin (≥800 µg folic acid)	71.4 (5)	54.6 (6)
Length of time taken (days)	32.8 ± 20.5	60.6 ± 66.1
Range of time taken (days)	7 - 64	4 - 168
Frequency taken (days/week)	7 ± 0	5 ± 2.1

^aNo significant differences at $p < 0.05$ for these parameters

Table 3: Mixed effects models for serum folate^a

	Group		Time		Group*Time		Variable ^d		Variable*time	
	F	p	F	p	F	p	F	p	F	p
Repeat Measures Analysis	3.16	0.091	0.19	0.668	2.67	0.118	-	-	-	-
Race/ethnicity	4.23	0.055	1.14	0.301	2.99	0.102	5.61	0.007	0.97	0.432
MTHFR genotype	2.10	0.165	1.45	0.244	2.48	0.133	0.26	0.773	2.27	0.132
Pre-pregnancy BMI (kg/m ²)	2.61	0.123	0.02	0.889	2.29	0.148	1.37	0.280	0.20	0.818
Length of supplementation (days) ^b	4.03	0.072	0.28	0.610	3.72	0.083	4.12	0.038	2.98	0.083
Gestational age (days) ^c	3.65	0.071	0.25	0.620	2.89	0.105	1.97	0.176	1.01	0.328

^aStatistically significant if $p < 0.05$ and in bold font. MTHFR, methyltetrahydrofolate reductase; BMI, body mass index.

^bFolic acid supplementation prior to study enrollment

^cAt enrollment into study

^dConfounding variable being adjusted for in the mixed effects model

Table 4: Mixed effects models for RBC folate

	Group		Time		Group*Time		Variable		Variable*time	
	F	p	F	p	F	p	F	p	F	p
Repeat Measures Analysis	1.60	0.221	5.14	0.035	1.77	0.198	-	-	-	-
Race/ethnicity	3.06	0.099	5.48	0.032	2.89	0.107	4.54	0.016	1.29	0.310
MTHFR genotype	2.14	0.161	6.63	0.019	1.28	0.272	0.88	0.431	1.01	0.383
Pre-pregnancy BMI (kg/m ²) ^b	1.20	0.287	2.49	0.132	1.88	0.187	0.03	0.973	0.50	0.614
Length of supplementation (days) ^b	1.60	0.235	6.20	0.032	3.12	0.108	2.26	0.144	3.80	0.047
Gestational age (days) ^b	1.77	0.200	5.04	0.037	1.79	0.197	0.93	0.347	0.09	0.763

^aStatistically significant if $p \leq 0.05$ and in bold font. RBC, red blood cell; MTHFR, methyltetrahydrofolate reductase; BMI, body mass index.

^bPre-pregnancy BMI (by weight classification), length of supplementation prior to study enrollment (0 days, <30 days, 30-60 days, or >60 days), gestation age at day of enrollment (< 56 days or >55 days)

Table 5: Spearman correlation of pre-pregnancy BMI and folate biomarkers

Variable	N	Pre-pregnancy BMI (kg/m ²)			
		Unadjusted		Adjusted ^a	
		r	p-value	r	p-value
Baseline Serum Folate (nmol/L)	22	-0.303	0.171	-0.194	0.414
Serum Folate Response (nmol/L) ^b	22	0.249	0.263	0.158	0.507
Baseline RBC Folate (nmol/L)	21	-0.088	0.704	-0.240	0.323
RBC Folate Response (nmol/L) ^b	22	-0.084	0.719	-0.064	0.796

^aAdjusted for race/ethnicity and MTHFR genotype. All parameters analyzed on the natural log scale to correct for non-constant variance observed on the original scale. Statistically significant if $p < 0.05$ and in bold font. BMI, body mass index; RBC, red blood cell.

^bFolate response defined as 28-week folate concentration - baseline folate concentrations

Supplemental Table 1: Raw data by participant

Participant	Group	Age (years)	Race	BMI (kg/m ²)	Genotype	RBC Folate Baseline (nmol/L)	RBC Folate 28 wks (nmol/L)	Serum Folate Baseline (nmol/L)	Serum Folate 28 wks (nmol/L)	Prior Supplement Use	Supplement Use Time (days)	Total Dietary Folate (DFE)	Dietary Folic Acid (µg)
FAP101	B	33	W	24.4	CC	1929.65	1998.6	62.49	63.04	prenatal	168	783.57	218.29
FAP102	B	21	AA	19.8	CC	-	819.8	18.08	21.24	-	-	-	-
FAP104	B	22	W	24.6	CC	1355.91	1667.0	66.73	58.22	prenatal	14	406.06	151.66
FAP105	B	35	H	24.8	TC	1044.21	1667.1	70.44	78.13	prenatal	4	516.83	193.66
FAP108	A	21	AA	34.4	CC	1044.79	999.3	39.03	44.95	prenatal	30	-	-
FAP110	B	35	H	34.2	CC	873.88	1147.9	38.67	57.22	none	0	370.90	80.67
FAP113	B	21	H	26.6	TT	988.81	1705.0	48.96	64.5	none	0	449.33	145.77
FAP119	B	31	W	19.9	CC	1643.39	1483.2	95.18	52.94	prenatal	75	664.65	247.39
FAP121	A	20	H	35.7	TC	1377.33	1116.3	44.55	34.44	none	0	440.16	121.33
FAP122	B	37	AA	32.1	CC	1124.97	1551.8	23.57	29.28	none	0	378.67	126.69
FAP123	A	32	W	22.3	CC	2741.52	3529.6	84.7	62.08	prenatal	28	625.72	139.65
FAP127	A	21	H	21.8	TT	967.57	1677.8	37.71	65.87	none	0	-	-
FAP128	A	24	Other	22.3	TC	1642.95	1601.2	107.83	87.53	prenatal	35	660.41	210.89
FAP131	A	26	H	24.4	CC	1522.28	2250.3	113.19	62.24	prenatal	7	-	-
FAP132	B	24	H	27.3	TT	814.09	810.2	52.20	47.20	multivitamin	-	-	-
FAP133	B	33	AA	23.9	CC	378.18	528.1	27.69	51.62	none	0	-	-
FAP134	B	34	W	30.7	CC	1584.86	1250.4	85.83	46.43	prenatal	-	588.25	124.39
FAP135	B	22	H	32.4	TT	891.6	1028.8	22.65	31.51	-	-	-	-
FAP137	B	24	W	35.7	TT	1182.6	1734.2	21.97	81.82	prenatal	42	352.69	168.07
FAP138	B	19	Other	23.7	TC	498.83	935.3	23.56	72.47	-	-	593.68	261.21
FAP142	A	33	W	22.6	CC	1634.47	1137.2	75.26	65.16	prenatal	64	664.02	218.60
FAP148	A	25	H	22.7	TC	707.51	650.1	46.93	39.47	-	-	-	-

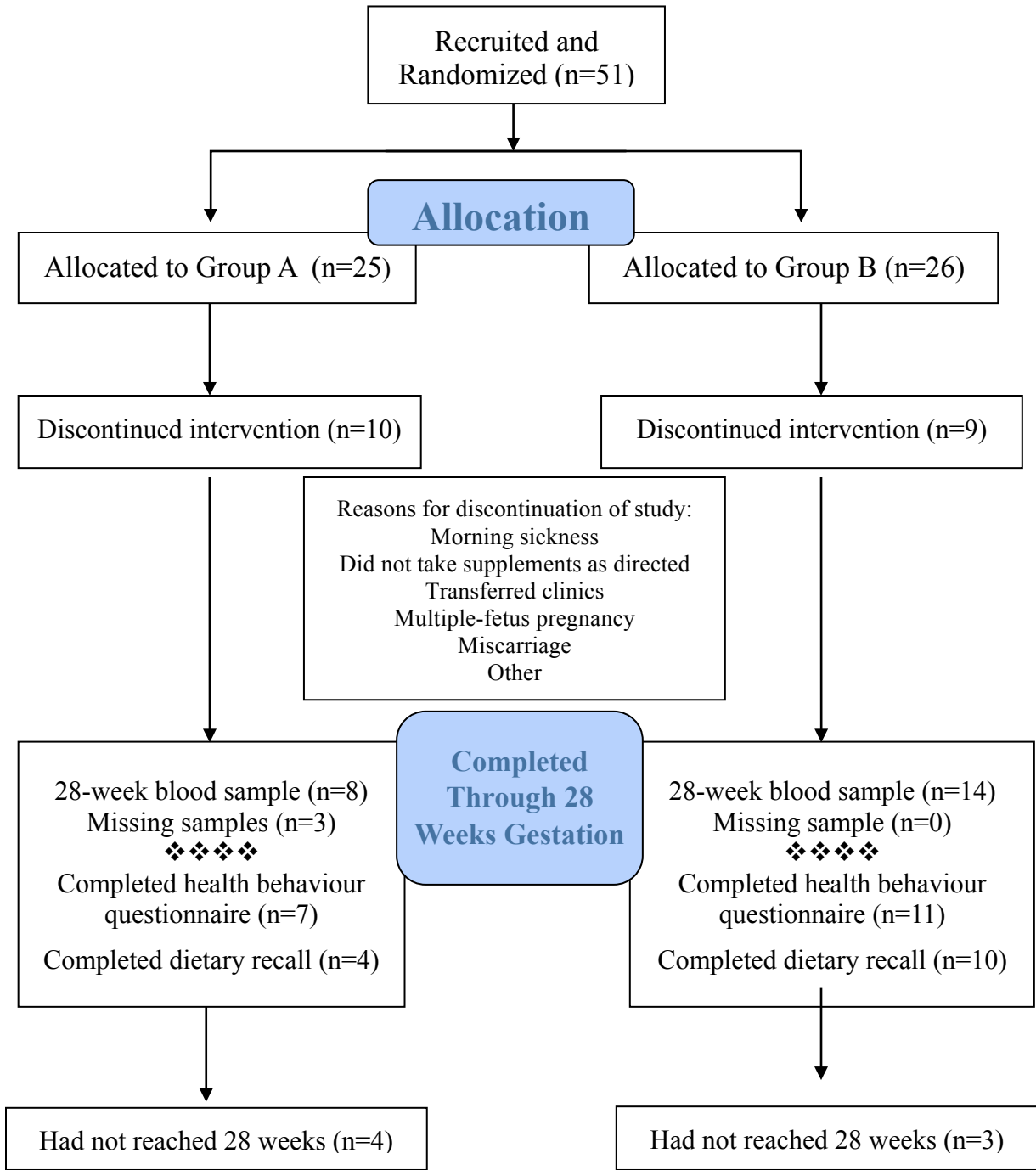


Figure 1: Recruitment and retention of study participants

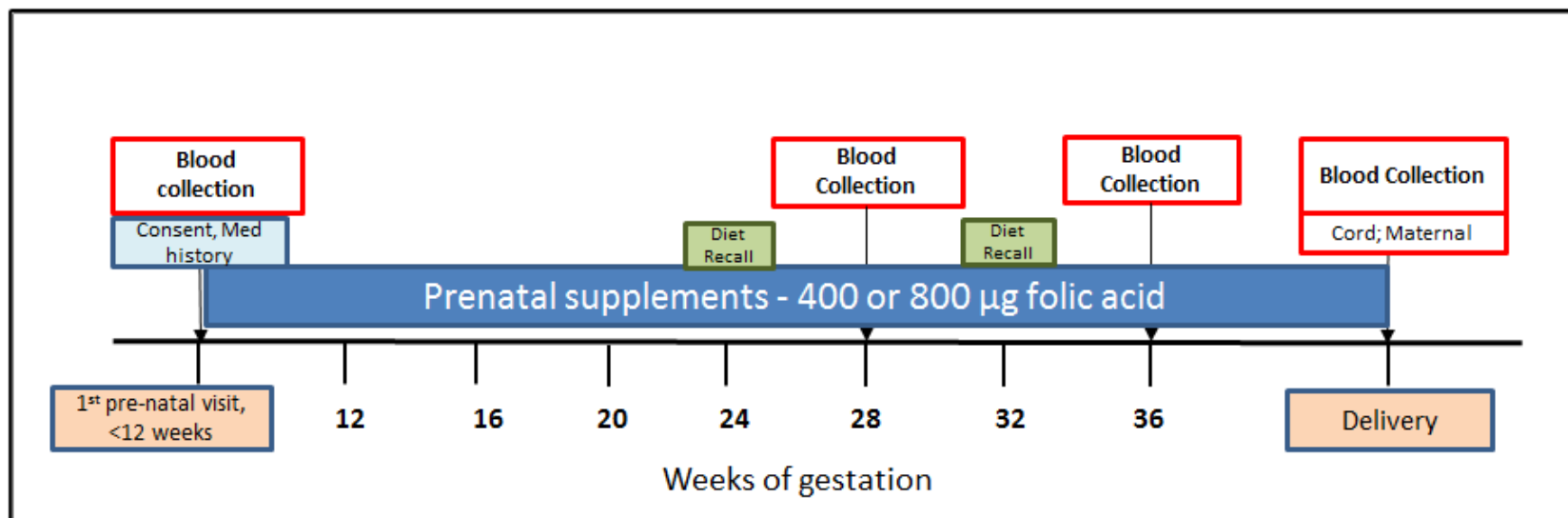


Figure 2: Protocol timeline

Serum folate concentration

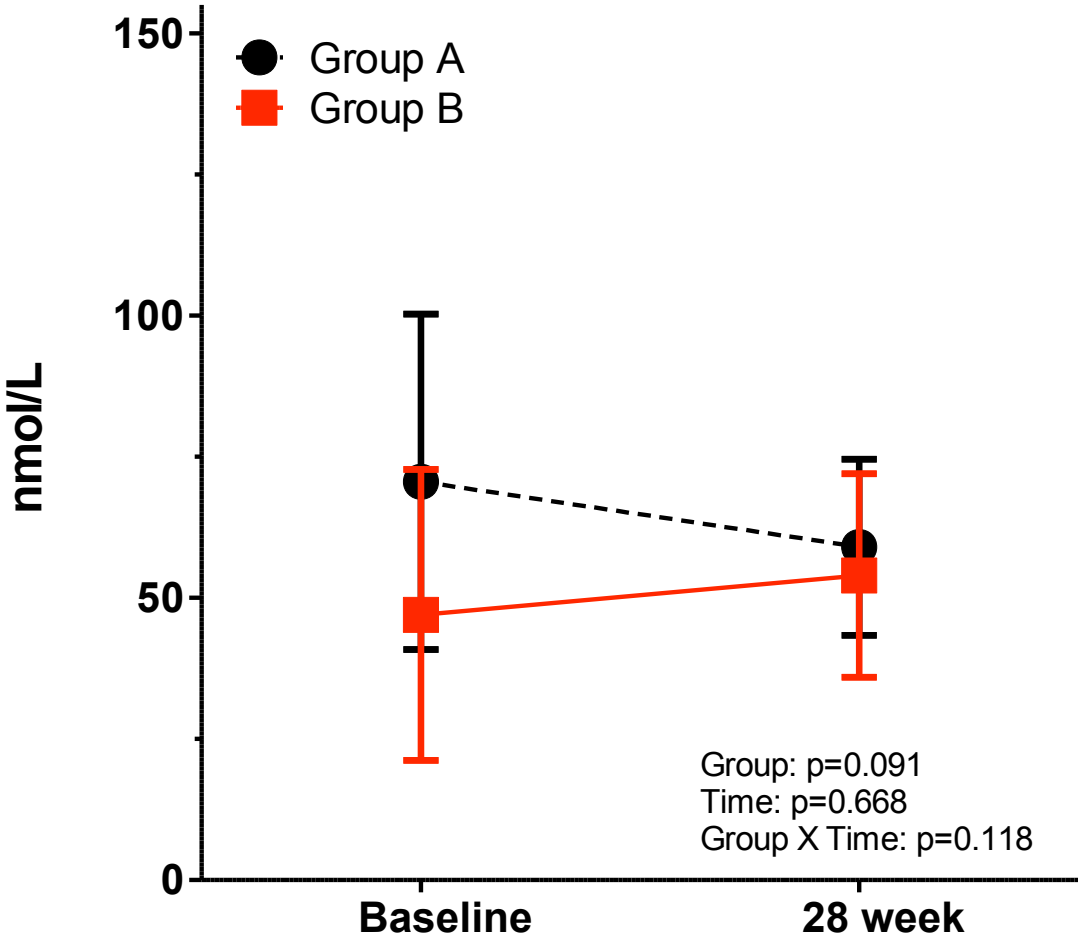


Figure 3: Serum folate response at 28 weeks gestation
Folate response analyzed using repeated measures analysis of variance. Values represent mean \pm standard deviation for $n=8$ (Group A) or $n=14$ (Group B) participants.

RBC folate concentration

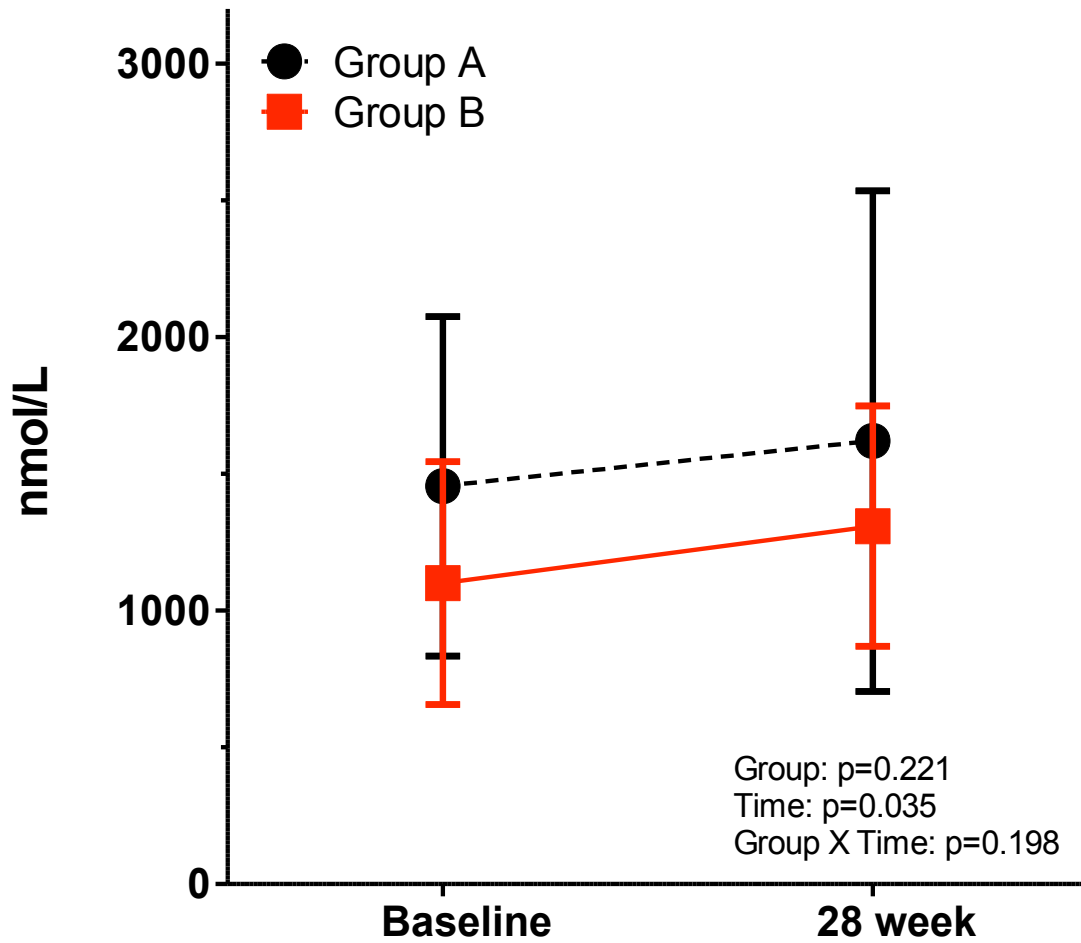


Figure 4: RBC folate response at 28 weeks gestation

Folate response analyzed using repeated measures analysis of variance. Values represent mean \pm standard deviation for $n=8$ (Group A) or $n=14$ (Group B) participants.

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CHAPTER 4

SUMMARY AND CONCLUSIONS

Folate, a water soluble B vitamin, is necessary for the production of red blood cells (RBCs) and for sufficient cell division and growth during periods of rapid growth, such as pregnancy (Bailey and Gregory 1999, Gropper et al 2009). The current Institute of Medicine recommendation for folate intake during pregnancy is 600 µg dietary folate equivalents (DFEs)/d, approximately equivalent to 400 µg folic acid (IOM, 1998). Despite a strong body of evidence supporting this recommendation (Caudill et al 1997, IOM 1998), most over-the-counter prenatal vitamins in the United State contain at least 800 µg folic acid, twice the recommendation. Additionally, previous research studies have identified significant differences in folate biomarker concentrations between normal weight and obese individuals (da Silva et al 2013), suggesting that individuals with a higher BMI may need larger doses of folic acid (Mojtabai 2004, Tinker et al 2012). Therefore the aim of the study was to investigate the difference in serum folate and RBC folate concentrations in response to daily supplementation with 400 or 800 µg folic acid throughout pregnancy, after adjusting for BMI and other potential confounders.

The current report is an interim analysis of serum and RBC folate response to supplementation in a subset of participants with available baseline and 28 week blood samples (n=22). In this subset, still blinded to treatment due to ongoing nature of study, significant differences in response to folic acid supplementation were not detected between group A and group B at 28 weeks gestation, likely due to high folate status and variability in

folate concentrations at baseline. Of the 18 participants who completed the Health Behavior Questionnaire at baseline, 11 participants reported prior use of a folic acid containing supplement for a range of time between 4 and 168 days prior to study enrollment. This variability in previous supplement use and the length of time of previous supplementation likely explains the observed significant time*supplementation interaction on RBC folate concentrations, as individuals that supplemented for <30 days prior to study enrollment had a greater increase in RBC folate concentration in response to the intervention than individuals that had taken folate supplements for ≥ 30 days prior to the intervention. Despite the variation in previous folic acid supplementation, a significant time effect was identified for RBC folate concentrations. This effect remained significant after individual adjustment for race/ethnicity, MTHFR genotype, length of prior folic acid supplementation, and gestational age at recruitment, but not after adjustment for pre-pregnancy BMI. Additionally, the mixed effects model showed a significant effect for race/ethnicity with lowest overall RBC folate concentrations observed for African American participants and highest overall RBC concentrations detected in non-Hispanic whites.

Upon analysis of serum folate concentrations, there were no significant differences found at baseline or 28 weeks gestation, however there was a significant effect for race/ethnicity identified in the mixed-effects model. This effect was likely because the African American participants tended to have much lower serum folate concentrations at baseline and 28-weeks gestation than the other race/ethnic groups. Additionally, there was a significant effect for length of previous supplementation on serum folate concentrations, with individuals previously taking supplements having a higher baseline serum folate concentration than those not supplementing prior to enrollment.

The data from the current study show no effect of BMI on folate biomarkers, contradicting findings of previous studies. If there is an effect of BMI on folate status, a response may not have been identified due to a small sample size, supplementation of folic acid prior to study enrollment, or the use of non-fasting blood samples.

To our knowledge, this is the first double blind randomized control trial to compare these two doses of folic acid in pregnant subjects from the first prenatal visit (~6-12 wk gestation) through delivery, while controlling for BMI. These data indicate prenatal supplementation with either 400 µg or 800 µg folic acid significantly increased RBC folate concentrations from baseline to 28 weeks. Analyses of these data did not identify an effect of BMI on RBC or serum folate concentrations, but there was a significant effect of race/ethnicity and previous supplementation on both RBC and serum folate. The finding of a time effect in the current study is not consistent with the study by Caudill et al (2007), which identified no significant increase in RBC folate concentration, but rather just a maintenance of RBC folate status with supplementation of 330 µg or 730 µg folic acid daily during the second trimester of pregnancy. The study by Caudill et al (2007) recruited at a later gestational age (14 weeks gestation), only supplemented through 25 weeks gestation, strictly controlled dietary intake, and required individuals to have normal blood folate concentrations at recruitment, which potentially led to the differences in findings between the two studies.

These data provide additional evidence that prior folic acid supplementation may confound any significant difference in folate biomarker response to these two folic acid doses and additionally may mask any BMI associations as have been previously reported. Completion of the study and further analysis is needed to

analyze the effects of folic acid dose on additional outcome parameters such as oxidized folic acid in the serum and changes in maternal and cord blood DNA methylation.

In order to determine if 400 µg and 800 µg supplemental folic acid leads to significant differences in RBC and serum folate during pregnancy and if BMI plays a mediating role, future studies should aim to overcome limitations of this study by: 1) performing the study in a population with no previous folic acid supplementation, 2) using a larger population to allow for a wider range of statistical analysis, and 3) obtain fasting blood samples to reduce variability in serum folate concentrations.

At baseline, there was a large range for serum folate concentrations (18.07 to 113.19 nmol/L) and RBC folate concentrations (378.18 to 2741.52 nmol/L), likely due to variability in supplementation prior to study enrollment. In addition, a significant prior supplementation effect was identified for serum folate analyses and a significant time*prior supplementation effect was indicated for RBC folate analyses. Future studies should be conducted in a population without prior supplementation in order to reduce baseline variability and this confounding variable. This type of intervention would provide a better understanding of the relationship between supplement dose, folate biomarker response, and the mediating effect of BMI.

Additionally, this study needs to be repeated in a larger population. Due to an unexpected delay in the start of the study, an initially slow rate of recruitment and a higher than anticipated dropout rate, baseline and 28 week were available from only 22 participants in time to complete this interim report. This limited the types of statistical analyses we could run and the effects we could identify. Nonetheless, the current data will be important for

power analysis to determine the number of participants needed in future, large scale studies, to detect a significant change in RBC and serum folate concentrations.

Finally, serum folate concentration is ideally measured using fasting blood samples, as this biomarker is sensitive to recent intake of folate containing foods and supplements (Shane 2011). As per the typical standard of care of the collaborating clinic, the pregnant participants in this study were not required to fast prior to blood collection. In future studies, researchers should consider asking participants to fast from midnight to early morning blood collection so as to obtain serum folate concentrations reflective of supplement dose and not confounded by recent dietary intake.

A larger study comparing the effects of 400 µg and 800 µg supplemental folic acid throughout pregnancy in a population without prior folic acid supplementation, using fasting blood samples would help determine if there is a dose response throughout pregnancy. In addition a larger sample size without prior supplementation would add to the evidence that response to supplemental folic acid may be mediated by BMI. The results of this study in addition to larger future studies may provide information about the relationship between folic acid dose and biomarker response in pregnant women and contribute to the evidence on which future recommendations for pregnant women can be based.

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APPENDICES

APPENDIX A

STUDY RECRUITMENT FLYER (ENGLISH)

Nutrition Research: Folic Acid Supplementation Study



Who Can Enroll:

- Healthy pregnant women ages 18-40 years old
- Less than 12 weeks pregnant
- **Enroll at 1st prenatal visit – ask midwife for details!**
- Normal weight to moderately obese
- No use of prescription drugs
- No use of alcohol or cigarettes

Benefits of Participation:

- Free prenatal supplements during entire pregnancy
- Nutrition analysis

Study Requirements:

- Use provided prenatal vitamins during pregnancy
- Additional blood taken during scheduled blood draws
- Complete 2 dietary records

Conducted by:

- Folate Research Team at UGA/ ARMC midwifery clinic
- Dr. Lynn Bailey (UGA), principal investigator
- 706-542-4256; folate@uga.edu



APPENDIX B

STUDY RECRUITMENT FLYER (SPANISH)

Investigación de Nutrición: Estudio de Suplemento de Ácido Fólico



Quienes Se Pueden Inscribir:

- Mujeres embarazadas con buena salud de 18 a 40 años de edad
- Embarazada por menos de 12 semanas
- **Inscripción durante primera visita prenatal- Favor preguntar a la partera por más detalles!**
- Peso normal o moderadamente obesa
- Que no use medicamento recetado
- Que no fume ni tome alcohol

Beneficios de Participación:

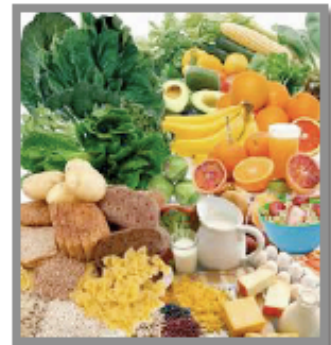
- Suplementos prenatales gratuitos durante el embarazo
- Análisis nutricional

Requisitos del Estudio:

- Tomar suplementos prenatales durante el embarazo
- Proveer una toma adicional de sangre en cada consulta
- Completar 2 registros dietéticos

Conducido Por:

- Grupo de Investigación de Folato UGA/ ARMC clínica de obstetricia
- Dra. Lynn Bailey (UGA), investigadora principal
- 706-542-4256; folate@uga.edu



APPENDIX C

STUDY CONSENT FORM (ENGLISH)

UNIVERSITY OF GEORGIA
RESEARCH PARTICIPANT INFORMED CONSENT
AND PRIVACY AUTHORIZATION FORM
Folic Acid Supplementation in Pregnant Women: Dose Response

Researcher's Statement

We are asking you to take part in a research study. Before you decide to participate in this study, it is important that you understand why the research is being done and what it will involve. This form is designed to give you the information about the study so you can decide whether to be in the study or not. Participation in the study is voluntary. Your decision to participate, or not, will not affect the services or standard of care provided during your prenatal clinical appointments. Please take the time to read the following information carefully. Please ask the researcher if there is anything that is not clear or if you need more information. When all your questions have been answered, you can decide if you want to be in the study or not. This process is called "informed consent." A copy of this form will be given to you.

Principal Investigator: Dr. Lynn B. Bailey
Department of Foods and Nutrition
Telephone: 706-542-4256
Email: folate@uga.edu

Purpose of the Study

Folate is a general term for a water-soluble vitamin especially important during pregnancy. Folic acid is a form of the vitamin that is used in supplements and fortified foods. Prenatal supplements often contain much different amounts of folic acid and yet scientists and medical practitioners don't know how specific amounts of folic acid affect the blood levels of pregnant women and their babies at birth. The purpose of this study is to determine how levels of folate and related indicators in your blood at different times during pregnancy and in your babies cord blood after birth differ in response to one of two different amounts of folic acid in prenatal supplements. The folic acid doses represent the current Recommended Dietary Allowance (RDA) for pregnant women and a higher dose typically found in over-the-counter prenatal vitamin supplements. Your participation in this study will help provide important new information that will not only inform scientists but will also help guide clinicians who routinely recommend prenatal supplements.

Eligibility

You are qualified to volunteer for the study if you are a pregnant patient at Athens Regional Midwifery Clinic and meet other requirements which include the following: (a) 18-40 yrs old; (b) body weight, normal to moderately obese; (c) less than twelve weeks pregnant; (d) carrying only one baby; (e) no history of chronic disease; (f) non-anemic; and (g) not taking prescription drugs. Eligibility for the study will be verified based on meeting the above criteria and perceived willingness to complete study procedures and questionnaires. You may be withdrawn from the study without regard to consent if it is determined that you are carrying more than one

baby, you develop pregnancy-associated complications such as gestational diabetes or hypertension, you fail to take prenatal supplements as directed or to complete other study related procedures, or if you discontinue prenatal care through ARMC midwifery clinic.

Study Procedures

If you agree to participate in the study, you will be asked to take part in the following study related procedures:

Blood collection – Blood will be collected during your scheduled pre-natal visits at times that samples are routinely drawn for diagnostics/pregnancy status monitoring. No additional needlesticks will be required for research purposes. At each collection, a small needle will be inserted into a vein in your arm and an additional 30 mL (about 6 teaspoons) of blood will be taken for research purposes. Blood will be collected during your initial prenatal visit, at clinic visits at 28 and 36 weeks gestation and at delivery and will only take a few minutes. This blood will be used to measure blood folate and related nutritional and genetic indicators. In addition, after your baby is born and the cord has been cut, a blood sample (approximately 5 ml or 1 teaspoon) will be collected from the cord for analysis of folate status indicators. Some of the blood measurements will be done by collaborators at other universities within the United States. The samples will be sent with a participant number code and our collaborators will not be given any information that would allow them to directly identify you. Any information that is discovered from testing of this blood is related to research only and will not be used as therapy or diagnostic testing. For possible analysis in the future of additional folate-related metabolic indicators, a portion of your blood will be saved. Blood samples will be stored with a number code and your personal information will not be associated with your sample. Storage of samples for potential future analysis is not a requirement for participation in this study and you have the right to ask that all of your samples be removed and/or destroyed once the current study is completed. Any unused portions of blood that are collected will be discarded 10 years after completion of the study, per safe handling of hazardous material as defined by The University of Georgia Hazardous Material Safety protocol.

Vitamin supplement protocol – As a participant in this study, you will take prenatal supplements that contain one of two doses of folic acid. One of folic acid doses represents the current RDA for pregnant women and the other is a higher dose as typically found in prenatal supplements. There are no known risks related to the consumption of either of the doses of folic acid included with the prenatal supplements. Both supplements contain the same vitamins, minerals, and DHA in amounts that are routinely provided in commercially available prenatal supplements. The only difference in the two supplements is the amount of folic acid. At your first prenatal visit, you will be provided the first four week supply of prenatal vitamins including one of two doses of folic acid, a prenatal multivitamin/mineral tablet and a DHA (nutrient important for brain development) supplement. The supplements will be packaged as individual daily supplies and you will take all supplements daily throughout your pregnancy from your first prenatal visit until delivery. You will take all the tablets for each day at once and at the same time each day, preferably with your evening meal. To insure that you remember to take your supplements, you will be instructed in the use of a compliance calendar and may receive telephone and/or ‘text’ message reminders from project staff. You will be asked to return your pill containers and any unused pills at

your next visit. You will receive a new supply of supplements every four weeks through the end of your pregnancy. You will be asked to follow your typical diet, but to refrain from consuming other dietary supplements, multivitamins, and highly fortified cereal products (containing > 100% the RDA for folate).

Medical records - Information regarding your age, ethnicity, medical history, physical exam findings and blood test results will be obtained from your medical records at ARMC to determine the effect of these factors on the response to the folic acid supplementation. Information will also be obtained from the medical records regarding the date and mode of delivery, gestational age, gender, measurements, Apgar score and blood test results of your baby to determine the effect of the folic acid supplementation on the growth and development of your baby. The privacy law, Health Insurance Portability & Accountability Act (HIPAA), protects your health information. Researchers may use or disclose protected health information for research purposes only if they have received your authorization for ARMC Midwifery Practice to disclose your information. The researchers will protect this information by using it only as permitted by this Authorization and as directed by state and federal law. If you have any questions and/or wish to revoke this Authorization in writing at any time, you can contact Dr. Lynn B. Bailey (see page 1). This Authorization expires ten years after the completion of the study. If you choose to participate in the research, you must sign this form so that your health information may be used for the research. Your decision to release or not to release this information will not affect the current or future services you receive from the ARMC Midwifery Practice; however, if you do not agree to this, you will not be able to participate in this study. The health information listed above may be disclosed for use in other projects related to pregnancy, nutrition, and infant health. While such disclosure is no longer protected by this authorization, the disclosure of your identifiable health information would only be to researchers who are members of the current research team and who obtain your written consent for involvement in such projects.

Study specific questionnaire - To obtain additional information not available on the medical records you will be contacted by telephone and asked to complete a brief questionnaire. The questions will include information regarding previous and current folic acid supplement use, regular consumption of cereals and other folic acid fortified foods, past and present smoking and alcohol habits, and other lifestyle factors. This questionnaire will be administered by University of Georgia research personnel and the telephone interview should take 15 minutes or less.

Food diaries/diet recalls – Your usual dietary intake at the various stages of pregnancy will be estimated through the use of food diaries and a computer-based diet recall program. You will be provided with Three Day Diet Recall Sheets on which you will record the foods you consume in a food diary format for assigned days at 24 and 36 weeks of gestation. These records will be returned via email or regular mail to UGA project staff. After receipt of the food diaries by study personnel, trained research staff will contact you via telephone to obtain additional and more specific information such as brands and amounts consumed. The research staff will enter the information obtained through the food diary and follow-up interview into an on-line program for subsequent analysis. It should take about an hour at each of the two collection points to record your information in the food diaries and for the

follow-up interview, for approximately two hours total.

Risks and discomforts

- **Blood draw:** Blood will be drawn for the purposes of this study only at times when samples are already being taken by the clinic for as part of your usual care. There is no additional risk for collecting extra blood for research purposes.

- **Questionnaires/Dietary Recall:** The discomfort or stress that you may face during this research may be associated with the disclosure of information concerning your dietary intake and health history; however it is important to share this information so that your health and nutritional status can be evaluated correctly. All individually-identifiable information will be kept strictly confidential and your name and other identifying information will be kept under lock and key, will not appear on project data files and will not be shared with anyone else.

Benefits

The information provided by this research study will help the researchers advance their knowledge about how different amounts of prenatal folic acid affect blood folate and other indicators of nutrition status in pregnant women. The study will provide data that will inform clinicians regarding the impact of the current recommended intake of folic acid for pregnant women compared to a higher dose often included in prenatal supplements on both your blood folate levels during pregnancy and the blood level of your baby as determined from your infants' cord blood at delivery. This new knowledge will help guide future decisions regarding the most appropriate dose of folic acid to recommend for prenatal patients. In addition, information regarding how nutrients from dietary sources are associated with nutritional status will provide new evidence for future guidance regarding prenatal dietary intake recommendations.

Incentives for participation

You will receive your prenatal supplements at no cost as part of the study protocol. Depending on gestational week at enrollment, you may receive prenatal supplements for up to eight months, representing a potential cost savings of up to \$240 (~ \$30 per month). The prenatal supplements will be packaged as four week supplies and will be provided for the duration of participation in the study. If you choose to withdraw from the study at any point or if you are withdrawn from the study without regard to your consent for circumstances as previously indicated, you will not be provided with additional supplements. We will also provide you with a dietary intake analysis and information on your blood folate levels at various stages of pregnancy.

Privacy/Confidentiality

Every effort will be taken to protect your identity. No individually-identifiable information about you, or provided by you during the research, will be shared with others without your permission, except if necessary to protect your rights or welfare (for example, if you are injured and need emergency care), or if required by law. Your participation results, which will include an assigned participant number, and your consent form will not be stored together. A separate list will be the only document linking your name and participant number, and it will be kept along with the consent forms in a locked file drawer, and

accessed only by Dr. Bailey and her immediate research team. This list will be destroyed ten years from the end of the study. All other documents, including questionnaires, diet assessment forms and blood sample submission forms will only include your participant number. This research includes testing for genetic differences that may influence individual response to folate supplementation. Any information obtained from this testing is related to research only, will not be used for diagnostic or therapeutic testing and will not be linked to any individually identifiable information. In the unlikely event that there is a violation in confidentiality, a recent federal law the Genetic Information Nondiscrimination Act (GINA) will help protect you from health insurance or employment discrimination based on genetic information potentially obtained through this research. This study will be registered at ClinicalTrials.gov, a Web-based publically-available resource that provides patients, healthcare professionals and researchers with information on clinical trials or intervention studies in human volunteers. Study results submitted to this database will be in the form of summary information and will not include any individual data. You will not be identified in this or any other report or publication of this study.

Taking part is voluntary

Your involvement in the study is voluntary, and you may choose not to participate or to stop at any time without penalty or loss of benefits to which you are otherwise entitled. If you decide to discontinue or withdraw from the study or if the investigator decides to terminate your participation without regard to your consent, the information/data collected from or about you up to the point of withdrawal will be kept as part of the study and may continue to be analyzed, unless you ask to have information that can be identified as yours returned to you, removed from the research records, or destroyed. If you withdraw or are withdrawn from the study, you also have the right to ask for your specimens to be removed from the study and/or destroyed.

If you are injured by this research

The researchers will exercise all reasonable care to protect you from harm as a result of your participation. If you think that you have suffered a research-related injury, you should seek immediate medical attention and then contact Dr. Bailey right away at (706)-542-4256.

Permission for photograph-taking:

Please provide initials below if you consent for photography and subsequent use of your image for research-related purposes, such as presentations and publications related to this UGA research study. You may still participate in this study even if you are not willing to have your photograph taken.

_____ I am willing to have my photograph taken and used as described above
_____ I do not want to have my photograph taken and/or used as described.

Permission for contact by UGA research personnel, now and in the future:

By signing my initials here, _____, I agree to allow the investigators of this study to contact me to obtain information required for the Study Specific Questionnaire as previously described.

By signing my initials here, _____, I agree to allow the investigators of this study to contact me in the future to request participation in future studies. I understand that at that time, I may refuse any further participation with no negative consequences.

My contact information is:

Telephone Number(s) _____ (home) _____ (cell)

Address: _____

Email: _____

If you have questions

The main researcher conducting this study is Dr. Lynn B. Bailey, a professor at the University of Georgia. Please ask any questions you have now. If you have questions later, you may contact Dr. Bailey at folate@uga.edu or at (706) 542-4256. If you have any questions or concerns regarding your rights as a research participant in this study, you may contact the Institutional Review Board (IRB) Chairperson at (706)-542-3199 or irb@uga.edu.

Research Subject’s Consent to Participate in Research:

To voluntarily agree to take part in this study, you must sign on the line below. Your signature below indicates that you have read or had read to you this entire consent form, and have had all of your questions answered.

Name of Researcher

Signature

Date

Name of Participant

Signature

Date

Please sign both copies, keep one and return one to the researcher.

APPENDIX D

STUDY CONSENT FORM (SPANISH)

UNIVERSIDAD DE GEORGIA
CONSENTIMIENTO INFORMADO Y AUTORIZACION PRIVADA
DE PARTICIPANTE DE INVESTIGACION

Suplementación del Ácido Fólico en Mujeres Embarazadas:
Respuesta a la Dosis

Declaración de la investigadora:

Estamos pidiendo que usted participe en una investigación. Es importante que entienda porque se hace la investigación y que involucrará antes de que decide participar. Este formulario está diseñado para darle información sobre la investigación para que pueda decidir si quiere participar o no. La participación en esta investigación es voluntaria. Su decisión participar o no participar no afectará los servicios o el nivel de atención ofrecido en sus citas clínicas prenatales. Por favor, toma el tiempo para leer la información siguiente con cuidado. Por favor, pregunte al investigador si hay algo que no está claro o si necesita más información. Cuando todas sus preguntas han sido contestadas, Ud. puede decidir si quiere estar parte de la investigación o no. Este proceso se llama “consentimiento informado.” Una copia de este formulario se le dará a usted.

Investigadora Principal: Dra. Lynn B. Bailey
Departamento de la Nutrición y la Comida
Teléfono: 706-542-4256
Correo Electrónico: folate@uga.edu

Propósito de la Investigación:

El folato es una palabra general para una vitamina soluble en agua que es importante especialmente durante el embarazo. El ácido fólico es una forma de la vitamina que se usa en suplementos y comidas fortificadas. Suplementos prenatales de menudo contienen cantidades del ácido fólico muy diferentes aún no saben ni los científicos ni los profesionales médicos como ciertas cantidades del ácido fólico afecta a los niveles en la sangre de mujeres embarazadas o sus infantes al nacer. El propósito de esta investigación es determinar como los niveles del folato e indicadores relacionados en su sangre durante varios tiempos del embarazo y en la sangre de cordón de su bebé al nacer sean diferentes en respuesta a una de dos cantidades del ácido fólico en suplementos prenatales. Las dosis representan la Ración Dietética Recomendada (RDR) para mujeres embarazadas y una dosis más alta que se encuentra frecuentemente en los suplementos prenatales sin receta. Su participación en esta investigación ayudará proveer información importante y nueva que no solo informará a los científicos sino también ayudará guiar a los médicos los cuales recomiendan los suplementos prenatales a menudo.

Elegibilidad

Usted está calificado ser voluntario para la investigación si eres un paciente embarazado de Athens Regional Midwifery Clinic (Clínica de las Parteras de Athens Regional) y cumple Ud. Otros requisitos los cuales incluyen el siguiente: (a) 18-40 años de edad; (b) peso de cuerpo normal a moderadamente obeso; (c) menos que doce semanas de embarazo; (d) llevando a solo un bebé; (e) sin historial médico de enfermedad crónica; (f) sin anemia; (g)

no tomando ningún medicamento recetado. La elegibilidad para la investigación se verificará con los requisitos anteriores y su disposición de completar los procedimientos del estudio y los cuestionarios. Se puede retirarse de la investigación sin tener en cuenta su consentimiento si se determinará que Ud. está llevando más que un bebé, si presenta con complicaciones de embarazo como diabetes gestacional o hipertensión, si no tomas los suplementos prenatales como dirigido o completar otros procedimientos del estudio, o si suspende su cuidado prenatal por la Clínica de las Parteras de ARMC.

Procedimientos de la Investigación

Si acepta Ud. participar, se le pedirá hacer los siguientes procedimientos del estudio:

Colección de sangre– Se recogerá la sangre durante sus citas prenatales preestablecidas en los mismos tiempos que lo hacen normalmente para los diagnósticos/ el monitoreo del estado del embarazo. No habrá pinchazos de aguja adicionales para fines de la investigación. En cada colección, un aguja pequeña se inserta en su vena y 30 mL (casi 6 cucharaditas) adicionales de sangre se recogerá para fines de la investigación. Se recogerá la sangre durante su visita prenatal inicial, en las visitas de 28 y 36 semanas de gestación y en el nacimiento y solo tomará unos pocos minutos. Esta sangre se usará para medir el folato de sangre y indicadores nutricionales y genéticos relacionados. Adicionalmente, después de nacer su bebé y cortar el cordón umbilical, un poco de sangre (5 mL/ 1 cucharadita) se recogerá del cordón umbilical para análisis de indicadores del estado de folato. Algunas de las medidas se hará por otros colaboradores en otras partes de los Estados Unidos. Los especímenes se enviará con un numero de participante, y nuestros colaboradores no recibirán ninguna información que se puede usar para identificarle directamente a usted. Cualquiera información que se descubre de la examinación de esta sangre es solamente para la investigación, y no se usará para tratamiento o exámenes diagnósticos. Se guardará un poco de su sangre para análisis de indicadores metabólicos del folato posible en el futuro. Especímenes de sangre se guardará con un código numérico y su información personal no se asocia con su espécimen. El almacenamiento de su sangre para investigación adicional no es un requisito de esta investigación, y Ud. tiene la derecha pedir que todas sus especímenes serán eliminados/destruidos después de que termina la investigación principal. Cualquiera especímenes de sangre no usadas serán descartadas después de 10 años del final de la investigación, según manejo seguro de materiales peligrosos, definido por el protocolo de Seguridad de Materiales Peligrosos de la Universidad de Georgia.

Protocolo de suplementación de vitaminas– Como un participante en esta investigación, Ud. tomará suplementos prenatales que contienen una de dos dosis del ácido fólico. Una de estas dosis representa la RDR corriente para mujeres embarazadas y la otra dosis es más alta y se encuentra típicamente en suplementos prenatales. No hay riesgos conocidos de consumir las dosis del ácido fólico incluido en las vitaminas prenatales. Los dos suplementos contienen las mismas vitaminas, minerales, y DHA en cantidades normalmente encontrado en suplementos prenatales comerciales. La única diferencia en los dos suplementos es la cantidad de ácido fólico. En su primera visita prenatal, se le proporcionará un suministro de 4 semanas de las vitaminas prenatales incluyendo una de dos dosis del ácido fólico, una pastilla de multivitamina/ minerales, y un suplemento de DHA (un nutritivo importante para el desarrollo cerebral). Los suplementos serán empaquetados en suministros diarios y usted

tomará los suplementos cada día durante su embarazo hasta el parto. Tomará cada tableta para cada día al mismo tiempo cada día, preferiblemente con la cena. Para asegurar que recuerda tomar los suplementos, se le indicará usar un calendario de conformidad y tal vez recibirá llamadas o mensajes de ‘texto’ como un recordatorio del personal de la investigación. Se le pedirá devolver sus contenedores de pastillas y cualquier pastilla no tomadas en su visita próxima. Recibirá un suministro nuevo de suplementos cada cuatro semanas hasta el final de su embarazo. Se le pedirá seguir su dieta normal y abstenerse de tomar otros suplementos de dieta, multi-vitaminas, o productos de cereal muy fortificados (conteniendo > 100% la RDR para el folato).

Registros médicos – Información con respecto a su edad, etnicidad, historia clínica, hallazgos del examen físico, y resultados del examen de sangre se obtendrá de sus registros en ARMC para determinar el efecto de estos factores en su reacción a la suplementación del ácido fólico. También se obtendrá información con respecto a la fecha y manera de parto, edad gestacional, género, medidas, la calificación de Apgar, y resultados de examinación de sangre de su niño para determinar el efecto de la suplementación del ácido fólico en el desarrollo y crecimiento de su hijo.

La ley de la vida privada, la Ley de “Portabilidad” y Responsabilidad del Seguro Médico (HIPAA), protege su información de la salud. Investigadores pueden usar o revelar información protegida solamente si han recibido su autorización que La clínica de las Parteras de ARMC puede revelar su información. Los investigadores protegerán esta información por usarla solo como ya está permitida con esta autorización y como dirigida por las leyes del estado y las leyes federales. Si tiene cualquier pregunta y/o quiere revocar esta autorización al escribir en cualquier momento, puede contactar a Dra. Lynn B. Bailey (ve la página 1). Esta autorización expira diez años después de que termina esta investigación. Si decide participar en esta investigación, hay que firmar este formulario para que se puede usar su información para la investigación. Su decisión dar o no dar a conocer esta información no afectará los servicios que recibes ahora o en el futuro de la clínica de las parteras de ARMC; sin embargo, si no le da permiso, no podrá participar en esta investigación. La información ya descrito puede ser revelado para el uso en otros proyectos sobre el embarazo, nutrición, y la salud infantil. Aún esta revelación no sea protegida por esta autorización, la revelación de su información identificable de la salud solamente sería a los investigadores quienes son miembros de este equipo de investigación y que obtengan su consentimiento escrito para su participación en estos proyectos.

Cuestionario específicamente para el estudio – Para obtener información adicional no contenido en los registros médicos, usted será contactada por teléfono y le pedirá cumplir un cuestionario pequeño. Las preguntas incluirán información con respecto a su uso actual y anterior de suplementos del ácido fólico, su consumo usual de cereales y otras comidas fortificadas con el ácido fólico, hábitos de fumar y tomar alcohol actuales y anteriores, y otros factores del estilo de vida. Este cuestionario se administrará por el personal de investigación de la Universidad de Georgia. La entrevista de teléfono debe tomar 15 minutos o menos.

Los diarios de comida/ Recordatorios de dieta– Su ingesta dietética usual en las varias etapas del embarazo se estimará por el uso de diarios de comida y un programa de

recordatorios de dieta basada en la computadora. Se le proporcionará con unos formularios de Recordatorios de Dieta de Tres Días en los cuales recordará las comidas que consume Ud. en el formato del diario de comida para días asignados en 24 y 36 semanas de gestación. Estos registros se devolverán por correo electrónico o normal al personal del UGA. Después de recibir los diarios de comida, personal capacitado le contactará por teléfono para obtener información adicional y más específica como nombres de marca y las cantidades consumidas. El personal de investigación pondrá la información del diario de comida y la entrevista siguiente en una programa en línea para análisis subsiguiente. Debe tomar una hora en cada de los dos puntos de colección para recordar toda su información en los diarios de comida y para la entrevista, aproximadamente dos horas en total.

Riesgos y molestias

- **Extracción de sangre:** Se le extraerá sangre para los propósitos de esta investigación solamente cuando ya la están extrayendo en la clínica como parte de su cuidado normal. No hay riesgo adicional de colectar sangre extra para los usos de investigación.
- **Cuestionarios/Recordatorios de la dieta:** La molestia o estrés que puede enfrentar durante esta investigación puede ser asociada con la revelación de información sobre su ingesta dietética e historia clínica; sin embargo es importante compartir esta información para que su estado de salud y nutrición puede ser evaluado apropiadamente. Toda la información que es individualmente identificable será mantenido estrictamente confidencial y su nombre y otra información personal será guardado bajo llave, no aparecerá en archivos de datos del proyecto, y no estarán compartidos con otras personas.

Beneficios

La información proporcionado por esta investigación ayudará a los investigadores avanzar su conocimiento sobre como cantidades diferentes del ácido fólico prenatal afecta folato de sangre y otros indicadores del estado nutricional de mujeres embarazadas. La investigación proveerá datos que informarán a los médicos según el impacto en su folato de sangre durante el embarazo y el nivel de folato de sangre de su bebé después del parto (según la sangre del cordón umbilical) de la RDR actual del ácido fólico comparado a un dosis mas alto normalmente encontrado en suplementos prenatales comerciales. Este conocimiento nuevo guiará decisiones futuras sobre el dosis más apropiado para pacientes prenatales. Adicionalmente, información sobre como los nutritivos de fuentes dietéticas afectan al estado nutricional proveerá evidencia nueva para dirección futura sobre recomendaciones de ingesta dietética prenatal.

Incentivos de participación

Recibirá usted sus suplementos prenatales gratis por ser parte del estudio. Tal vez recibirá suplementos prenatales por 8 meses, dependiente en su semana de gestación al inscribirse en la investigación. Esto representa un ahorro potencial de \$240 (~\$30 al mes). Los suplementos prenatales serán empaquetados como suministros de cuatro semanas y se proveerán para la duración entera de su participación en el estudio. Si quiere retirarse de la investigación en cualquier momento o si está retirado sin respecto a su consentimiento para las razones indicadas previamente, no se le proporcionará con suplementos adicionales. También se le proporcionará con un análisis de ingesta dietética e información de sus niveles del folato de sangre en varias etapas del embarazo.

Privacidad/Confidencialidad

Se hará todo lo posible para proteger su identidad. Ninguna información individualmente identificable sobre usted, o provecho por Ud. durante la investigación, se compartirá sin su permiso, a menos que sea necesario para proteger sus derechos o su bienestar (por ejemplo, si está herida y necesita cuidado de emergencia), o si requerido por la ley. Sus resultados de participación, los cuales incluirán un número de participante asignado, y su formulario de consentimiento no se guardarán juntos. Una lista separada será el único documento que enlace su nombre y número de participación, y se guardará con los documentos de consentimiento en un cajón de archivo bloqueado; a lo cual solamente Dra. Bailey y su equipo de investigación tendrá acceso. Esta lista se destruirá diez años después de que termina la investigación. Todos los otros documentos, incluyendo los cuestionarios, formularios de dieta, y formularios de envío de muestras de sangre solamente incluirán su número de participante.

Esta investigación incluye una examinación de diferencias genéticas que pueden afectar la reacción individual a la suplementación del folato. Cualquiera información que sea obtenida por esta examinación se relaciona solamente con la investigación, y así no será usado para exámenes diagnósticos ni terapéuticos y no serán relacionados con ninguna información individualmente identificable. En el caso improbable de que haya una violación de confidencialidad, una ley federal, el Acto de No Discriminación de Información Genética (GINA) le protegerá de la discriminación en su trabajo o del seguro de salud basada en información posiblemente obtenida por esta investigación.

Este estudio será registrado en ClinicalTrials.gov, un recurso público de internet el cual provee información sobre estudios intervencionistas y ensayos clínicos a los pacientes y profesionales de la salud. Los resultados de este estudio que se presentarán a esta base de datos serán en forma resumida y no incluirán datos individuales. No se le identificará a usted en este o cualquier otro informe o publicación de este estudio.

Participar es voluntario

Su participación en la investigación es voluntaria, y puede decidir no participar o retirar en cualquier momento sin penalización o pérdida de beneficios a los cuales usted tiene derecho. Si decide retirar del estudio o si el investigador decide terminar su participación sin respecto a su consentimiento, la información/los datos recopilados de usted hasta el punto de retiro se mantendrá como parte de la investigación y pueden ser analizados a menos que usted pide que la información suya se devolverá a Ud., quitada de los registros de investigación, o eliminada. Si retira o está retirada del estudio, tiene el derecho de pedir que las especímenes suyas sean eliminadas del estudio o destruidas.

Si le causa daño esta investigación

Los investigadores harán todo lo posible y razonable para protegerle del daño como resultado de su participación. Si piensa Ud. que a sufrido un daño relacionado a la investigación, debe buscar atención médica inmediatamente, y después llama a Dra. Bailey (706)-542-4256.

Permiso para sacar fotos:

Por favor, firme sus iniciales abajo si Ud. da su consentimiento de ser fotografiada y el uso

después de su imagen para usos relacionados a la investigación, como presentaciones o publicaciones relacionadas con esta investigación de UGA. Puede participar en el estudio aún si no quiere ser fotografiada.

_____ Doy mi consentimiento ser fotografiada y que mi imagen será usada como ya descrito.
_____ No quiero que saquen mi foto ni que la usen como ya descrito.

Permiso para el contacto por el personal de investigación de UGA, ahora y en el futuro:
Con mi firma de iniciales aquí, _____, permito que me pueden contactar los investigadores de este estudio para obtener información necesario para el Cuestionario Específicamente para el Estudio como ya descrito.

Con mi firma de iniciales aquí, _____, les permito a los investigadores de este estudio contactarme en el futuro para solicitar mi participación en estudios futuros. Entiendo que en aquel momento, puedo negar participación adicional sin consecuencias negativas.

Mi información de contacto es:

Número(s) de teléfono: _____ (Casa) _____ (Móvil)

Dirección: _____

Correo Electrónico: _____

Si tiene preguntas:

La investigadora principal es Dra. Lynn B. Bailey, una profesora en la Universidad de Georgia. Por favor, haga cualquieras preguntas ahora. Si tiene preguntas luego, puede contactar a Dra. Bailey por correo electrónico en folate@uga.edu o por teléfono en (706)-542-4256. Si tiene cualquiera pregunta sobre sus derechos como un participante en esta investigación, puede contactar al presidente de la Junta de Revisión Institucional (IRB) por teléfono (706)-542-3199 o correo electrónico: irb@uga.edu.

Consentimiento del Sujeto de Investigación a Participar en la Investigación:

Para acordar voluntariamente a participar en este estudio, tiene que firmar en la línea abajo. Su firma indica que ha leído este formulario de consentimiento entero, o que ha sido leído para usted, y que todas sus preguntas han sido contestadas.

Nombre del Investigador

Firma

Fecha

Nombre de Participante

Firma

Fecha

Por favor, firme las dos copias, guarde una y devuelva la otra al investigador.

APPENDIX E

HEALTH BEHAVIOR QUESTIONNAIRE (ENGLISH)

Folic Acid Supplementation in Pregnancy: Health Behavior Questionnaire

Date: _____ Time: _____ Telephone interview completed by: _____

Participant number: _____

- Have you been taking your prenatal supplements every day as directed? ___Yes ___No

- Have you experienced any problems with the supplements? ___Yes ___ No
 - If yes, what? _____

- Do you have any questions about the supplements or other aspects of the study?

- Before enrolling in this study, had you heard of folic acid? ___Yes ___ No
 - If yes, how? ___magazine/newspaper/internet ___ radio/TV ___ schooling
___ doctor/nurse/health professional ___ family/friends ___ other

- Did you take a multivitamin during the month(s) just before you got pregnant?
 - ___Yes ___ No
 - If yes, what brand? _____
 - How often? ___1- 3 times/wk ___4 - 6 times/wk ___every day
 - If no, why didn't you take vitamins? ___didn't think I needed ___too expensive
___vitamins gave me side effects ___ did not plan to get pregnant
_____other

- Did you take a supplement that just contained folic acid during the month(s) just before you got pregnant?
 - ___Yes ___ No
 - If yes, why did you take it? _____
 - How often? ___1- 3 times/wk ___4 - 6 times/wk ___every day
 - If no, why didn't you folic acid? ___didn't think I needed ___too expensive
___supplements five me side effects ___ did not plan to get pregnant
_____other

- After finding out you were pregnant or may be pregnant and before receiving the supplements from the clinic, did you take any multivitamins or prenatal vitamins? ___Yes ___ No

- If yes, what brand? _____
- For what period of time? _____
- How often? _____

- After finding out you were pregnant or may be pregnant and before receiving the supplements from the clinic, did you take a supplement that just contained folic acid? ___Yes ___ No

- If yes, what brand or amount? _____
- For what period of time? _____
- How often? _____

- Did you take any dietary or herbal supplement other than multivitamins or folic acid at any time just before or during this pregnancy?

- ___Yes ___ No
- If yes, what kind of supplements, what brand & how often? _____

- Do you regularly consume any of the following?

- Ready-to-eat breakfast cereal ___Yes ___No _____ Brand ___times/wk
- Meal replacement drinks/bars ___Yes ___No _____ Brand ___times/wk
- Energy drinks ___Yes ___No _____ Brand ___times/wk
- Protein shakes ___Yes ___No _____ Brand ___times/wk
- Snack bars ___Yes ___No _____ Brand ___times/wk
- Spinach, kale or other leafy greens ___Yes ___No _____times/wk
- Orange juice ___Yes ___No _____ Brand ___times/wk

- In the past, did you ever smoke? _____Yes _____No
If yes, for how long? _____When did you quit smoking? _____

- In the past, did you ever regularly drink more than one serving of alcoholic beverages a day?
 - _____Yes _____No
 - Is yes, how often do you drink 2 or more alcoholic beverages a day? _____

- Do you currently drink more than one serving of alcoholic beverages a day?
 - _____Yes _____No
 - If yes, how often do you drink 2 or more alcoholic beverages a day? _____

- Over the past week, about how much time did you spend engaging in physical activities (exercise, walking, gardening, vacuuming, etc)? _____(hrs/min) per (day/wk)

- Were your activities spent ___ mainly indoors ___ mainly outdoors ___ half indoors & half outdoors

- Was this similar level of physical activity similar to that before your pregnancy? _____ Yes
 _____No, more active before pregnancy _____No, more active now

- Over the past month, how much time did you usually spend outside each day between sunrise & sunset? _____

- Is this amount of time spent outdoors in the sun fairly typical for you at this time of year?
 _____yes _____ no

- When outside in the sun, did you usually wear a hat, sunscreen or other sun protection?
 _____yes _____ no

APPENDIX F

HEALTH BEHAVIOR QUESTIONNAIRE (SPANISH)

Suplementación del Ácido Fólico durante el Embarazo: Cuestionario del Comportamiento de Salud

Fecha: _____ Tiempo: _____ Entrevista de teléfono hecho por: _____

Número de participante: _____

- ¿Ha tomado sus suplementos prenatales cada día como dirigida?? Sí No

- ¿Ha tenido una problema con los suplementos? Sí No
 - ¿Cuáles? _____

- ¿Tiene unas preguntas sobre los suplementos o otros aspectos del estudio?

- ¿Antes de inscribirse en este estudio, había oído del ácido fólico? Sí No
 - ¿Cómo? revista/periodico/internet radio/Televisión escuela médico/enfermera/profesional de salud familia/amigo otro

- ¿Tomaba una vitamina durante el mes antes de la concepción de su hijo?
 - Sí No
 - ¿Cuál marca? _____
 - ¿Con que frecuencia? 1- 3 veces a la semana 4 - 6 veces a la semana cada día
 - Si contestó “no”, ¿por qué no tomaba vitaminas? no pensaba que era necesario demasiado caro las vitaminas me causaban efectos negativos no intenté ser embarazada otro

- ¿Tomaba un suplemento del ácido fólico durante el mes antes de la concepción de su hijo?
 - Sí No
 - ¿Cuál marca? _____
 - ¿Con que frecuencia? 1- 3 veces a la semana 4 - 6 veces a la semana cada día

- Si contestó “no”, ¿por qué no tomaba el ácido fólico? ___no pensaba que era necesario ___demasiado caro ___ me causaba efectos negativos ___ no intenté ser embarazada _____otro
- Después de realizar que estaba embarazada y antes de recibir los suplementos de la clínica, ¿tomabas unas vitaminas o suplementos prenatales? ___Sí ___ No
 - ¿Cuál marca? _____
 - ¿Qué período de tiempo? _____
 - ¿Con qué frecuencia? _____
- Después de realizar que estaba embarazada y antes de recibir los suplementos de la clínica, ¿tomabas un suplemento que solo fue ácido fólico? ___Sí ___ No
 - ¿Cuál marca? _____
 - ¿Qué período de tiempo? _____
 - ¿Con qué frecuencia? _____
- ¿Tomaste un suplemento dietético o herbal que no fue vitaminas o el ácido fólico en cualquier momento durante o antes de este embarazo?
 - ___Sí ___ No
 - ¿Cuáles tipos, Cuál marca, y con qué frecuencia? _____
 - _____
 - _____
- ¿Consumes normalmente unos de los siguientes?
 - Cereal del desayuno _____Sí ___No _____ Marca __veces/sem
 - Bebidas o barras de reemplazo de comida ___Sí ___No _____ Marca __veces/sem
 - Bebidas de energía _____Sí ___No _____ Marca __veces/sem
 - Batido de proteína _____Sí ___No _____ Marca __veces/sem
 - Bar (de bocadillo) _____Sí ___No _____ Marca __veces/sem
 - Espinacas, col rizada, otras verduras de hoja verde ___Sí ___No _____veces/sem
 - Jugo de naranja _____Sí ___No _____ Marca __veces/sem
- ¿En el pasado, jamás ha fumado? _____Sí _____No
 ¿Por cuánto tiempo?_____¿Cuándo lo dejó?_____

- ¿En el pasado, jamás tomaba más que una bebida alcohólica al día?
 - _____ Sí _____ No
 - ¿Con qué frecuencia tomabas 2 o más bebidas alcohólicas en un día? _____

- ¿Ahora toma mas que una bebida alcohólica al día?
 - _____ Sí _____ No
 - ¿Con qué frecuencia tomas 2 o más bebidas alcohólicas al día? _____

- ¿En la semana pasada, cuánto tiempo pasaba Ud. haciendo actividades físicas (ejercicio, caminando, haciendo quehaceres, etc)? _____ min/día o _____ horas/semana

- ¿Fueron estas actividades (elige uno): ___ adentro ___ afuera ___ ½ adentro y ½ afuera?

- ¿Esto es un nivel de actividad similar a la suya antes del embarazo? _____ Sí _____ No, fue más activa antes _____ No, soy más activa ahora

- En el mes pasado, cuánto tiempo usualmente pasaba afuera entre la salida y la puesta del sol? _____

- ¿Es esta cantidad típica para Ud. en este estación del año? _____ Sí _____ no

- ¿Cuando pasa Ud. tiempo afuera, usualmente usaba una gorra o protección del sol? _____ sí _____ no

APPENDIX G

THREE DAY DIETARY RECALL FORM (ENGLISH)

THREE DAY DIET RECALL: FOLIC ACID SUPPLEMENTATION IN PREGNANCY STUDY

ID# _____

Instructions:

- 1) Please use the attached sheets to record all that you eat for three days during the week of _____.
- 2) You will receive a reminder from the research staff when it is time to complete the forms
- 3) Record the information for foods eaten during three 24 hour periods on non-consecutive days, including one weekend day - indicate the date of the recall and day of the week on each sheet
- 4) Use a separate line for each food item. Form can be handwritten – no need to type
- 5) Indicate the time and place (home, work, restaurant, etc.) that the food was eaten and whether it was a snack or part of a meal. (see sheet 1 for a few examples)
- 6) List the food item and approximately how much of it you ate (cups, pieces, etc.).
- 7) Record details, when appropriate, for each food item, including:
 - cooking method (grilled, baked, fried, etc)
 - brand name
 - condiments added (ketchup, salad dressing, butter, etc.)
- 8) Please answer the general questions related to physical and outdoor activities below.
- 9) When the recall sheets for all three days have been completed, please return all four pages (instruction page and three recall pages) to the UGA Folate Team using the self-addressed stamped envelope.
- 10) For questions or assistance please contact: The Folate Research Lab at 706-542-7689

Health Behavior Questionnaire – follow-up:

- 1) Have you been taking your prenatal supplements every day as directed? ___Yes ___No
- 2) Have you experienced any problems with the supplements? ___Yes ___No
If yes, what? _____
- 3) Over the past week, about how much time did you spend engaging in physical activities (exercise, walking, gardening, vacuuming, etc)? _____min/day or _____ hrs/wk
- 4) Were your activities spent (check one): ___ mainly indoors ___ mainly outdoors ___ half indoors & half outdoors
- 5) Over the past month, how much time did you usually spend outside each day between sunrise & sunset? _____
- 6) When outside in the sun, did you usually wear a hat, sunscreen or other sun protection?
____yes ____no

APPENDIX H

THREE DAY DIETARY RECALL FORM (SPANISH)

RECORDATORIO DE DIETA DE 3 DIAS: ESTUDIO DE LA SUPLEMENTACIÓN DEL FOLATO DURANTE EL EMBARAZO

ID# _____

Instrucciones:

- 1) Por favor, usa los formularios adjuntos para recordar todo lo que come Ud. para tres días durante la semana de _____.
- 2) Recibirá un recuerdo del personal de la investigación cuando el tiempo de llenar el formulario llega.
- 3) Escribe la información para las comidas consumidas durante tres periodos de 24 horas, en días no consecutivos, incluyendo un día de fin de semana- indica la fecha del recordatorio y cual día de la semana en cada hoja.
- 4) Usa una línea diferente para cada ítem de comida. El formulario puede ser escrito con mano- no es necesario escribir a máquina.
- 5) Indica el tiempo y lugar (casa, trabajo, restaurante, etc.) que se comió la comida y si fue parte del desayuno, el almuerzo, la cena, o un bocadillo. (Hay unos ejemplos en página 1)
- 6) Escribe la comida y aproximadamente cuanto comió Ud. (tazas, pedazos, etc.)
- 7) Escribe detalles, cuando son a propósito, para cada comida, incluyendo:
 - método de cocinar (a la parrilla, de horno, frito, etc.)
 - nombre de la marca
 - condimentos usados (salsa de tomate, mantequilla, salsas, etc.)
- 8) Por favor, conteste las preguntas generales abajo sobre actividades físicas y afueras.
- 9) Cuando los formularios para cada de los tres días están cumplidos, por favor devuélvalos (4 páginas, página de instrucciones y tres recordatorios) al Equipo del Folato de UGA usando el sobre con sello que ya tiene dirección.
- 10) Si tiene preguntas o necesita ayuda, por favor llama:
El laboratorio de Investigación del Folato 706-542-7689

Cuestionario del Comportamiento de Salud- seguimiento:

- 1) ¿Ud. ha tomado sus suplementos prenatales cada día como dirigido? _____ Sí _____ No
- 2) ¿Ha tenido una problema con los suplementos? _____ Sí _____ No
¿Cuáles? _____
- 3) ¿En la semana pasada, cuánto tiempo pasaba Ud. haciendo actividades físicas (ejercicio, caminando, haciendo quehaceres, etc)? _____ min/día o _____ horas/semana
- 4) ¿Fueron estas actividades (elige uno): _____ adentro _____ afuera _____ ½ adentro y ½ afuera?
- 5) ¿En el mes pasado, cuánto tiempo usualmente pasaba afuera entre la salida y la puesta del sol?

- 6) ¿Cuando pasaba Ud. tiempo afuera, usualmente usaba una gorra o protección del sol? _____ sí
_____ no

APPENDIX I

ASA-24 PARTICIPANT INSTRUCTIONS



ASA24™

ASA24 Automated Self-administered 24-hour Recall

Your usual dietary intake and intake of specific nutrients, including folate, will be estimated using the multi-pass Automated Self-administered 24-hour Recall (ASA24™) system hosted through the National Cancer Institute website. This methodology uses multiple probes to capture types and amounts of foods eaten, time and occasion of eating, and additional details related to preparation methods and additions such as condiments. Information from three non-consecutive days including one weekend day is generally required to provide an indication of ‘typical intake’.

Log-in information for the program is provided below. When you log-in you will be asked to supply information about all the food that you ate the previous day (e.g. Monday if log-in is on Tuesday). Once you log-in, you will have until the end of that day to complete the recall – you can come back to it if you are interrupted. You will be asked to complete two sets of dietary recalls – one at 24 weeks of gestation and one at 32 weeks. Study personnel will contact you when it is time to complete each set of recalls. For each set of dietary recalls, you will supply information for three days (non-consecutive) including one weekend day. [You will need to log-in separately to complete each days recall.]

Log-in information:

Website: <https://asa24.nci.nih.gov>

Username: FAPREG121

Password: Folic#669

If you experience problems logging in or using the program please contact:

Folate Research Team: folate13@uga.edu or 706-542-7689

Dr. Dorothy Hausman: dhausman@uga.edu or 706-542-4871

A summary of your dietary intake information will be sent upon completion of the study.

APPENDIX J

BLOOD SAMPLE COLLECTION FORM

Folic Acid Supplementation in Pregnancy Study: Blood Sample Collection & Transmittal Form

Folic Acid Study ID # **FAP #154** 1st visit 28 wk 36 wk Delivery

TIME OF LAST MEAL/SNACK: _____ **Birthdate** _____

Blood Drawn: Date: _____ Time: _____

Lavender-top ___ (1 x 10 ml)___ Mix well; Wrap in foil; Place in refrigerator/cooler

Lavender-top ___ (1 x 10 ml)___ Mix well; Wrap in foil; Place in refrigerator/cooler

Red-top ____ (1 x 9 ml)____ Mix well; Wrap in foil; Place in refrigerator/cooler

Comments: _____

Midwife / Phlebotomist: _____

Delivered to: _____ Time: _____

Please call **Folate Research Team (706-247-4244 or 706-247-4381)** for sample pick-up.

APPENDIX K

INITIAL CHECKLIST

Initial Checklist

**TITLE OF STUDY: Folic Acid Supplementation in Pregnant Women:
Dose Response**

PRINCIPAL INVESTIGATOR: Dr. Lynn B. Bailey, University of Georgia

IRB PROJECT NUMBER: STUDY00000506 (UGA)

Date _____
Name _____
Best time to call _____
Participant Number _____
ARMC Midwife _____

INCLUSION CRITERIA (ALL of below should be checked)

___ Age (18-40yrs)
___ Week of Gestation (< 12 week)
___ BMI ___ (18.5-35.0)
 Height: ___ ft. ___ in.
 Weight: ___ kg.

EXCLUSION CRITERIA (NOT ELIGIBLE if checked)

___ Chronic disease (diabetes, hypertension, epilepsy, cancer, kidney disease,
 cardiovascular disease)
___ Use of anticonvulsive drugs

NEED INFORMATION ON THE FOLLOWING:

___ Anemia
___ Current illness (pneumonia, urinary tract infection, mononucleosis)
___ Smoking
___ Alcohol consumption (2 or more drinks per day)
___ Vegan dietary regime (excludes all animal products from diet)
___ *in vitro* fertilization treatment
___ Use of other prescription drugs _____
___ Use of antibiotics in past 2 weeks

CHECK LIST

___ Blood Drawn (3 tubes)
___ Provide Pill-Box
___ Provide Diet Recall Form/Remind contact from UGA Folate Research Team
___ Notify Dr. Hea Jin Park (706-248-4153) of sample collection
 • Dr. Park (or other Folate Team member) will pick-up sample within 90 min.
 of collection

APPENDIX L

MONTHLY CHECKLIST

Visit Checklist (at every visit)

**TITLE OF STUDY: Folic Acid Supplementation in Pregnant Women:
Dose Response**

**PRINCIPAL INVESTIGATOR: Dr. Lynn B. Bailey, University of Georgia
IRB PROJECT NUMBER: STUDY00000506 (UGA)**

Date (Gestational weeks) _____
Participant Number _____
ARMC Midwife _____

CHECK POINTS

___ Anemia

___ Pregnancy-associated complications (gestational diabetes, pre-eclampsia)

___ Acute illness (pneumonia, urinary tract infection, mononucleosis)

___ Use of prescription drugs

Name, duration

(_____)

___ Use of antibiotics in past 2 weeks

Name, duration

(_____)

If any of above is checked, please contact the UGA Folate Research Team (706-248-4153). Drop decision will be made by the UGA Folate Research Team and ARMC staff will be notified and to inform the participant before next visit.

CHECK LIST

___ Exchange Pill-Box

APPENDIX M

28 WEEK CHECKLIST

28 Weeks Checklist

**TITLE OF STUDY: Folic Acid Supplementation in Pregnant Women:
Dose Response**

**PRINCIPAL INVESTIGATOR: Dr. Lynn B. Bailey, University of
Georgia**

IRB PROJECT NUMBER: STUDY00000506 (UGA), ZZZZ (ARMC)

Date _____

Participant number _____

ARMC Midwife _____

CHECK POINTS

____ Carrying more than one fetus

____ Anemia

____ Pregnancy-associated complications (gestational diabetes, pre-eclampsia)

____ Acute illness (pneumonia, urinary tract infection, mononucleosis)

____ Use of prescription drugs

Name, duration ()

____ Use of antibiotics in past 2 weeks

Name, duration ()

If any of above is checked, please contact the UGA Folate Research Team (706-542-7689, folate13@uga.edu). Drop decision will be made by the UGA Folate Research Team and ARMC staff will be informed to notify the participant.

CHECK LIST

____ Blood Drawn (Lavender-top 1)

____ Blood Drawn (Lavender-top 2)

____ Blood Drawn (Red-top)

____ Exchange Pill-Box

____ Provide Diet Recall Form/Remind contact from UGA Folate Research Team

APPENDIX N

36 WEEK CHECKLIST

36 Weeks Checklist

**TITLE OF STUDY: Folic Acid Supplementation in Pregnant Women:
Dose Response**

**PRINCIPAL INVESTIGATOR: Dr. Lynn B. Bailey, University of
Georgia**

IRB PROJECT NUMBER: STUDY00000506 (UGA), ZZZZ (ARMC)

Date _____

Participant Number _____

ARMC Midwife _____

CHECK POINTS

___ Anemia

___ Pregnancy-associated complications (gestational diabetes, pre-eclampsia)

___ Acute illness (pneumonia, urinary tract infection, mononucleosis)

___ Use of prescription drugs

Name, duration (_____)

___ Use of antibiotics in past 2 weeks

Name, duration (_____)

If any of above is checked, please contact to UGA Folate Research Team (706-542-7689, folate13@uga.edu). Drop decision will be made by the UGA Folate Research Team and ARMC staff and will be informed and will notify the participants.

CHECK LIST

___ Blood Drawn (Lavender-top 1)

___ Blood Drawn (Lavender-top 2)

___ Blood Drawn (Red-top)

___ Exchange Pill-Box

APPENDIX O

DELIVERY CHECKLIST

Delivery Checklist

**TITLE OF STUDY: Folic Acid Supplementation in Pregnant Women:
Dose Response**

**PRINCIPAL INVESTIGATOR: Dr. Lynn B. Bailey, University of
Georgia**

IRB PROJECT NUMBER: STUDY00000506 (UGA), ZZZZ (ARMC)

Date

Participant Number

ARMC Midwife

Maternal Blood

___ Blood Drawn (Red-top) – Top priority

___ Blood Drawn (Lavender-top 1) – Second priority

___ Blood Drawn (Lavender-top 2)

APPENDIX P

LAB PROCESSING OF BLOOD SAMPLES PROTOCOL

**TITLE OF STUDY: Folate Supplementation in Pregnant Women: Dose
Response**

PRINCIPAL INVESTIGATOR: Dr. Lynn B. Bailey

IRB PROJECT NUMBER: STUDY00000506

Collection of blood samples (Athens Regional Midwifery Practice):

- Participant will not be requested to fast prior to sample collection

- Draw samples for research needs:
 - **One 9 ml red/grey top tube**
 - Invert the tube about 5 times after obtaining the sample
 - ***Protect from exposure to light - keep covered with foil.***
 - Place immediately in refrigerator until pickup by research team

 - **Two 10 ml purple EDTA tubes**
 - Immediately after drawing, invert both tubes 5 times to mix sample with EDTA.
 - ***Protect from exposure to light - keep covered with foil.***
 - Place immediately in refrigerator until pickup by research team

- Complete Folic Acid Supplementation in Pregnancy: Blood Sample Collection form
 - Note time of last meal or snack and time of blood collection

Pick of blood and transport of blood samples

- Notify Dr. Hea Jin Park (706-542-5093) of sample collection
- Dr. Park (or other Folate Team member) will travel to ARMC to collect sample within 90 min. of sample collection
- Samples will be transferred to chilled Chameleon Coolers within sealed Biosafety Transport containers for transport to Dawson Hall
- Also collect copies of Consent Form and Blood Sample Collection Form

TITLE OF STUDY: Folate Supplementation in Pregnant Women: Dose Response

PRINCIPAL INVESTIGATOR: Dr. Lynn B. Bailey

IRB PROJECT NUMBER: STUDY00000506

Processing of blood samples (in Dawson 269 Lab):

Required reagents:

1% ascorbic acid solution (RBC folate) – add 1 g L-ascorbic acid (176.13 FW) / 100 ml deionized water; store at 4°C.

7% w/v ascorbic acid solution (serum folate) – pre-weigh 70 mg L-ascorbic acid aliquots, add exactly 1 ml of deionized water, mix gently by inversion to dissolve the ascorbic acid, will take 10 min to completely dissolve; **PREPARE FRESH DAILY**

10 mg ascorbic acid (folic acid) – pre-weighed in one of the two ‘folic acid’ storage vials

DMSO (buffy coat) – no dilution or preparation required

1 M acetic acid (SAM/SAH/Hcy) – slowly add 5.75 ml of glacial acetic acid to ~90 ml of deionized water while stirring, bring to a final volume of 100 ml. Store at RT.

Processing of individual blood collection tubes:

- 9 ml **red/grey top** (serum folate, folate forms, inflammatory markers, etc.)
 - Allow the tube to sit at room temperature to clot for 30 minutes
 - Centrifuge at room temperature. Recommended time – BD website: 15 minutes at 1100-1300 g for fixed angle centrifuge; 10 minutes at same g for swing-head units.
 - For Serum Folate: carefully remove 1 ml serum from the tube and add to a vial labeled ‘serum folate’ containing 71.4 ul of 7% w/v ascorbic acid solution (prepared fresh daily); mix well; remove a ~500 mL a second ‘serum folate’ vial.
 - For Folic Acid/Folate Forms: carefully remove an additional 1 ml serum from the tube, add to a vial labeled ‘folic acid’ containing 10 mg ascorbic acid; mix well; remove a ~500 mL a second ‘folic acid’ vial.
 - For Inflammatory Markers: carefully remove 500 µl serum from the tube, add to the vial labeled ‘Inflammatory’
 - For Vitamin D: carefully remove 200 µl serum from the tube, add to the vial labeled ‘Vitamin D’
 - For CMP (Comprehensive Metabolic Panel): carefully remove 500 µl serum from the tube, add to the vial labeled ‘CMP’. Place in participant-specific sample collection bag, keep at room temperature for Lab Corp pick-up (see below).
 - For Extra Serum: carefully residual serum from the tube as 500 µl aliquots, taking care not to draw up excess red blood cells; add to one or more vials labeled ‘extra serum’

- Sort samples by assay and separate duplicates prior to freezing at -80° C
- Store primary samples in the -80° freezer in 269 Dawson; the duplicate set in the -80° freezer in 301 Dawson (Pazdro lab)
- Store the blood clot at -20°.

- 10 ml **purple EDTA** tube (Whole blood, plasma, buffy coat)
 - Whole Blood Samples (prior to centrifugation)
 - For RBC folate analysis:
 - In an appropriately labeled cryovial (RBC folate), dilute 100 µl EDTA whole blood with 1 ml of 1% ascorbic acid solution (1 g/dl; ascorbic acid – FREE; room temp.), This corresponds to a 1:11 dilution.
 - Put the tubes on a rotator (**keep protected from light**) and let them sit out for 30 minutes (room temperature) to ensure hemolysis before freezing.
 - After 30 minutes, remove a 500 µl aliquot from the tube and add to a second screw cap cryovial.
 - Place in storage box(es) labeled for RBC folate
 - Freeze samples at -80° C
 - For Genotyping (by UGA Genomics Facility):
 - Transfer a 500 µl aliquot of whole blood a cryovial labeled with participant number, 'Genotyping' and other storage information
 - Store at -80° C
 - For CBC (Complete Blood Count – by Lab Corp)
 - Remove 500 µl whole blood to the vial labeled 'CBC'. Place in participant-specific sample collection bag, keep at room temperature for Lab Corp pick-up (see below).
 - Plasma (after centrifugation)
 - After removal of whole blood samples, centrifuge at 2200 PRM for 15 min. at 4°C.
 - For Choline: carefully remove 500 µl of plasma from the tube, add to a vial labeled 'choline'
 - For SAM/SAH/Hcy: carefully remove an additional 500 µl of plasma from the tube, add to a vial labeled 'SAM/SAH/Hcy'; Add 50 µl of 1 M acetic acid; vortex gently
 - For Vitamin B12: carefully remove an additional 500 µl of plasma from the tube, add to a vial labeled 'vitamin B12'
 - For Extra Plasma: carefully residual plasma from the tube as 500 µl aliquots, taking care not to draw up the white buffy coat layer; add to one or more vials labeled 'extra plasma'
 - Sort samples by assay and separate duplicates prior to freezing at -80° C
 - Store primary samples in the -80° freezer in 269 Dawson; the duplicate set in the -80° freezer in 301 Dawson (Pazdro lab)

- Buffy Coat (after centrifugation and removal of plasma)
 - Add 50 µl DMSO to vial labeled 'Buffy Coat'
 - Using a wide bore pipet tip (cut end from 1 ml tip), draw up as much of the buffy coat as possible. You will get red cells as well.
 - Place this material into the vial with DMSO (50 µl).
 - Mix by inversion (do not vortex) and store at -80° C
- 10 ml **purple EDTA** tube (store for subsequent DNA methylation analysis)
 - Upon return to the lab, invert sample gently 5-6 times to assure that it is well mixed.
 - Aliquot to two 5 ml cryogenic vials/tubes, labeled for DNA methylation
 - Store one tube in the -80° freezer in 269 Dawson; the second in the -80° freezer in 301 Dawson (Pazdro lab)

Lab Corp samples –

- Place serum sample labeled "CMP" and whole blood sample labeled "CBC" in biohazard sample bag
 - Zip shut to seal, parafilm wrapping for tubes
- Check sample requisition form to assure that the following information has been added:
 - Complete in advance
 - Report sending info - box on upper right
 - ✓ "Account Bill" - near top left
 - Subject number (patient name), "F" (sex) & Fasting "No"
 - Dr. Bailey's name and signature
 - Diagnosis – "Fasting Baseline Values – Nutrition Research Study"
 - ✓ 322000 Comprehensive Metabolic Panel
 - ✓ 005009 CBC w Diff w Plt
 - Complete day of collection
 - Collection time and date
 - Date of birth
- Place completed requisition form in outer pocket of biohazard sample bag
- Place samples in Lab Corp dropbox
- Call for sample pick-up: **1-800-621-8037**, #1, ext. **3600**
 - Account # is **10551575**
 - Pick-up location is: **305 Sanford Drive**, Athens, GA 30602
 - Please specify if samples will be: **Pick up after 6:30pm, outside**
 - Inside - by 269 Dawson (if early afternoon)
 - Outside – put dropbox by center Dawson doorway
 - Record confirmation number (to check on sample pick-up if needed)